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Distinct Domains in ORF52 Tegument Protein Mediate Essential Functions in Murine Gammaherpesvirus 68 Virion Tegumentation and Secondary Envelopment

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Epstein-Barr virus and Kaposi’s sarcoma-associated herpesvirus are etiologically associated with several types of human malignancies. However, as these two human gammaherpesviruses do not replicate efficiently in cultured cells, the morphogenesis of gammaherpesvirus virions is poorly understood. Murine gammaherpesvirus 68 (MHV-68) provides a tractable model to define common, conserved features of gammaherpesvirus biology. ORF52 of MHV-68 is conserved among gammaherpesviruses. We have previously shown that this tegument protein is essential for the envelopment and egress of viral particles and solved the crystal structure of ORF52 dimers. To more closely examine its role in virion maturation, we performed immunoelectron microscopy of MHV-68-infected cells and found that ORF52 localized to both mature, extracellular virions and immature viral particles in the cytoplasm. ORF52 consists of three α-helices followed by one β-strand. To understand the structural requirements for ORF52 function, we constructed mutants of ORF52 and examined their ability to complement an ORF52-null MHV-68 virus. Mutations in conserved residues in the N-terminal α-helix and C terminus, or deletion of the α2-helix, resulted in a loss-of-function phenotype. Furthermore, the α1-helix was crucial for the predominantly punctate cytoplasmic localization of ORF52, while the α2-helix was a key domain for ORF52 dimerization. Immunoprecipitation experiments demonstrated that ORF52 interacts with another MHV-68 tegument protein, ORF42; however, a single point mutation in R95 in the C terminus of ORF52 led to the loss of this interaction. Moreover, the homologues of MHV-68 ORF52 in Kaposi’s sarcoma-associated herpesvirus and Epstein-Barr virus complement the defect in ORF52-null MHV-68 and interact with MHV-68 ORF52. Taken together, these data uncover the relationship between the α-helical structure and the molecular basis for ORF52 function. This is the first structure-based functional domain mapping study for an essential gammaherpesvirus tegument protein.

Herpesviruses constitute an ancient virus family consisting of three subfamilies, Alpha-, Beta-, and Gammaherpesvirinae. The herpesvirus virion consists of four morphologically distinct components: the double-stranded DNA genome in the core, an icosahedral capsid shell, the outer lipid-glycoprotein envelope, and an electron-dense tegument between the capsid and the envelope. Virion morphogenesis for herpesviruses is a multi-step process that is generally classified into four distinct stages: nucleocapsid assembly in the nucleus, primary envelopment at the nuclear membrane followed by de-envelopment and egress into the cytoplasm, secondary envelopment, and egress from the cell. Most studies on herpesvirus morphogenesis have focused on alphaherpesviruses, including herpes simplex virus 1 (HSV-1) and pseudorabiesvirus (PrV). In contrast, relatively little is known about the morphogenesis of beta- and gammaherpesviruses. Productive replication (i.e., the complete lytic phase) of two human gammaherpesviruses, Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV), is limited in cultured cells. Murine gammaherpesvirus-68 (MHV-68) is closely related to KSHV and EBV (8, 20, 23); approximately 90% of MHV-68 genes have homologous counterparts in KSHV and EBV, particularly lytic genes (26). MHV-68 can establish productive infections in a variety of fibroblast and epithelial cell lines, so it provides an excellent model for investigating the basic biology of gammaherpesviruses (20). By using MHV-68 genome cloned as a bacterial artificial chromosome (BAC), the functions of MHV-68 proteins have been studied in the context of viral infection via a genetics approach (1, 4, 22, 28).

Compared with capsids and glycoproteins, little is known about the structure and composition of the virion tegument proteins. Tegument proteins are also less conserved, as a herpesvirus in each subfamily encodes a number of tegument proteins that are absent in other subfamilies. In recent years, emerging evidence has suggested that the tegument is an organized structure built through specific protein-protein interactions (7, 13, 19, 27). Furthermore, tegumentation is a key step for virion maturation, during which nascent nucleocapsids are wrapped and bud into the lumen of cytoplasmic compartments, forming a nearly complete virion within the lumen. Mature virions are then released into the extracellular space in a manner resembling exocytosis (10). Although specific tegument proteins apparently play an important role in the herpesvirus tegumentation and envelopment process, the molecular mechanisms responsible for the assembly of tegu-
ment proteins into nascent herpesvirus particles are poorly understood.

