Self-Assembly of Epstein-Barr Virus Capsids[▽]

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Epstein-Barr virus (EBV), a member of the *Gammaherpesvirus* family, primarily infects B lymphocytes and is responsible for a number of lymphoproliferative diseases. The molecular genetics of the assembly pathway and high-resolution structural analysis of the capsid have not been determined for this lymphocryptovirus. As a first step in studying EBV capsid assembly, the baculovirus expression vector (BEV) system was used to express the capsid shell proteins BcLF1 (major capsid protein), BORF1 (triplex protein), BDLF1 (triplex protein), and BFRF3 (small capsid protein); the internal scaffold protein, BdRF1; and the maturational protease (BVRF2). Coinfection of insect cells with the six viruses expressing these proteins resulted in the production of closed capsid structures as judged by electron microscopy and sedimentation methods. Therefore, as shown for other herpesviruses, only six proteins are required for EBV capsid assembly. Furthermore, the small capsid protein of EBV (BFRF3), like that of Kaposi's sarcoma-associated herpesvirus, was found to be required for assembly of a stable structure. Localization of the small capsid protein to nuclear assembly sites required both the major capsid (BcLF1) and scaffold proteins (BdRF1) but not the triplex proteins. Mutational analysis of BFRF3 showed that the N-terminal half (amino acids 1 to 88) of this polypeptide is required and sufficient for capsid assembly. A region spanning amino acids 65 to 88 is required for the concentration of BFRF3 at a subnuclear site and the N-terminal 65 amino acids contain the sequences required for interaction with major capsid protein. These studies have identified the multifunctional role of the gammaherpesvirus small capsid proteins.

Epstein-Barr virus (EBV) is a gammaherpesvirus that infects greater than 90% of the general population and causes several lymphoproliferative diseases. Primary infection in adolescents with EBV results in infectious mononucleosis (46). EBV is also oncogenic and is associated with malignancies such as Burkitt's lymphoma (4), nasopharyngeal carcinoma, and posttransplant lymphoproliferative disease (47).

Herpesviruses assemble icosahedral capsid structures in the nuclei of infected cells; six proteins are required for assembly of these structures. The capsid shell is largely composed of multimers of the major capsid protein, which are linked by the triplex structure (trimer of the two triplex proteins) and decorated by the small capsid protein. The internal scaffold protein directs the closure of the shell into an icosahedral structure (reviewed in references 17, 35, and 41). The EBV serine protease is synthesized as a 605-amino-acid polypeptide, which cleaves itself between amino acids Ala235 and Ser236 (release [R] site) and also between amino acids Ala568 and Ser569 (maturation [M] site). The catalytic domain resides in the N-terminal 235 amino acids (13). The six EBV capsid proteins are BcLF1 (major capsid protein), BORF1 (triplex 1), BDLF1 (triplex 2), BdRF1 (scaffold protein), BVRF2 (protease), and BFRF3 (small capsid protein) (Table 1). BVRF2 and BdRF1 are synthesized from the same open reading frame (ORF), and consequently BdRF1 overlaps the C-terminal 261 to 605 amino acids of BVRF2.

Protein-protein interactions drive the self-assembly of herpesvirus capsid structures. Capsid assembly for herpesviruses begins in the cytoplasm. Cellular localization studies show that the major capsid protein is transported into the nucleus by the scaffold protein (not cleaved at the maturation site), and triplex protein 2 (Table 1) is transported efficiently in the presence of triplex protein 1 (1, 29, 36). The full-length protease can also interact with the major capsid protein, and this may be one mechanism it uses to localize to the preassembly complex (12, 39). The small capsid protein requires both the major capsid and the scaffold protein for its concentration in the nuclear assembly sites (9, 36). Complexes formed between the two triplex proteins—between the major capsid protein, scaffold protein, and probably the small capsid protein—come together in the nucleus to form the capsid shell (40).

The first closed structure formed in the nucleus is the spherical procapsid, which serves as the substrate for the association with the DNA packaging complex and is filled with a genome complement of DNA (26, 37, 45). Within this procapsid, the protease cleaves the scaffold protein at the C terminus, which allows the scaffold protein to be released from the shell during DNA packaging. Thus, the protease is essential for the maturation of this structure from that containing a scaffold to one containing DNA (15).

The assembly of herpesvirus capsids and determination of their structures are biased toward the studies of herpes simplex virus (HSV) capsids. Very little information on EBV capsid structure or the molecular pathways of the assembly process is available because of a lack of a tractable system to isolate EBV capsids in quantities sufficient for biochemical and structural analyses. Both Tatman et al. (42) and Thomsen et al. (43) have shown self-assembly of HSV-1 capsids using the baculovirus

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TABLE 1. EBV capsid protein ORFs

EBV ORF ^a	Function	Predicted mass (kDa)	Homolog	
			KSHV	HSV
BcLF1	Major capsid	154	ORF25	VP5
BORF1	Triplex 1	39	ORF62	VP19C
BDLF1	Triplex 2	34	ORF26	VP23
BdRF1	Scaffold	36	ORF17.5	p22a
BVRF2	Protease	64	ORF17	VP24/p21
BFRF3	Small capsid	18	ORF65	VP26

^a B95-8 sequence (8).

expression vector (BEV) system and Newcomb et al. (27) have demonstrated self-assembly using purified components. Recently we were able to assemble the capsids of Kaposi's sarcoma-associated herpesvirus (KSHV), another gammaherpesvirus (gamma-2-herpesvirus), using the BEV system (31). Many aspects of the assembly pathway of this virus were similar to those discovered for HSV-1, with the exception of the finding that the small capsid protein (pORF65) was essential for KSHV capsid assembly (31). In order to shed light on EBV assembly, we undertook a similar study using baculovirus expression of EBV capsid proteins and assembly of the icosahedral structure in coinfecting insect cells. Self-assembly of EBV capsids was accomplished using the BEV system, and therefore this is the first report of EBV capsid assembly using an in vitro method.

MATERIALS AND METHODS

Cell lines and antibodies. *Spodoptera frugiperda* (Sf9 and Sf21) cells were grown in Grace's insect cell medium, supplemented with 10% fetal calf serum (Gibco-Invitrogen). Sf9 and Sf21 cells were grown and passaged as described by Okoye et al. (30). The green fluorescent protein (GFP) rabbit polyclonal antibody was purchased from Molecular Probes. Mouse monoclonal antibody to hemagglutinin (HA) was purchased from Sigma.

Plasmids. The six EBV capsid protein ORFs coding for BcLF1, BORF1, BDLF1, BVRF2, BdRF1, and BFRF3 were PCR amplified with high-fidelity polymerase *Pfu* Ultra (Stratagene). The template for these amplifications was a BAC-cloned copy of the EBV Akata genome (25). The PCR primer pairs used to amplify each ORF are listed in Table 2, and the published sequence of EBV strain B958 was used to design the primer pairs (8). The ORFs were cloned in the baculovirus transfer vector pFastBac 1 (pFB1) (Invitrogen) (23). All of the genes were cloned as EcoRI-HindIII fragments, except for the BcLF1 and BORF1 genes, which were cloned as SpeI-XbaI and BamHI-HindIII fragments, respectively. The cloned genes were sequenced to check for correct amplification. Confirmed plasmids were designated by the transfer plasmid abbreviation and gene name: for example, pFB1-BcLF1. The pFB1-BFRF3-EGFP plasmid was made by first amplifying BFRF3 using a reverse primer that lacked a stop codon. This gene was cloned as an EcoRI-HindIII fragment into pFB1 (pFB1-BFRF3^{amb-}). The enhanced GFP (EGFP) ORF was amplified from pEGFP-N2 (Clontech) and cloned as a HindIII fragment into pFB1-BFRF3^{amb-} in the correct orientation. The plasmid was sequenced for the correct ORF amplification.

BFRF3 mutant genes were made with PCR assays, using pFB1-BFRF3 as a template. The primers used to synthesize the mutant genes are also shown in Table 2. The forward primer specified an EcoRI site and the reverse primer specified a SpeI site. The PCR products were cloned as EcoRI-SpeI fragments into pFB1-CHA and pFB1-CEGFP. The latter plasmids were made by insertion of a double-stranded DNA oligonucleotide encoding the HA epitope or a PCR product of EGFP (Clontech) as SpeI-HindIII inserts. The resulting BFRF3 mutant gene plasmid would create a C-terminal in-frame fusion with the Flu HA or EGFP sequence.

Generation of recombinant baculoviruses using the Bac-to-Bac system. The pFB:EBV gene expression cassette was transferred into the baculovirus genome harbored in *Escherichia coli* cell line DH10BAC according to the manufacturer's protocol and also as described by Okoye et al. (30).

TABLE 2. Oligonucleotide primer sequences

Primer name	Sequence ^a
BcLF1/F/SpeI.....	<u>GGACTAGT</u> GACCATGGCCTCA AATGAGGGTGTG
BcLF1/R/XbaI	<u>GTCTAGAT</u> CAAAAAACCACCT TATTTCC
BORF1/F/BamHI.....	GGGATCCGACCATGAAGGTC CAGGGGTCCGTC
BORF1/R/HindIII.....	<u>GGAAGCTT</u> CTAGAGAGAATC ACCTCCAGTC
BDLF1/F/EcoRI.....	<u>GGAATTC</u> ACCATTGGATTGAA AGTGGTAGTG
BDLF1/R/HindIII	<u>GGAAGCTT</u> TTATCTTAACCAG CAAGTGGCCG
BVRF2/F/EcoRI	<u>GGAATTC</u> ACCATTGGTGCAGG CACCGTCTGTATAC
BVRF2-BdRF1/R/HindIII	<u>GGAAGCTT</u> TCAAGCCACGCG TTTATTCAGC
BdRF1/F/EcoRI	<u>GGAATTC</u> ACCATTGCTATCAGG TAACGCAGGAG
BFRF3/F/EcoRI	<u>GGAATTC</u> ACCATTGGCACGCCG GCTGCCAAG
BFRF3/R/HindIII	<u>GGAAGCTT</u> CTACTGTTTCTTA CGTGCCCC
BFRF3/R ^{amb-} /HindIII	<u>GGAAGCTT</u> CTGTTTCTTACGT GCCCGCG
BFRF3/F/EcoRI	<u>GGAATTC</u> ACCATTGGCACGCCG GGCTGCCAAGCCC
BFRF3-89/F/EcoRI	<u>GGAATTC</u> ACCATTGGCACACCT TGGCGGGTCATCCGCC
BFRF3-65/R/SpeI.....	<u>GGACTAGT</u> AAAAGTCCTCTG CACGTACTCTTC
BFRF3-88/R/SpeI.....	<u>GGACTAGT</u> ATGAGCACCAGC CCCAGCCACAC
BFRF3-110/R/SpeI.....	<u>GGACTAGT</u> GGTCCCAGCGGA TGCGGCGGCCTG
BFRF3-125/R/SpeI.....	<u>GGACTAGT</u> GGACTGGGCTAC GGCCGTGGACGG
BFRF3-176/R/SpeI.....	<u>GGACTAGT</u> CTGTTTCTTACGT GCCCGCGGTGG

^a Restriction enzyme sites are underlined, and the start codon (ATG) for each gene is in boldface.

Baculovirus transfection and virus amplification. Sf9 cells were used for bacmid DNA transfections and virus amplifications. The procedures used are described in Okoye et al. (30) and Perkins et al. (31). Two independent bacmid clones were transfected for each gene.

Western blot analysis. Sf21 cells were seeded in 12-well trays (12 × 10⁶ cells per tray), and for each well, 50 µl of baculovirus and 50 µl of phosphate-buffered saline were added. Sf21-infected cells were harvested 72 h postinfection, and the extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membranes (Millipore) in Tris-glycine buffer using a Bio-Rad mini-transblot apparatus. SDS-PAGE analysis was performed as described by Person and Desai (24). Transfer buffer and procedures were used according to the manufacturer's protocol. Western blots were carried out using ¹²⁵I-labeled protein A for detection as described by Okoye et al. (30).

Sample preparation for TEM. Sf21 cells were used for almost all transmission electron microscopy (TEM) experiments. A total of 1 × 10⁷ cells in 100-mm dishes were coinfecting with 250 µl of each of the six baculoviruses, and infected cells were processed for EM 68 h after infection. Infected cells were processed for conventional thin-section TEM as described by Huang et al. (18) and Perkins and McCaffery (32). Negative-stained capsids were prepared essentially as described by Perkins et al. (31), using 1% phosphotungstic acid as the staining solution.

TEM. Samples were examined using Phillips EM 410, EM 420, and Tecnai 12 transmission electron microscopes (FEI, Hillsboro, OR); images were captured with an SIS Megaview III or FEI Eagle 2k camera.

FIG. 1. Expression of the EBV capsid proteins in insect cells using recombinant baculoviruses. Sf21 cells (1×10^6) were infected with each baculovirus (50 μ l) expressing the six EBV capsid proteins. Infected cells were harvested 72 h after infection, and the extracts prepared were analyzed by SDS-PAGE (15% acrylamide). The gel was stained with Coomassie brilliant blue to reveal the polypeptides. The position of the EBV capsid protein is indicated with an asterisk to the right of each lane. BAC is a baculovirus that does not contain a gene insert, and mock-infected (MI) cells are also shown. The protein standards are in lane M, and the size of each band in kDa is shown to the left.

Sedimentation analysis. Sf21 cells were used for all sedimentation experiments. The infection methods and sedimentation procedures are described by Perkins et al. (31).

Confocal analysis. Sf21 cells seeded in a 35-mm FluoroDish (1×10^6 cells) were infected with 35 μ l of each baculovirus. The infected cells were analyzed in a Zeiss LSM 510 confocal microscope. Images were collected with a pinhole set between 1 and 1.5 Airy units.

Data and figure preparation. Autoradiographs were scanned at 300 dots per inch using Adobe Photoshop. Electron micrographs were taken as either 12- or 16-bit images, exported as 16-bit tiff files, and adjusted for brightness/contrast, and figures were compiled using Canvas 9.0. Images of EBV capsid bands in sucrose gradients were taken by John Letos (School of Medicine, Johns Hopkins University).