MHV-68 ORF52 encodes a tegument protein that is abundantly present in virions (5). ORF52 has no homologue in the alpha- or betaherpesviruses and thus is unique to gammaherpesviruses. By constructing an ORF52-null MHV-68/BAC (52S BAC) genome, we have previously shown that ORF52 encodes a highly expressed late protein, with an essential function after viral genome replication, viral DNA cleavage/packaging, and nucleocapsid assembly in the nucleus but prior to complete virion tegumentation and envelopment in the cytoplasm and egress of infectious virions from the cell (4). Without the ORF52 protein, cytoplasmic viral particles cannot form mature virions and be released from the cell. Partially tegumented capsids produced by the ORF52-null mutant contain all of the capsid proteins and some inner tegument proteins (e.g., ORF64 and ORF67) but fail to acquire outer tegument proteins (e.g., ORF45). In addition, ORF52 localizes in the cytoplasm to a distinct compartment that is reminiscent of the secretory pathway (4). Collectively, these findings indicate that ORF52 is essential for the morphogenesis of infectious MHV-68 particles in the cytoplasm and likely acts via the cellular secretory pathway.

The MHV-68 ORF52 gene encodes a 135-amino-acid protein. To gain insight into the mechanism of ORF52 function, we have previously solved its protein crystal structure, which contains three α-helices and one β-strand (3). Among these domains, the N-terminal α1-helix is extended as an arm in the dimer form, while the α2-helix, a very small α3-helix, and the β-strand form a hydrophobic core. There are five strictly conserved sites within ORF52. One of them (Arg95) is localized in the middle of the hydrophobic core. There are five strictly conserved sites within ORF52. In addition, ORF52 localizes in the cytoplasm to a distinct compartment that is reminiscent of the secretory pathway (4). Collectively, these findings indicate that ORF52 is essential for the morphogenesis of infectious MHV-68 particles in the cytoplasm and likely acts via the cellular secretory pathway.

In this work, we determined that MHV-68 tegument protein ORF52 associates with viral particles undergoing the tegumentation and secondary envelopment stage of virion morphogenesis. In order to further understand the molecular functions of MHV-68 ORF52, we have specifically dissected the role of ORF52 domains and critical amino acids based on structural information and identified domains important for localization and dimerization as well as interaction with another tegument protein, ORF42.

**MATERIALS AND METHODS**

**Viruses and cells.** Wild-type (WT) MHV-68 was originally obtained from the American Type Culture Collection (ATCC; VR1465), and the working stock was generated by infecting BHK-21 cells at a multiplicity of infection (MOI) of 0.02 PFU per cell. Viral titer was determined by plaque assay in BHK-21 cells as previously described (measured in PFU) (29). BHK-21 and 293T cells were cultured in complete Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The Flp-In-FRT293-FLAG ORF52 cell line was generated based on the Flp-In system manual (Invitrogen). Briefly, Flp-In-293 cells were maintained in 15 μg/ml blasticidin and 100 μg/ml zeocin. pcDNA5-FRT-TO-FLAG ORF52 plasmid and pOG44 plasmid (a 5.8-kb Flp recombinase expression vector designed for use with the Flp-In system) were cotransfected into Flp-In 293 cells with Lipofectamine 2000 transfection reagent (Invitrogen) and screened with 200 μg/ml hygromycin for cells stably expressing FLAG-ORF52.

**Plasmid construction.** To facilitate the manipulation of MHV-68 genes in the context of the viral genome, the whole genome of MHV-68 was cloned into a bacterial artificial chromosome (BAC). The construction of MHV-68 BAC plasmid (WT BAC) and the ORF52-null MHV-68 BAC plasmid (52S BAC) has been described previously (4, 28). Expression plasmids of FLAG-tagged ORF52 were generated by PCR amplification from genomic MHV-68 DNA and cloning in frame into pFLAG-CMV2 (Sigma) at BglII and XbaI sites. Hemagglutinin (HA)-tagged ORF52 was expressed using the pCMV-HA vector (Clontech). Coding sequences for full-length ORF52 and a mutant with deletion of the N-terminal 33 amino acids (aa) (N33del-ORF52) were amplified from wild-type MHV-68 BAC by PCR. Plasmids expressing the single-site mutants (R95A, L20A, E23A, N24A, L27A) and α2-helix-deleted ORF52 (Mdel-ORF52) were created using a two-step oligonucleotide-directed PCR mutagenesis method. The coding sequence for KSHV-ORF52 or EBV BLRF2 was amplified using total cellular DNA extracted from the latently infected cell line BCBL-1 or B95-8 as the template. The PCR fragments were cloned into the EcoRI and KpnI sites of pCMV-HA and verified by sequencing. The sequences of primers used for plasmid construction are available upon request.