RESULTS

Cloning and expression of the EBV capsid proteins. The six ORFs of the EBV capsid proteins (shown in Table 1) were cloned into the baculovirus transfer vector pFastBac1 (pFB1). The cloned genes were sequenced to check for correct amplification and then transferred into the baculovirus genome using bacmid technology (23). In order to examine expression of the six EBV capsid proteins, Sf21 cells were infected with the recombinant baculoviruses and the infected cells were harvested at 72 h postinfection. The lysates were analyzed by SDS-PAGE, and following electrophoresis, the gel was stained with Coomassie brilliant blue. The results of this experiment are shown in Fig. 1. Accumulation of significant quantities of five capsid proteins was seen in the insect cells, and the polypeptides were of the correct molecular mass as judged by their mobility in the gel (see Table 1). The predicted size of the scaffold protein (BcRF1) is 36 kDa, but based on its migration in the gel, it appeared to have a higher molecular mass. One explanation for this could be the observed posttranslational modifications of herpesvirus scaffold proteins (5, 16). Accumulation of the protease (BVRF2) was not discernible in these experiments. Another baculovirus was made in which an N-terminal HA tag was fused to BVRF2. The expression of both

the full-length protease and the N-terminal catalytic domain (cleavage at the release site) in infected Sf21 cells was detected by Western blot methods (data not shown). This indicated that the EBV protease was expressed in insect cells and was proteolytically active.

EBV capsid self-assembly. To determine whether EBV self-assembly could be achieved in this system, Sf9 cells were coinfecting with all six viruses expressing the capsid proteins. Infected cells were processed for conventional EM (TEM) 68 h after infection. Many closed capsid structures were observed in the nuclei of cells coinfecting with recombinant baculoviruses (Fig. 2A and B). Some of these capsids contained a large internal core structure (2), which is the characteristic scaffold structure prior to cleavage of the scaffold protein by the protease (Fig. 2B). Capsids could also be isolated by sucrose gradient sedimentation methods. For this experiment, Sf21 cells were coinfecting with all six viruses and lysates were prepared from infected cells 68 h after infection. The lysates were then sedimented on sucrose gradients. A light-scattering band was visualized using reflected light (Fig. 2C; EBV-ALL). This band was harvested by side puncture, and the material was examined by TEM following negative staining. Icosahedral closed capsid structures were evident, and the capsomeres made up of major capsid protein (BcLF1) were clearly visible (Fig. 2D). The diameter of negatively stained EBV capsids was calculated to be 137.4 ± 1.85 nm ($n = 25$). We also measured similarly stained KSHV capsids assembled in insect cells and HSV-1 capsids isolated from infected cells and obtained similar numbers. Similar preparations of capsids were concentrated by centrifugation, and the pellet was resuspended in Laemmli sample buffer. The protein composition of the BEV-derived EBV capsids was examined by Nu-Page analysis followed by Coomassie staining (Fig. 2E). The five abundant EBV capsid proteins detected are indicated on the right of the gel. For BcRF1, two bands were detected which could be due to proteolytic cleavage by BVRF2 or phosphorylation. There are additional minor polypeptide species in the preparations, which are probably of baculovirus or cell origin, that cosediment with the capsids. Thus, EBV self-assembly can be achieved by the coexpression of six proteins only as shown for other herpesviruses (22, 31, 42, 43).

Requirements for EBV self-assembly. In order to determine which EBV capsid protein was required for self-assembly, similar coinfections were carried out as described above, but in each case a virus expressing one of the six capsid proteins was omitted from the infection. Initial experiments always include the virus expressing BVRF2, the maturational protease. However, it became apparent, from several thin-section TEM experiments, that it was easier to find capsids in the cells when the protease was not present. The reason for this was not clear: it could be that the protease is toxic to the cells or that premature cleavage of the scaffold protein by BVRF2 prevented the major capsid-scaffold protein interaction. The TEM experiments were therefore carried out in the absence of the protease-expressing virus. For comparison, Sf21 cells coinfecting with all six viruses are shown in Fig. 3A. Numerous capsids were observed in the nuclei of cells coinfecting with the remaining five viruses expressing EBV capsid proteins (Fig. 3B and inset). As expected, capsids were not found in Sf21 cells if the viruses expressing the major capsid protein (Fig. 3E) or the

FIG. 2. Self-assembly of EBV capsids. (A) Sf9 cells were coinfecting with all six baculoviruses expressing the EBV capsid proteins, and the infected cells were examined by conventional EM 68 h after infection. Numerous EBV closed capsid structures were evident in the nucleus of the infected cells (white arrowheads). (B) The EBV capsids contained characteristic internal scaffold protein core structures. (C) Sf21 cells were coinfecting with baculoviruses expressing all six capsid proteins (EBV-ALL), and the lysates sedimented through sucrose gradients. Reflected light was used to visualize the EBV capsid band. HSV-1 strain KOS-infected cell lysates were also analyzed, and A and B capsids were visible. (D) Material harvested from sucrose gradients was negatively stained and examined by EM. (E) Polypeptide composition of sucrose gradient-purified capsids was examined using a 4 to 12% Nu-Page Bis-Tris gel using MES (morpholineethanesulfonic acid) buffer (Invitrogen). The five abundant capsid proteins are indicated to the right. Protein standards are in lane M, and the sizes in kDa (estimated in MES buffer) of the bands are to the left of the lane. There is usually a small size discrepancy with our protein samples in Nu-Page gels due to our Laemmli buffer. Scale bar, 500 nm; inset bar, 100 nm. The nuclear envelope (ne) and mitochondria (m) are indicated. The locations of baculovirus particles are indicated by black arrowheads in panel A.

triplex proteins (Fig. 3D) were not added to the cells. Open capsid shells were evident in cells when the virus expressing the scaffold protein was omitted (Fig. 3C), indicating the importance of the scaffold protein for the closure of the capsid shell.

In order to isolate capsids using sucrose gradients, the virus expressing protease (BVRF2) had to be included in the coinfection. A light-scattering capsid band was clearly visible when lysates derived from cells infected with all six viruses were

sedimented, but a band was not visible when the protease-expressing virus was left out of the coinfection (data not shown). When material from the gradient lacking BVRF2 was examined, although there were some closed structures, many more open shell structures were present (data not shown). This result was similar to that seen in HSV-1-infected cells. Procapsids, which form in the absence of functional protease, are sensitive to cold and sedimentation but are readily seen in thin sections of infected cells by TEM experiments (28, 37).

FIG. 3. The major capsid and triplex proteins are essential for EBV self-assembly. (A) Sf21 cells were coinfecting with all six baculoviruses expressing the EBV capsid proteins, and thin sections were examined by TEM. In similar infections, the virus expressing BVRF2 protease (B to E) was omitted from the infection as well as the virus expressing the BDRF1 scaffold (C), both BORF1 and BDLF1 triplex proteins (D), and the BcLF1-major capsid protein (E). White arrowheads indicate the location of closed EBV capsid structures, and white arrows indicate open capsid shells. Black arrowheads mark baculovirus particles, and the nuclear envelope (ne) and plasma membrane (pm) are indicated where visible. Scale bars, 500 nm; inset bar, 100 nm.

EBV small capsid protein, BFRF3, is required for capsid assembly. Recent studies have shown the small capsid protein (pORF65) is essential for self-assembly of KSHV capsid structures (31). To determine whether the gamma-1 small capsid protein was also required for capsid assembly, Sf21 cells were coinfecting with all six viruses expressing the capsid proteins (EBV-ALL), and in similar infections the virus expressing BFRF3 was omitted from the coinfection (–BFRF3). Following infection, cells were harvested and the extracts were sedimented through sucrose gradients as described above. Light-scattering particles were observed by reflected light in the EBV-ALL gradient but not in the –BFRF3 gradient (Fig. 4A). Material was also harvested from the –BFRF3 gradient by side puncture from the same position where the EBV-ALL capsids sediment and examined by EM following negative staining. Assembled structures were not evident in the sample (data not shown). TEM analysis of thin sections of similarly infected cells was also carried out in the absence of the virus expressing BFRF3. Again coinfections were carried out in the absence of the virus expressing the protease, and although many capsids were evident in the presence of BFRF3 (Fig. 4B),

capsids were not detected in the absence of BFRF in all thin sections of infected cells examined (Fig. 4C). Hence, the EBV small capsid protein is also essential for capsid assembly.

Localization of BFRF3-GFP to subnuclear sites. Previously we fused EGFP to the HSV-1 small capsid protein, VP26, and found that this fusion protein localized to nuclear assembly sites, required major capsid protein (VP5) for this localization, and was incorporated into the capsid shell and mature virion (11). A similar approach was used to analyze the localization of BFRF3 in coinfecting Sf21 cells. The EGFP ORF was amplified and cloned at the C terminus of BFRF3. This gene fusion cassette was transferred into the baculovirus genome, and cells infected with this virus displayed green fluorescence when viewed under the microscope (Fig. 5; BFRF3-EGFP). Interestingly, the fluorescence begins to accumulate in a subnuclear structure that is likely the nucleolus (Fig. 5, BFRF3-EGFP inset). Sf21 cells were also coinfecting with this virus as well as the viruses expressing BcLF1, BDRF1, BORF1, and BDLF1 (EBV-ALL). The fluorescence was observed to relocalize to nuclear puncta (Fig. 5, EBV-ALL inset) as early as 24 h after infection, suggesting localization to sites of capsid protein ac-

FIG. 4. The EBV small capsid protein (BFRF3) is required for capsid assembly. (A) Sf21 cells were infected with the six baculoviruses expressing EBV capsid proteins (EBV-ALL), and similar infections were carried out in the absence of the virus expressing BFRF3 (–BFRF3). The lysates were sedimented in sucrose gradients and viewed using reflected light. A light-scattering band corresponding to EBV capsids (indicated by arrow) was visible in the EBV-ALL gradient but not in the –BFRF3 gradient. (B) Sf21 cells were coinfected with the baculoviruses expressing BcLF1, BORF1, BDLF1, BdRF1, and BFRF3. Thin sections of infected cells were examined by TEM, and many closed EBV capsid structures were evident in the nucleus (white arrowheads). (C) Similar infections were carried out as described for panel B, except that the virus expressing BFRF3 was left out of the infection. Scale bar, 1,000 nm. The black arrowheads indicate baculovirus particles, and the nuclear envelope (ne) and mitochondria (m) are also marked.

cumulation and thus possibly capsid assembly. In similar infections, viruses expressing the individual capsid proteins were omitted from the infection to determine the protein-protein interactions involved in this relocalization. Nuclear capsid protein localization sites (fluorescent puncta) were evident in the absence of either triplex protein (–BORF1 and –BDLF1) or both (data not shown); however, in the absence of the major capsid protein (–BcLF1) or its interactive partner BdRF1 (scaffold protein), the fluorescence observed was diffusely distributed throughout the cell. Therefore, like HSV-1 VP26, the EBV BFRF3 most likely binds to the major capsid protein, which is transported into the nucleus by virtue of its interaction with the scaffold protein (11, 36). This allows for the accumulation of this tripartite complex within the nucleus at sites of

virus assembly. The fluorescent puncta are probably not indicative of mature capsid formation because they are still observed in the absence of the triplex proteins, which are essential for assembly of the closed mature structure (10, 33). Hence, these fluorescent puncta likely represent sites of capsid protein complex accumulation or viral “factories.”

Assembly and subnuclear localization domains of BFRF3. In order to identify functional domains of BFRF3 for both capsid assembly and virus factory localization, a series of truncation mutations of BFRF3 were made. The six plasmids made specified the N-terminal 65, 88 (N-terminal half), 110, and 125 amino acids of BFRF or the C-terminal half (amino acids 89 to 176) as well as the full-length polypeptide (176 amino acids) (Fig. 6A). These sequences were fused at the C terminus to an

FIG. 5. Localization of BFRF3 to the capsid assembly sites requires both BcLF1 and BdRF1. Sf21 cells were infected with the virus expressing BFRF3-EGFP. In EBV-ALL infections, the viruses expressing BcLF1, BdRF1, BORF1, and BDLF1 were also added to the cells (EBV-ALL). In similar infections, the virus expressing the major capsid protein (–BcLF1), scaffold protein (–BdRF1), triplex 1 protein (–BORF1), or triplex 2 protein (–BDLF1) was left out of the infection. The cells were imaged using a confocal microscope (Zeiss LSM510) 24 h postinfection. The objective was $\times 40$. Scale bar, 50 μm ; inset scale bar, 10 μm .

HA tag sequence to monitor mutant polypeptide accumulation or to EGFP to monitor nuclear localization. The specific mutants were made to terminate in unstructured regions of BFRF3 so as to prevent secondary structure perturbation. The recombinant baculoviruses made were used to infect Sf21 cells to determine accumulation of the mutant proteins. All six proteins, whether fused to HA or GFP, were stably made and accumulated in insect cells as judged by Western blot methods using anti-HA or anti-GFP antibodies (Fig. 6B). The BFRF3-65-HA and 89-176-HA polypeptide levels are lower than those of the other mutant polypeptides. It is possible that there is

some loss of these small proteins during cell lysis after the prolonged duration of infection. In addition, multiple bands were observed for some polypeptide species, which could be a result of phosphorylation modifications that the herpesvirus small capsid proteins undergo and which has been demonstrated for BFRF3 (19). Limited proteolytic degradation of some BFRF3 polypeptides was also observed.