**Production of monoclonal anti-ORF52 antibodies.** Rabbit cells were infected with MHV-68 at an MOI of 2 and collected at 48 h postinfection. Viral antigens were then provided to Epitomics, Inc. (Burlingame, CA) to generate monoclonal rabbit hybridomas against a panel of MHV-68 lytic antigens. His-tagged ORF52 was expressed in 293T cells and purified by use of a histidine-Ni column. Purified His-ORF52 protein was then used to screen hybridoma cell lines that produced anti-ORF52 antibodies. Rabbit hybridoma cells were further selected for single clones that produced high-affinity anti-ORF52 antibodies and maintained with RPMI medium supplemented with 1% 2-mercaptoethanol, 10% fetal bovine serum, and 10% Epitomics rabbit hybridoma supplement A under hypoxanthine-aminopterin-thymidine (HAT) selection. Rabbit hybridoma cells were proliferated in Epitomics hybridoma growth medium, and the antibodies were prepared in Epitomics serum-free medium.

**Immunoprecipitation and immunoblotting.** 293T cells seeded onto a 6-cm plate (0.8 × 10⁵ per plate) were transfected with 4.5 μg of total DNA by the calcium phosphate method. Thirty-six hours after transfection, cells were washed once with ice-cold phosphate-buffered saline (PBS) and then solubilized in EBC buffer (50 mM Tris-Cl [pH 7.4], 120 mM NaCl, 1% NP-40, 0.25% deoxycholic sodium, 1 mM EDTA) with protease inhibitors. Lysates were centrifuged at 13,000 rpm, 15 min × 2. Ten percent of the supernatant was used as an input control. Soluble proteins were mixed with 10 μl anti-FLAG M2 agarose (Sigma) and rotated at 4°C overnight. Beads were washed five times with EBC buffer before use. Immunocomplexes were washed five times in NETN buffer (20 mM Tris-Cl [pH 8.0], 1 mM EDTA, 0.5% NP-40, 120 mM NaCl), and supernatant was depleted. Bound proteins were recovered by boiling in SDS sample buffer for 10 min. The protein samples were separated on a 12% SDS-polyacrylamide gel, and proteins were transferred onto nitrocellulose membrane (Millipore). The nitrocellulose was blocked by boiling in SDS sample buffer for 10 min. The protein samples were separated on a 12% SDS-polyacrylamide gel, and proteins were transferred onto nitrocellulose membrane (Millipore). The nitrocellulose was blocked.
phosphate-buffered saline at 4°C for 12 h, postfixed in 1% OsO4, dehydrated, and embedded in Epon. Approximately 60- to 70-nm-thick sections were stained with 2% uranyl acetate and 0.3% lead citrate and examined at 200 kV on an FEI Tecnai 20 transmission electron microscope according to published protocols (25).

Immunogold TEM. BHK cells were seeded a day before infection with MHV-68 at 50% confluence in 10-cm plates. The cells were infected at an MOI of 3. Sample preparation and immunostaining were performed as described previously (21). Briefly, at 18 h after infection, the cells were washed twice with PBS and fixed with 2% paraformaldehyde plus 0.01% glutaraldehyde in 0.1 M phosphate buffer (PB) for 2 h at 4°C on the plate and embedded in 12% gelatin in 0.1 M PB. Small blocks were infiltrated in 2.3 M sucrose plus 20% polyvinylpyrrolidone (PVP) in PB overnight at 4°C and quickly plunged into liquid nitrogen. Sections approximately 60 nm thick were cut with a Leica UC6/FC6 ultramicrotome and picked up with 2.3 M sucrose.