The HA-tagged BFRF3-expressing viruses were used in the capsid assembly assay to identify the BFRF3 assembly domain. Sf21 cells were coinfecting with the five baculoviruses expressing BcLF1, BORF1, BDLF1, BVRF2, and BdRF1 and with the

FIG. 6. The assembly domain of BFRF3 maps to the N-terminal half of the polypeptide. (A) The 176-amino-acid sequence of BFRF3 (strain B958) is shown as well as the locations (arrow) of the N-terminal truncation mutants. The C-terminal half of the polypeptide starts at amino acid 89 (gray arrow). (B) Sf21 cells were infected with the baculoviruses expressing HA- or EGFP-tagged BFRF3 mutant polypeptides. Cells were harvested 72 h after infection, and the lysates were analyzed by SDS-PAGE (17% acrylamide for HA or 12% acrylamide for EGFP) and Western blotting methods using anti-HA (α HA) or anti-GFP (α GFP) antibodies. 14 C-labeled protein standards are in lane M, and the sizes of the bands in kDa are shown to the left. (C) Sf21 cells were coinfecting with baculoviruses expressing BcLF1, BORF1, BDLF1, BdRF1, and BVRF2 and also with the BFRF3 wild-type (176) or mutant HA-tagged polypeptides. Cells were harvested 68 h after infection, and the lysates prepared were sedimented through sucrose gradients. The material at the position where capsids sediment was harvested from each gradient and examined by EM. The data shown are compiled from three independent experiments for most BFRF3 mutants. Scale bar, 100 nm.

virus expressing wild-type (176-HA) or mutant BFRF3. Capsid assembly was determined by harvesting infected cells 68 h after infection and sedimentation through sucrose gradients. The material from the position at which capsids sediment in the sucrose gradient was harvested from each gradient and analyzed by EM following negative staining (Fig. 6C). Capsids were detected for wild-type BFRF3 (176) and for BFRF3-125, BFRF3-110, and also BFRF3-88. Assembled structures were not evident for BFRF3-65 or BFRF3-89-176 gradients (data not shown). The experiments were repeated at least three times each for all mutants, with the same result. The number of cells used in each experiment for the BFRF3-65, BFRF3-88 and BFRF3-89-176 mutants was also increased (1.5 $\times$) to ensure we would not miss detecting low numbers of assembled capsids. From these experiments, it appears that the N-terminal half of BFRF3 is sufficient for capsid assembly. Using the current assembly assay, it was hard to quantitatively determine accurately the numbers of assembled capsids made by each mutant; however, only BFRF3-176 and BFRF3-125 gave capsids in quantities that were easily visualized by reflected light. For BFRF3 mutants 110 and 88, this was not the case and capsids were only evident by EM methods.

Sf21 cells were also infected with the viruses expressing EGFP tagged BFRF3. First cells were infected with the virus just expressing BFRF3. For wild-type BFRF3 (176), BFRF3-125, BFRF3-110, and BFRF3-88, the GFP signal was seen to accumulate primarily at a subnuclear structure (Fig. 7A). BFRF3-65 and BFRF3-89-176 displayed diffuse GFP distribution in the cell: that is, a distribution both cytoplasmic and nuclear but not concentrated in the subnuclear structure. For all mutants except BFRF3-89-176, fluorescence was also shown to accumulate late in infection as tiny bright puncta in the cytoplasm. These could be aggregates or higher-order multimers of BFRF3 that cannot enter the nucleus. From these data, it appears that a sequence between amino acids 65 and 88 is required to target BFRF3 to a subnuclear structure.

Because the relocation of BFRF3-GFP to virus factory sites could be seen by the addition of just the BcLF1- and BdRF1-expressing viruses, the GFP localization of the BFRF3 mutants to nuclear assembly sites was examined in the presence of just these two proteins. In cells expressing wild-type BFRF3 (176) or BFRF3-125, BFRF3-110, and BFRF3-88, the GFP signal relocated to nuclear assembly sites within 24 h after coinfection. BFRF3-65-EGFP also relocated to nuclear sites, indicating this polypeptide can still interact with major capsid protein. The mutant BFRF3-89-176 polypeptide did not relocate to nuclear assembly sites, and the fluorescence observed was diffusely distributed within the cell, similar to that seen when the mutant protein was expressed by itself in Sf21 cells. The results obtained with both wild-type and the mutant BFRF3 polypeptides were similar when the viruses expressing the triplex proteins or when the virus expressing BVRF2 was included in the coinfection (data not shown). The only difference was the reduction in the number of cells displaying nuclear puncta, presumably due to the decrease in the number of cells successfully coinfecting with all six viruses.

Assembly of EBV capsids with HA and GFP tags. The addition of the HA and EGFP tags at the C terminus of BFRF3 was used to monitor protein accumulation in infected cells and nuclear localization of the protein. Capsids did assemble when

the BFRF3 polypeptide specified a C-terminal HA tag (Fig. 6C); however, it was not clear that a large tag such as GFP (27 kDa) could be tolerated by an essential capsid protein, especially one that is smaller than the tag itself. Capsid assembly was hence examined with the BFRF3-EGFP- and BFRF3-HA-expressing viruses using sedimentation methods described above. A light-scattering band was visible in the sucrose gradients, albeit it was much weaker in intensity than the BFRF3-HA capsid band. The material from the sucrose gradient was harvested and examined by EM. Fully assembled capsids were detected with BFRF3-EGFP (Fig. 8B) and were of similar appearance to the BFRF3-HA capsids (Fig. 8A). Therefore, this fusion protein can support capsid assembly, although probably at a reduced level. Immuno-EM methods using HA- or GFP-specific antibodies were also used to analyze the BFRF3-HA and BFRF3-EGFP capsids. The results demonstrated that the tags are exposed on the surface of the capsid, as expected, and are accessible to the gold-bound antibody (data not shown).

DISCUSSION

In a recent study, icosahedral capsids of KSHV, a gamma-2-herpesvirus, were assembled in insect cells upon coexpression of six proteins using recombinant baculoviruses (31). This paper demonstrates that self-assembly of EBV can also be achieved by similar methods—the first example of self-assembly of a gamma-1-herpesvirus. Coexpression of BcLF1 (major capsid), BORF1 (triplex 1), BDLF1 (triplex 2), BdRF1 (scaffold), BVRF2 (protease), and BFRF3 (small capsid) proteins was sufficient for production of EBV angular capsids. EBV capsids can be isolated and purified from baculovirus-infected insect cells and can now be used to define structural features of this capsid as well as to determine the molecular genetics of the EBV assembly pathway.

The requirements for EBV self-assembly are similar to those found for the HSV-1 and KSHV assembly pathways. The major capsid and triplex proteins are essential for assembly; in their absence, capsid structures do not form. The importance of the scaffold protein for closure of the EBV capsid shell into an icosahedral structure was also demonstrated for EBV by TEM analysis.

The role of the EBV protease in assembly was complicated. Capsid-containing cells were more abundant when examined by thin-section TEM analysis when the protease-expressing virus was omitted from the coinfection. The protease could, by virtue of its activity and the ability to cleave the scaffold protein at its C terminus (the C-terminus domain of the scaffold is required for binding to major capsid protein), decrease the efficiency of the assembly process. This has been shown in HSV-1 by experimentally removing the C-terminal domain of the scaffold protein (21). In the presence of the protease-expressing virus, the few cells that contain capsids are harder to find by conventional EM methods, but capsids are much easier to see when concentrated in a single band by sedimentation methods. In order to isolate capsids by sucrose gradient sedimentation methods, it was always necessary to include the virus expressing the protease. When this virus was omitted, a light-scattering band in the gradient was not seen. The observation that EBV capsids can be formed in the absence of the protease

FIG. 7. Subnuclear and assembly site localization of BFRF3 mutant polypeptides. (A) Sf21 cells were infected with the baculovirus expressing EGFP-tagged BFRF mutants to determine the domains required for subnuclear localization of the BFRF3 mutant polypeptides. (B) Similar infections were performed as in panel A, but this time the viruses expressing major capsid (BcLF1) and scaffold (BdRF1) proteins were also included to investigate BFRF3 localization to assembly sites in the nucleus. In both cases, cells were imaged live by confocal microscopy at 29 h (A) and between 24 and 27 h (B) after infection. The magnification was $\times 40$. Scale bar, 50 μm .