Immunostaining was performed as follows: the sections were washed in PBS buffer (1% BSA and 0.15% glycine in PBS) followed by blocking in normal rabbit serum (1:20 dilution in BSA buffer) for 30 min. Subsequently, the sections were reacted for 2 h at room temperature with rabbit-derived anti-ORF52 monoclonal antibody (or BSA buffer as negative controls) and then for 1 h at room temperature with goat anti-rabbit IgG conjugated with 15-nm colloidal gold particles (Sigma). After a brief wash in PBS buffer and PB, the sections were treated with 2.5% glutaraldehyde for 5 min. Finally, the sections were stained with 2% neutral uranyl acetate and 4% uranyl acetate and sealed with methyl cellulose. The sections were examined with an FEI Tecnai Spirit operated at 80 kV.

RESULTS
ORF52 is involved in tegumentation and secondary envelopment. Our previous studies indicated that, in the absence of ORF52, although MHV-68 viral DNA replication in the nucleus was normal, virion morphogenesis and egress were affected. Statistical data showed that the proportions of capsids in the nuclei to total particles were similar in WT BAC- and 52S BAC (ORF52-null virus)-transfected cells, but an obvious block of viral particle morphogenesis was observed in the cytoplasm (4). To observe this blockage in greater detail, we collected 52S BAC- or WT BAC-transfected cells 6 days posttransfection and examined the subcellular localization of viral particles in these cells through thin-section TEM. In the cytoplasm of 52S BAC-transfected cells, large numbers of immature viral particles surrounded cytoplasmic vesicles without entry, forming a garland-like structure. Moreover, no viruses were found outside the cells (Fig. 1A). However, DNA
replication and capsid assembly in the nucleus appeared to be normal. All 3 types of intracellular capsids were identified: A capsids (empty), B capsids (containing scaffold proteins but no DNA), and C capsids (containing packaged genomic DNA) (Fig. 1B). Compared with 52S BAC-transfected cells, few immature viruses were retained in the cytoplasm in WT BAC-transfected cells. Viruses could be found inside intracytoplasmic vesicles; some virions were fixed at the point of envelopment within vesicles. An electron-dense tegument layer was found between the virus and the vesicle, suggesting active protein-protein interactions (Fig. 1C). This result, together with our previous data (4), strongly suggested that ORF52 is involved in tegumentation and/or secondary envelopment.

The subcellular localization of tegument proteins is closely related to their functions (14, 15). Previous immunofluorescence studies have shown that transfected ORF52 protein localized predominantly to distinct puncta in the cytoplasm. ORF52 partially colocalized with p115, a cytoplasmic marker for Golgi-derived compartments and vesicles in the secretory pathway (4); however, the exact localization of ORF52 in relation to vesicles involved in secondary envelopment could not be ascertained by immunofluorescence. We therefore performed immunogold TEM to acquire detailed localization data on ORF52 in wild-type MHV-68-infected cells. By using a primary anti-ORF52 monoclonal antibody, gold particles were observed on extracellular virions (Fig. 2A), confirming that ORF52 was a virion tegument protein. ORF52 was also found on the membrane of cytoplasmic vesicles (Fig. 2B), viruses that had entered vesicles (Fig. 2C), and immature viral particles (Fig. 2D), suggesting that ORF52 localizes to nascent viral particles prior to or during tegumentation and secondary envelopment. In addition, gold particles were also localized to the Golgi complex (Fig. 2D), consistent with the colocalization of ORF52 with p115 (4). No gold particle was found on negative control images (Fig. 2E and F). Quantitation and statistical analysis of the distribution of gold particles (n = 96; enumerated from samples treated with anti-ORF52 primary antibody) from 20 immuno-EM images revealed that ORF52 was localized to intracellular immature virions (43%) and extracellular virions (19%) as well as the membrane of cytoplasmic vesicles (24%), consistent with ORF52’s suggested role in tegumentation and the secondary envelopment of immature viral particles (no gold particles were found in 8 negative control images).