(as judged by thin-section TEM) but could not be isolated by sedimentation methods is consistent with HSV data showing that capsids can be formed in the absence of the protease (28, 37). Purification of HSV-1 capsids was only achieved by immunoprecipitation with an antibody to major capsid protein (28). The spherical procapsid structures, which accumulate in

the absence of the protease, were shown to be sensitive to cold and sedimentation conditions (28, 37).

It is clear from this study and those on KSHV self-assembly (31) that the small capsid protein, far from being a decorative protein, is an essential component of the gammaherpesvirus capsid shell. The small capsid proteins likely perform stabiliz-

FIG. 7—Continued.

ing functions that facilitate the assembly of the capsid shell. EBV small capsid protein is essential for self-assembly, as judged by both sedimentation and ultrastructural analysis of infected cells. In this paper, the assembly domain of this protein was shown to reside in the N-terminal half of the polypeptide. The BFRF3 polypeptide containing the first 88 amino acids was sufficient to form stable capsids. The C-terminal half of BFRF3 encoded by amino acids 89 to 176 was not able to form capsids. Even though the levels of accumulation of this

polypeptide were lower than those of BFRF3-88, the EM method used is sensitive enough to discover very low concentrations of assembled structures. In three independent experiments with both mutant polypeptides, we never found any EBV capsids with BFRF3-89-176. Data from the KSHV self-assembly system have also shown that the N-terminal 86 amino acids of pORF65 can support capsid assembly, whereas the C-terminal half cannot assemble capsids (unpublished observations). Although assembled particles were readily observed

FIG. 8. Assembly of EBV capsids with EGFP-tagged BFRF3. Sf21 cells were coinfectd with viruses expressing BcLF1, BORF1, BDLF1, BdRF1, and BVRF2 and the virus expressing BFRF3-HA (A) or BFRF3-EGFP (B). Cell lysates were sedimented through sucrose gradients, the band visualized by reflected light was harvested, and the material was examined by EM. Scale bars, 100 nm.

by EM methods using the BFRF3-88 mutant polypeptide, only the 125-amino-acid polypeptide gave levels of capsids that were discernible as a light-scattering band in sucrose gradients. Thus, there is a requirement for a longer BFRF3 polypeptide for efficient self-assembly in the BEV system. The additional sequences could aid the stabilizing functions of BFRF3 or facilitate interactions with the capsid shell. The congruence of the data shows that the key functional residues of the gamma-herpesvirus small capsid proteins reside in the N-terminal region. Subsequent studies are directed toward uncovering these residues for both BFRF3 and ORF65.

The EGFP tag was used primarily to follow the localization of BFRF3 in the presence of the other capsid proteins. The results show that BFRF3 is relocalized to nuclear assembly sites only in the presence of major capsid protein and scaffold protein. These data are similar to what has been reported for HSV-1 VP26 (9, 36) and also our unpublished observations with KSHV ORF65. The scaffold protein for all these viruses specifies a nuclear localization signal (NLS) that is essential for nuclear localization of the scaffold-major capsid preassembly complex (29, 34). The small capsid protein by virtue of its ability to bind major capsid protein is also transported to these assembly sites or virus factories. The BFRF3-65 mutant polypeptide in cellular localization studies displays a diffuse distribution in insect cells and cannot support capsid assembly. Nevertheless, it relocalizes to nuclear assembly sites in the presence of major capsid and scaffold proteins. This suggests that the 65 amino acids contain the information necessary for

interaction with major capsid protein but not enough to assemble a stable structure. The distribution of BFRF3-89-176 mutant polypeptide is also diffuse in the cell, and this distribution does not change in the presence of the major capsid and scaffold proteins. This result indicates that the 89-176 region of BFRF3 does not contain binding sites for the major capsid protein. These two observations suggest that the inability of BFRF3-89-176 to assemble capsids is not due to the fact that it does not become concentrated in the nucleus.

The herpesvirus small capsid proteins, even with fluorescent tags, are small enough to diffuse through the nuclear pore complex and are thus present in both cytoplasmic and nuclear compartments of cells. HSV-1 VP26-GFP displays a diffuse cellular distribution in both transfected Vero cells (9) and baculovirus-infected Sf9 cells (unpublished observations). A similar distribution was reported for the human cytomegalovirus small capsid protein fused to GFP when expressed in the absence of other viral proteins (3). Recently it was shown that the varicella-zoster virus (VZV) small capsid protein contains an NLS, localizes to the nucleus, and in transfection experiments is able to transport the VZV major capsid protein into the nucleus. This suggests that VZV may have evolved two mechanisms to ensure nuclear translocation of major capsid protein (6).

The BFRF3-EGFP fusion protein is 45 kDa and so is still able to cross the nuclear envelope in the absence of active transport. It localizes to the nucleus of insect cells and becomes concentrated in a subnuclear structure that is likely the nucleolus, although this has not been formally shown. The mutational analysis shows that a region between amino acids 65 and 88 is at least partly responsible for this phenotype. This region contains a number of basic residues (Fig. 6A), more than any other region of this protein. Arginine-rich stretches can act as potential NLSs (1, 14, 44). The polypeptide sequence of BFRF3 does not contain a recognizable NLS, as judged by prediction software programs, although there is a sequence, KRQR, spanning amino acids 76 to 79 (Fig. 6A) that fits the consensus monopartite NLS (K-K/R-X-K/R) (7, 20). The BFRF3-65 polypeptide does not contain this sequence and does not become concentrated in the nucleus. To determine whether BFRF3 contains a functional NLS, we engineered a BFRF3-mRFP-EGFP fusion polypeptide. A recombinant baculovirus was isolated that expressed this dual-fluorescent protein and produced a larger polypeptide in infected cells that should not be able to diffuse through the nuclear pore. When Sf21 cells infected with this virus were examined by fluorescence microscopy, the protein as judged by both green and red fluorescence, was retained primarily outside of the nucleus (data not shown), but there was weak fluorescence in the subnuclear site. The normal BFRF3-EGFP fusion polypeptide became primarily concentrated in the subnuclear structure as shown in Fig. 5. One caveat to this observation is that the double fusion could affect the folding of BFRF3 and hence its ability to correctly translocate into the nucleus. Formal proof that there is an NLS in BFRF3 would be provided by fusion of the sequence between amino acids 65 and 88 to GFP and would show that the protein becomes localized to the nucleus. When the viruses expressing major capsid and scaffold proteins were added to cells, the BFRF3-mRFP-EGFP polypeptide re-

localized to the nucleus, indicating this protein is still able to interact with major capsid protein.