**ORF52 mutants display dominant negative effects.** Previously, we examined ORF52 deletion mutants, including a 33-amino acid deletion of the N-terminal α1-helix (N33del-ORF52) and a point mutation at amino acid 95 from arginine to alanine (R95A-ORF52), in complementation assays. Our findings suggested that the α1-helix and Arg95 are both essential for the function of ORF52 (2). Considering the structural independence of different domains, we hypothesized that ORF52 function may require coordination of multiple domains; thus, some mutants of ORF52 might have a dominant negative effect on wild-type ORF52. WT BAC and plasmids encoding ORF52 mutants were cotransfected into cells to examine whether these mutants affect the lytic replication and/or release of the MHV-68 virus. Four days posttransfection, supernatants were analyzed by real-time PCR to measure MHV-68 genomes in released viral particles. For the ORF52 mutants, viruses released into the supernatant were significantly reduced to approximately 17% (N33del) or 25% (R95A) compared to WT BAC transfectant, suggesting that both the N33del-ORF52 and the R95A-ORF52 mutations exerted a dominant negative effect on wild-type ORF52 expressed from the viral genome (Fig. 3A). To confirm this result, N33del-ORF52 or R95A-ORF52 was cotransfected with 52S BAC into a cell line (Flp-In-293-FLAG-ORF52) that constitutively expresses wild-type ORF52. Similarly, viruses released into the supernatant were reduced to approximately 33% (N33del) or 50% (R95A) of those of 52S BAC transfectant (Fig. 3B). The mild differences in values between these assays may be due to constitutive expression of ORF52 in the stable cell line, which may take part in virus assembly before a high level of mutant ORF52 synthesis. These results indicate that N33del-ORF52 and R95A-ORF52 display a dominant negative effect on wild-type ORF52 and that the α1-helix and Arg95 may exert independent effects on ORF52 function.

**MHV-68 ORF52 and its homologous proteins in KSHV and EBV share similar functions.** As a model of gammaherpesviruses, MHV-68 is phylogenetically related to KSHV and EBV, both of which are human pathogens. The homologues of MHV-68 ORF52 in KSHV and EBV are KSHV ORF52 (KORF52) and BLRF2, respectively. According to the amino acid alignment (Fig. 4A), KORF52 and BLRF2 share a high degree of similarity to MHV-68 ORF52. KORF52 and BLRF2 share 28% identity and 40% identity,
respectively, with MHV-68 ORF52 (12, 30). To investigate whether these two proteins possess functions similar to those of MHV-68 ORF52, we tested their ability to complement the null mutation of ORF52 in the 52S BAC MHV-68 genome. We performed a complementation assay in 293T cells by cotransfecting 52S BAC genome and KORF52 or BLRF2 expression plasmid. Both proteins rescued the virus propagation to nearly the same level as MHV-68 ORF52 (Fig. 4B). Our previous data demonstrated that MHV-68 ORF52 can self-associate as a homodimer or tetramer. To determine whether this feature was conserved among gammaherpesviruses, we performed communoprecipitation (co-IP) experiments and examined the ability of KORF52 and BLRF2 to form hybrid dimers with MHV-68 ORF52. Both KORF52 and BLRF2 interacted with MHV-68 ORF52 (Fig. 4C), possibly as a hybrid dimer. Taken together, these experiments suggest that KORF52 and BLRF2 share sequence homology that translates to a common structure and function.

The N-terminal α1-helix determines the localization of ORF52. In order to further evaluate the role of ORF52 in tegumentation and secondary envelopment, we explored functions of important domains identified in our previous ORF52 structural study (3). There are five strictly conserved sites within the MHV-68 ORF52 protein (Fig. 4A). Four of the five residues (Leu20, Glu23, Asn24, and Leu27) are located in the α1-helix. As these residues are conserved among gammaherpesviruses, we reasoned that these residues may play significant roles in ORF52 function. To explore the importance of these residues, four plasmids expressing point mutants of ORF52, HA-L20A-ORF52 (L20A), HA-E23A-ORF52 (E23A), HA-N24A-ORF52 (N24A), and HA-L27A-ORF52 (L27A), were constructed (Fig. 5A). Complementation assays were performed by cotransfecting 52S BAC and each ORF52 mutant plasmid individually into 293T cells. A single point mutation in any of these conserved sites was sufficient to abolish the function of ORF52 (Fig. 5B). Thus, all four N-terminal conserved sites (Leu20, Glu23, Asn24, and Leu27) are important for the function of ORF52. Because these sites are located within the α1-helix of ORF52, we next examined the localization of the ORF52 point mutants in the context of infection. As shown in Fig. 5C, wild-type ORF52 was distributed predominantly in a distinct perinuclear, punctate pattern in the cytoplasm. When the α1-helix was deleted, ORF52 displayed a dispersed distribution in the cytoplasm and prominent nuclear localization. Interestingly, a single point mutation altered the ORF52 protein distribution severely: L20A-ORF52 was found both in the cytoplasm and in the nucleus; N24A-ORF52 and L27A-ORF52 were still retained in the cytoplasm but in a dispersed pattern; and E23A-ORF52 retained a distinct punctate pattern in the cytoplasm, but it also showed some dispersal from the mainly perinuclear pattern of the wild-type ORF52 protein. Taken together, our data show that the α1-helix, particularly the four conserved sites (Leu20, Glu23, Asn24, and Leu27) play an important role in ORF52 protein localization.

α2-Helix contributes to the dimerization of ORF52. Our previous structural study indicated that ORF52 could form a dimer, and α2-helix appeared to be a key domain responsible for dimerization (3). Purification of His-tagged ORF52 expressed in Escherichia coli also showed that ORF52 could dimerize independently in vitro (data not shown). To evaluate the importance of the α2-helix in mediating protein dimerization, we constructed a plasmid expressing an ORF52 mutant with amino acids 48 to 69 deleted (Mdel-ORF52), as shown in Fig. 6A. Using this mutant, we performed a co-IP experiment to see if the ability to dimerize was dependent on the α2-helix. While both wild-type ORF52 and N33del-ORF52 formed dimers with wild-type ORF52, the Mdel-ORF52 failed to do so (Fig. 6B). In addition, previous studies have shown that the point mutant R95A can also form dimers (3). These results indicate that the α2-helix of ORF52 is a key domain for dimerization. Immunofluorescence assay shows that the localization of the Mdel-ORF52 mutant is similar to that of wild-type ORF52 (Fig. 6D). To evaluate the effect of ORF52 dimerization on viral proliferation, we performed a complementation assay to determine whether this mutant rescues the ORF52-null (52S BAC) virus. Mdel-ORF52 was unable to complement the dysfunction of 52S BAC (Fig. 6C). These results indicate that α2-helix is responsible for ORF52 dimerization, which, in turn, is critical to MHV-68 ORF52 function.

ORF52 interacts with ORF42, and the C-terminal conserved R95 is critical for this interaction. As the tegumentation of nascent virions is likely driven by specific protein-protein interactions, it is possible that ORF52 interacts with other viral proteins during secondary envelopment. We thus tested a panel of structural proteins for their interactions with ORF52, with an emphasis on tegument proteins. This panel included ORF19, ORF33, ORF38, ORF42, ORF63, ORF64, ORF67, ORF75a, ORF75b, ORF75c, ORF25, ORF26, ORF43, ORF62, ORF65, ORF39, and ORF53. Some of the large proteins were broken into two or several domains to achieve better expression. Overall, expression of pro-
tein or protein domains was detected from approximately 75% of the clones (data not shown). We further examined the physical interaction between ORF52 and each of these expressed proteins/protein domains by using co-IP and found that ORF52 interacts with ORF42 (Fig. 7A, lane 1). ORF42 has also been suggested to be a tegument protein of MHV-68 (2). To examine which domains of ORF52 are responsible for ORF42 interaction, we cotransfected 3FLAG-tagged ORF42 and HA-tagged ORF52 mutants into 293T cells. Under the same conditions described above, ORF42 was found to interact with Mdel-ORF52 (Fig. 7A, lane 2) and N33del-ORF52 (Fig. 7A, lane 3) but not R95A-ORF52 (Fig. 7A, lane 4). These results indicate that Arg95 was an essential site for ORF52 to interact with ORF42. According to the analysis of ORF52 quaternary structure, Arg95 is localized to the surface of the β1-strand and is highly hydrophilic. While our complementation assay results indicate that R95A point mutation affects ORF52 function (3), there is no effect on dimerization (3) or localization (Fig. 7B). Furthermore, the interaction between Mdel-ORF52 and ORF42 was severely attenuated in comparison to that observed between wild-type ORF52 and ORF42, suggesting that the C-terminal domain and the dimerization state of ORF52 affect the interaction with ORF42.