The nucleolar localization may or may not be important for capsid assembly. The BFRF3-65 polypeptide lacks this region and displays a diffuse distribution in cells, but it can still interact with major capsid protein and localizes to assembly sites in the nucleus. It is possible that as BFRF3 diffuses in and out of the different cellular compartments, it becomes retained in the nucleolus by some unknown mechanism. The presence of BFRF3 at this site therefore maybe coincidental. Clearly the interaction of BFRF3 with major capsid protein and localization to assembly sites are dominant over its localization to the nucleolus.

We use the metaphor of the automobile assembly line to describe the organization of viral factories. As in the case of a factory assembly line, the many components necessary for capsid formation arrive at the viral factory site, are assembled into subcomplexes, and finally are joined into a fully assembled viral capsid. We have followed the transit of one such polypeptide, BFRF3-EGFP, from the cytosol to the viral factories and into a "finished product" capsid. An important question remains as to whether the presence of virus factories is a phenotype of overexpression of these proteins in a baculovirus system. The localization of these proteins in EBV-infected cells has yet to be determined, although proteomic analysis of some of the EBV capsid proteins has classified BFRF3's localization in 293T cells as pan-nuclear (38). In HSV-1-infected cells, the small capsid protein (VP26) GFP tag is used as a reporter of capsid assembly. In cells infected with viruses that assemble capsids or capsid shells (scaffold-null mutant), VP26GFP localizes to nuclear puncta (11 and unpublished observations). However, in cells infected with viruses that cannot assemble capsids (VP5, VP19C, or VP23 null), VP26GFP fluorescence is diffuse (9; unpublished observations).

One additional result of this study was the ability to assemble capsids with EGFP-tagged BFRF3, and this could potentially be a useful application for live virus imaging. A similar tag on HSV-1 VP26 has been extensively utilized to monitor HSV-1 entry and transport of capsids within cells and neurons and mature virions during egress to the cell surface (11). Capsid assembly with BFRF3-EGFP was slightly inefficient compared with that of the HA-tagged BFRF3 polypeptide, probably due to a steric hindrance from the large EGFP tag. Nevertheless, these results could form the basis of using this type of fusion protein to follow the EBV virion during lytic replication by incorporating this technology into the EBV genome.

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ADDENDUM IN PROOF

We have fused a sequence of BFRF3 encoding amino acids 66 to 82 to the EGFP ORF. A virus expressing this fusion

protein was isolated, and when Sf21 cells were infected with this virus, the fluorescence observed was present throughout the cell. Thus, this sequence by itself cannot localize EGFP to the subnuclear site.

REFERENCES

- Adamson, W. E., D. McNab, V. G. Preston, and F. J. Rixon. 2006. Mutational analysis of the herpes simplex virus triplex protein VP19C. *J. Virol.* **80**:1537–1548.
- Addison, C., F. J. Rixon, J. W. Palfreyman, M. O'Hara, and V. G. Preston. 1984. Characterisation of a herpes simplex virus type 1 mutant which has a temperature-sensitive defect in penetration of cells and assembly of capsids. *Virology* **138**:246–259.
- Borst, E.-M., S. Mathys, M. Wagner, W. Muranyi, and M. Messerle. 2001. Genetic evidence of an essential role for cytomegalovirus small capsid protein in viral growth. *J. Virol.* **75**:1450–1458.
- Brady, G., G. J. MacArthur, and P. J. Farrell. 2007. Epstein-Barr virus and Burkitt lymphoma. *J. Clin. Pathol.* **60**:1397–1402.
- Casaday, R. J., J. R. Bailey, S. R. Kalb, E. J. Brignole, A. N. Loveland, R. J. Cotter, and W. Gibson. 2004. Assembly protein precursor (pUL80.5 homolog) of simian cytomegalovirus is phosphorylated at a glycogen synthase kinase 3 site and its downstream "priming" site: phosphorylation affects interactions of protein with itself and with major capsid protein. *J. Virol.* **78**:13501–13511.
- Chaudhuri, V., M. Sommer, J. Rajamani, L. Zerboni, and A. M. Arvin. 2008. Functions of varicella-zoster virus ORF23 capsid protein in viral replication and the pathogenesis of skin infection. *J. Virol.* **82**:10231–10246.
- Chelsky, D., R. Ralph, and G. Jonak. 1989. Sequence requirements for synthetic peptide-mediated translocation to the nucleus. *Mol. Cell. Biol.* **9**:2487–2492.
- de Jesus, O., P. R. Smith, L. C. Spender, C. Elgueta Karstegl, H. H. Niller, D. Huang, and P. J. Farrell. 2003. Updated Epstein-Barr virus (EBV) DNA sequence and analysis of a promoter for the BART (CST, BARF0) RNAs of EBV. *J. Gen. Virol.* **84**:1443–1450.
- Desai, P., J. C. Akpa, and S. Person. 2003. Residues of VP26 of herpes simplex virus type 1 that are required for its interaction with capsids. *J. Virol.* **77**:391–404.
- Desai, P., N. A. DeLuca, J. C. Glorioso, and S. Person. 1993. Mutations in herpes simplex virus type 1 genes encoding VP5 and VP23 abrogate capsid formation and cleavage of replicated DNA. *J. Virol.* **67**:1357–1364.
- Desai, P., and S. Person. 1998. Incorporation of the green fluorescent protein into the herpes simplex virus type 1 capsid. *J. Virol.* **72**:7563–7568.
- Desai, P., and S. Person. 1996. Molecular interactions between the HSV-1 capsid proteins as measured by the yeast two-hybrid system. *Virology* **220**:516–521.
- Donaghy, G., and R. Jupp. 1995. Characterization of the Epstein-Barr virus proteinase and comparison with the human cytomegalovirus proteinase. *J. Virol.* **69**:1265–1270.
- Donnelly, M., J. Verhagen, and G. Elliott. 2007. RNA binding by the herpes simplex virus type 1 nucleocytoplasmic shuttling protein UL47 is mediated by an N-terminal arginine-rich domain that also functions as its nuclear localization signal. *J. Virol.* **81**:2283–2296.
- Gao, M., L. Matusick-Kumar, W. Hurlburt, S. F. DiTusa, W. W. Newcomb, J. C. Brown, P. J. McCann III, I. Deckman, and R. J. Colonno. 1994. The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth. *J. Virol.* **68**:3702–3712.
- Gibson, W., and B. Roizman. 1972. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. *J. Virol.* **10**:1044–1052.
- Homa, F. L., and J. C. Brown. 1997. Capsid assembly and DNA packaging in herpes simplex virus. *Rev. Med. Virol.* **7**:107–122.
- Huang, E., E. M. Perkins, and P. Desai. 2007. Structural features of the scaffold interaction domain at the N terminus of the major capsid protein (VP5) of herpes simplex virus type 1. *J. Virol.* **81**:9396–9407.
- Johannsen, E., M. Luftig, M. R. Chase, S. Weicksel, E. Cahir-McFarland, D. Illanes, D. Sarracino, and E. Kieff. 2004. Proteins of purified Epstein-Barr virus. *Proc. Natl. Acad. Sci. USA* **101**:16286–16291.
- Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino acid sequence able to specify nuclear location. *Cell* **39**:499–509.
- Kennard, J., F. J. Rixon, I. M. McDougall, J. D. Tatman, and V. G. Preston. 1995. The 25 amino acid residues at the carboxy terminus of the herpes simplex virus type 1 UL26.5 protein are required for the formation of the capsid shell around the scaffold. *J. Gen. Virol.* **76**:1611–1621.
- Kut, E., and D. Rasschaert. 2004. Assembly of Marek's disease virus (MDV) capsids using recombinant baculoviruses expressing MDV capsid proteins. *J. Gen. Virol.* **85**:769–774.
- Luckow, V. A., S. C. Lee, G. F. Barry, and P. O. Olins. 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J. Virol.* **67**:4566–4579.

24. McNab, A. R., P. Desai, S. Person, L. L. Roof, D. R. Thomsen, W. W. Newcomb, J. C. Brown, and F. L. Homa. 1998. The product of the herpes simplex virus type 1 UL25 gene is required for encapsidation but not for cleavage of replicated viral DNA. *J. Virol.* **72**:1060–1070.
25. Molesworth, S. J., C. M. Lake, C. M. Borza, S. M. Turk, and L. M. Hutt-Fletcher. 2000. Epstein-Barr virus gH is essential for penetration of B cells but also plays a role in attachment of virus to epithelial cells. *J. Virol.* **74**:6324–6332.
26. Newcomb, W. W., F. L. Homa, D. R. Thomsen, F. P. Booy, B. L. Trus, A. C. Steven, J. V. Spencer, and J. C. Brown. 1996. Assembly of the herpes simplex virus capsid: characterization of intermediates observed during cell-free capsid formation. *J. Mol. Biol.* **263**:432–446.
27. Newcomb, W. W., F. L. Homa, D. R. Thomsen, B. L. Trus, N. Cheng, A. Steven, F. Booy, and J. C. Brown. 1999. Assembly of the herpes simplex virus procapsid from purified components and identification of small complexes containing the major capsid and scaffolding proteins. *J. Virol.* **73**:4239–4250.
28. Newcomb, W. W., B. L. Trus, N. Cheng, A. C. Steven, A. K. Sheaffer, D. J. Tenney, S. K. Weller, and J. C. Brown. 2000. Isolation of herpes simplex virus procapsids from cells infected with a protease-deficient mutant virus. *J. Virol.* **74**:1663–1673.
29. Nicholson, P., C. Addison, A. M. Cross, J. Kennard, V. G. Preston, and F. J. Rixon. 1994. Localization of the herpes simplex virus type 1 major capsid protein VP5 to the cell nucleus requires the abundant scaffolding protein VP22a. *J. Gen. Virol.* **75**:1091–1099.
30. Okoye, M. E., G. L. Sexton, E. Huang, J. M. McCaffery, and P. Desai. 2006. Functional analysis of the triplex proteins (VP19C and VP23) of herpes simplex virus type 1. *J. Virol.* **80**:929–940.
31. Perkins, E. M., D. Anacker, A. Davis, V. Sankar, R. F. Ambinder, and P. Desai. 2008. Small capsid protein pORF65 is essential for assembly of Kaposi's sarcoma-associated herpesvirus capsids. *J. Virol.* **82**:7201–7211.
32. Perkins, E. M., and J. M. McCaffery. 2008. Conventional and immunoelectron microscopy of mitochondria. *Methods Mol. Biol.* **372**:467–483.
33. Person, S., and P. Desai. 1998. Capsids are formed in a mutant virus blocked at the maturation site of the UL26 and UL26.5 open reading frames of herpes simplex virus type 1 but are not formed in a null mutant of UL38 (VP19C). *Virology* **242**:193–203.
34. Plafker, S. M., and W. Gibson. 1998. Cytomegalovirus assembly protein precursor and proteinase precursor contain two nuclear localization signals that mediate their own nuclear translocation and that of the major capsid protein. *J. Virol.* **72**:7722–7732.
35. Rixon, F. J. 1993. Structure and assembly of herpesviruses. *Semin. Virol.* **4**:135–144.
36. Rixon, F. J., C. Addison, A. McGregor, S. J. Macnab, P. Nicholson, V. G. Preston, and J. D. Tatman. 1996. Multiple interactions control the intracellular localization of the herpes simplex virus type 1 capsid proteins. *J. Gen. Virol.* **77**:2251–2260.
37. Rixon, F. J., and D. McNab. 1999. Packaging-competent capsids of a herpes simplex virus temperature-sensitive mutant have properties similar to those of in vitro-assembled procapsids. *J. Virol.* **73**:5714–5721.
38. Salsman, J., N. Zimmerman, T. Chen, M. Domagala, and L. Frappier. 2008. Genome-wide screen of three herpesviruses for protein subcellular localization and alteration of PML nuclear bodies. *PLoS Pathog.* **4**:e1000100.
39. Sheaffer, A. K., W. W. Newcomb, J. C. Brown, M. Gao, S. K. Weller, and D. J. Tenney. 2000. Evidence for controlled incorporation of herpes simplex virus type 1 UL26 protease into capsids. *J. Virol.* **74**:6838–6848.
40. Spencer, J. V., W. W. Newcomb, D. R. Thomsen, F. L. Homa, and J. C. Brown. 1998. Assembly of the herpes simplex virus capsid: preformed triplexes bind to the nascent capsid. *J. Virol.* **72**:3944–3951.
41. Steven, A. C., and P. G. Spear. 1996. Herpesvirus capsid assembly and envelopment, p. 312–351. *In* W. Chiu, R. Burnett, and R. Garcea (ed.), *Structural biology of viruses*. Oxford University Press, Oxford, United Kingdom.
42. Tatman, J. D., V. G. Preston, P. Nicholson, R. M. Elliott, and F. J. Rixon. 1994. Assembly of herpes simplex virus type 1 capsids using a panel of recombinant baculoviruses. *J. Gen. Virol.* **75**:1101–1113.
43. Thomsen, D. R., L. L. Roof, and F. L. Homa. 1994. Assembly of herpes simplex virus (HSV) intermediate capsids in insect cells infected with recombinant baculoviruses expressing HSV capsid proteins. *J. Virol.* **68**:2442–2457.
44. Truant, R., and B. R. Cullen. 1999. The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin β -dependent nuclear localization signals. *Mol. Cell. Biol.* **19**:1210–1217.
45. Trus, B. L., F. P. Booy, W. W. Newcomb, J. C. Brown, F. L. Homa, D. R. Thomsen, and A. C. Steven. 1996. The herpes simplex virus procapsid: structure, conformational changes upon maturation, and roles of the triplex proteins VP19c and VP23 in assembly. *J. Mol. Biol.* **263**:447–462.
46. Vetsika, E. K., and M. Callan. 2004. Infectious mononucleosis and Epstein-Barr virus. *Expert Rev. Mol. Med.* **6**:1–16.
47. Young, L. S., and P. G. Murray. 2003. Epstein-Barr virus and oncogenesis: from latent genes to tumours. *Oncogene* **22**:5108–5121.