DISCUSSION

ORF52 is a tegument protein conserved in gammaherpesviruses. It exists in abundance in MHV-68 virions and plays an essential role in viral proliferation (5). However, because ORF52 has no homologue in alpha- or betaherpesviruses, the mechanisms of ORF52 function cannot be inferred from studies on known tegument proteins of alpha- or betaherpesviruses. In this report, we provide evidence of MHV-68 ORF52 involvement in virion tegumentation and secondary envelopment and gained detailed functions of different domains of ORF52 in the MHV-68 model. We show that α1-helix is crucial for the localization of ORF52. Single point mutation of the four conserved sites inside the α1-helix severely disrupted ORF52 localization. The α2-helix is indeed the key domain mediating ORF52 dimerization, and Arg95 is important for the interaction of ORF52 with another tegument protein, ORF42 (Fig. 8). To our knowledge, this is the first structure-based functional do
main mapping study for an essential gammaherpesvirus tegument protein, and it may provide a deeper insight into the process of herpesvirus assembly and egress.

The morphogenesis of herpesviruses is very complex, following an envelopment–de-envelopment–reenvelopment process (14, 16, 17). TEM imagery of MHV-68 virion morphogenesis in the cytoplasm (4) primed us to explore the function of ORF52 in tegumentation and secondary envelopment. In ORF52-null (52S BAC-transfected) cells, garland-like clusters of immature viral particles accumulated in the cytoplasm in close proximity to intracellular vesicles (Fig. 1). In agreement with our previous observations for MHV-68 infection in the absence of ORF52 (4), we noticed occasional single viral particles in the concave recess of a vesicle membrane partly surrounded by an electron-dense, tegument-like layer but no fully enveloped virions (Fig. 1A). In 52S BAC-transfected cells where a significant number of viral particles had completed primary envelopment–de-envelopment and entered the cytoplasm, although the cytoplasmic face of some vesicles was occupied by these immature virions, no concave recess was found, suggesting that these immature virions might compete for the vesicle membrane (Fig. 1A). However, without ORF52, the formation of a complete tegument and envelope could not be achieved, indicating specific vesicle compositions and/or morphologies might facilitate tegumentation and secondary envelopment in an ORF52-mediated manner. Meanwhile, in WT BAC-transfected cells, at the interacting surface of immature virions and vesicles in the cytoplasm, an electron-dense proteinaceous structure was found, suggesting that active protein-protein interactions occur during tegumentation and secondary envelopment. These interactions may contribute to the fission of fully enveloped virions into the vesicle. By immunoelectron microscopy, we detected ORF52 both within the tegument of mature virions and in this electron-dense proteinaceous material proximal to nascent viral particles (Fig. 2). A prior cryoelectron tomography study showed that the MHV-68 virion contains inner and outer tegument layers (7), thereby providing details regarding tegument interactions and suggesting a complex tegument organization in which ORF52 plays a vital role bridging tegument proteins and/or host proteins.

Previous immunofluorescence experiments have demonstrated that ectopically expressed ORF52 colocalized partially with Golgi-derived vesicles (4). In this study, immunoelectron microscopy also showed that native ORF52 expressed from viral genome is found on the Golgi complex and cytoplasmic vesicles in the context of infection (Fig. 2). Moreover, statistical analysis of ORF52 distribution revealed that, in addition to association with immature and mature virions, a significant proportion of ORF52 (24%) was localized to the membrane of cytoplasmic vesicles (see above), suggesting that these vesicles...
could be a major site for the secondary envelopment of MHV-68 virions. Collectively, these results suggest that MHV-68 virion morphogenesis may involve the Golgi complex and related components of the host secretory pathway.

Building on previous structural studies (3), we found that two disparate mutants of ORF52 had a dominant negative effect on wild-type ORF52 (Fig. 3). This suggests that different domains of ORF52 may exert distinct or independent func-

FIG 6 α2-Helix is crucial for ORF52 dimerization. (A) Schematic presentation of ORF52 and Mdel-ORF52. Mdel-ORF52 is an ORF52 mutant with the α2-helix (aa 48 to 69) deleted. (B) Mdel-ORF52 could not form dimer. 293T cells were cotransfected with FLAG-ORF52 and HA-tagged ORF52 or ORF52 mutants. After 36 h, the cells were solubilized and the lysates were immunoprecipitated with anti-FLAG beads. The samples were analyzed by Western blotting (WB) with anti-HA antibody. (C) Mdel-ORF52 could not substitute for the function of wild-type ORF52. 293T cells were cotransfected with S2sBAC and Mdel-ORF52 or empty vector. Four days after transfection, viral DNA was extracted from the supernatant and genome copy numbers were analyzed by real-time PCR. The results were representative of three independent experiments. (D) The localization of Mdel-ORF52 was normal. Vero cells on coverslips were transfected with Mdel-ORF52 plasmid by using jet-PEI. The cells were treated as described for Fig. 5C.

FIG 7 ORF52 interacts with ORF42, and amino acid R95 is crucial for this interaction. (A) 293T cells were cotransfected with 3FLAG-tagged ORF42 and HA-tagged ORF52 construct. Thirty-six hours after transfection, cell lysates were collected and immunoprecipitated with anti-FLAG beads. The samples were analyzed by Western blotting (WB) with anti-HA antibody. (B) The localization of R95A-ORF52 was normal. Vero cells on coverslips were transfected with R95A-ORF52 plasmid by using jet-PEI. The cells were treated as described for Fig. 5C.

FIG 8 Summary of ORF52 domains and their functions. The figure depicts a dimer of MHV-68 ORF52 (3). Based on its crystal structure and the results herein, the functions of ORF52 domains are summarized as follows: the N-terminal α1-helix is required for correct protein localization, the α2-helix plays a crucial role in ORF52 dimerization, and the conserved site Arg95 in the C terminus is critical for mediating ORF52 interaction with MHV-68 tegument protein ORF42.

MHV-68 ORF52 Tegument Protein
tions. We thus investigated the functions of MHV-68 ORF52 in detail by domain mapping. The results of these analyses are summarized in Fig. 8: the N-terminal α1-helix is required for the correct subcellular localization of ORF52; the central α2-helix plays a critical role in ORF52 dimerization; and the conserved site Arg95 in the C-terminal β-strand of ORF52 is essential for interaction with another MHV-68 protein, ORF42. Thus, the domains of ORF52 are relatively independent structures that mediate critical aspects of ORF52 function. For example, deleting the N-terminal 33 amino acids led to a drastic change in localization of the ORF52 protein (Fig. 5C) and abolished its function in virion assembly and egress (Fig. 5B). However, this N33del-ORF52 retained the ability to dimerize with WT ORF52 (3) and interact with ORF42 (Fig. 7A). Thus, N33del-ORF52 would compete with WT ORF52 in binding to ORF52 or ORF42, exert a dominant negative effect on WT ORF52, and as a result, drastically inhibit viral egress (Fig. 3). Similarly, the R95A mutant, although incapable of interacting with ORF42 (Fig. 7A), retained normal localization (Fig. 7B) and the ability to associate with WT ORF52 (3) and hence interfered with the normal function of WT ORF52 (Fig. 3). It is noted that four strictly conserved sites in the α1-helix are critical for the essential function of ORF52 in MHV-68 replication (Fig. 5B), thereby highlighting the importance of this domain. Several other hydrophobic residues in the α1-helix are also conserved. According to the structural model (Fig. 8), as the α1-helix extends away from the rest of the dimer, several highly hydrophobic patches will be exposed. The four strictly conserved sites and several other amino acids within the α1-helix may thus provide a hydrophobic cluster ideal for interaction with additional viral and/or cellular proteins, as suggested by yeast two-hybrid analysis of KSHV ORF52 (19, 24). Interestingly, the point mutants result in subcellular distributions different from those seen for the α1-helix deletion mutant, thereby suggesting other interacting partners for the α1-helix. However, the identity of cellular proteins interacting with ORF52 needs further exploration.

In this study, we find that in MHV-68, ORF52 interacts with another tegument protein, ORF42. Until now, little research has been performed on MHV-68 ORF42. Thus, the interaction between ORF42 and ORF52 provides a clue for studying MHV-68 virion assembly in the cytoplasm. For ORF42, we have found an interacting cellular protein involved in the secretory pathway and recycling (data not shown). Through interacting with tegument protein ORF42, ORF52 may serve as one bridge between tegument proteins and cellular proteins that facilitate secondary envelopment. By studying interacting cellular and viral partners of ORF42 and ORF52, we can work toward building a proteome to help explain the mechanism(s) of tegumentation and secondary envelopment of gammaherpesvirus virions. Considering that the lytic phase of gammaherpesvirus infection is crucial for the spread of virus from initially infected tissues to B cells or lymphatic epithelial cells (6, 11) and that the lytic phase has been implicated in tumor establishment and progression (9), the mapping of a protein interaction network involved in virion assembly and egress, particularly virus-host protein interactions, may shed light on the regulation of virion assembly and provide insights into developing therapeutic treatments in EBV- and KSHV-associated diseases.

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