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Identification of Proteins Associated with Murine Gammaherpesvirus 68 Virions

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Murine gammaherpesvirus 68 (MHV68 [also known as γHV-68]) is distinguished by its ability to replicate to high titers in cultured cells, making it an excellent candidate for studying gammaherpesvirus virion composition. Extracellular MHV68 virions were isolated, and abundant virion-associated proteins were identified by mass spectrometry. Five nucleocapsid protein homologues, the tegument protein homologue encoded by open reading frame (ORF) 75c, and envelope glycoproteins B and H were detected. In addition, gene products from MHV68 ORF20, ORF24, ORF28, ORF45, ORF48, and ORF52 were identified in association with virions, suggesting that these gammaherpesvirus genes are involved in the early phase of infection or virion assembly and egress.

The herpesvirus virion is composed of an icosahedral nucleocapsid surrounded by a proteinacious layer of tegument, which in turn is enclosed by a glycoprotein-containing lipid envelope (50). The structure and protein composition of the nucleocapsid have been shown to be conserved among the three subfamilies (α−, β−, and γ−) of herpesviruses (11, 14, 62–64, 72, 74). The icosahedral nucleocapsid contains at least four integral structural proteins (the major capsid protein, triplex-1 protein, triplex-2 protein, and small capsid protein) surrounding a core of viral DNA (11, 14, 27, 42, 56, 62, 72, 76). The other components of the virion, the envelope and the tegument in particular, are less well understood (38). The envelope contains viral glycoproteins critical for virion binding, entry, and signaling upon infection of a host cell (4, 15, 26, 34, 55, 67). The tegument is the electron-dense component of the virion surrounding the capsid and interacting with the envelope (14, 38, 75). While the tegument component of alphaherpesviruses and betaherpesviruses is known to contain a number of gene products involved in assembly and egress of infectious virus (38) or modulation of the host cell environment upon initial infection (10, 13, 21, 25, 30, 40), little is known about the protein composition of the gammaherpesvirus tegument nor about the functions of gammaherpesvirus tegument proteins immediately after infection of the cell.

Study of the functions of tegument proteins in the two human gammaherpesviruses, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), is hampered by the lack of cell culture systems capable of supporting productive replication of these viruses. However, murine gammaherpesvirus 68 (MHV68, or γHV-68) is not constrained in this manner, replicating to high titers in conventional tissue culture systems. MHV68 is a model for studying de novo gammaherpesvirus infection and pathogenesis (16, 20, 36, 66, 73). The virus is found in wild murid rodents and is capable of infecting laboratory strains of mice (8, 39, 48). MHV68 establishes productive infection in lung epithelia and a latent infection in splenocytes, macrophages, dendritic cells, and lung epithelial cells (23, 48, 57, 61, 69).

The MHV68 virion exhibits morphological similarity to the virion organization of other gammaherpesviruses (35, 48, 59). The viral genome encodes canonical capsid, tegument, and glycoprotein homologues found in gammaherpesviruses (66). The transcriptome of predicted MHV68 genes has been studied (3, 20, 36); however, the proteins encoded by most of these genes have not yet been identified in infected cells or in association with virions. In addition, the functional roles of conserved gammaherpesvirus virion proteins can be addressed by mutagenesis of the corresponding viral genes (2). These features make MHV68 an excellent model for studying gammaherpesvirus virion structure, composition, and assembly. However, these studies cannot proceed without a systematic identification of the viral proteins associated with virion particles. Therefore, we set out to identify and characterize proteins associated with the MHV68 virion.

Purification of extracellular MHV68. To obtain extracellular MHV-68 virions, 293T or NIH 3T3 cells were infected with wild-type MHV68 at a multiplicity of infection of 0.1. Supernatants were collected when cultures exhibited 90% cytopathic effect and were cleared of large cellular debris twice by centrifugation (1,000 × *g*, 15 min, 4°C). Extracellular virus was pelleted by ultracentrifugation through a 5% sucrose cushion

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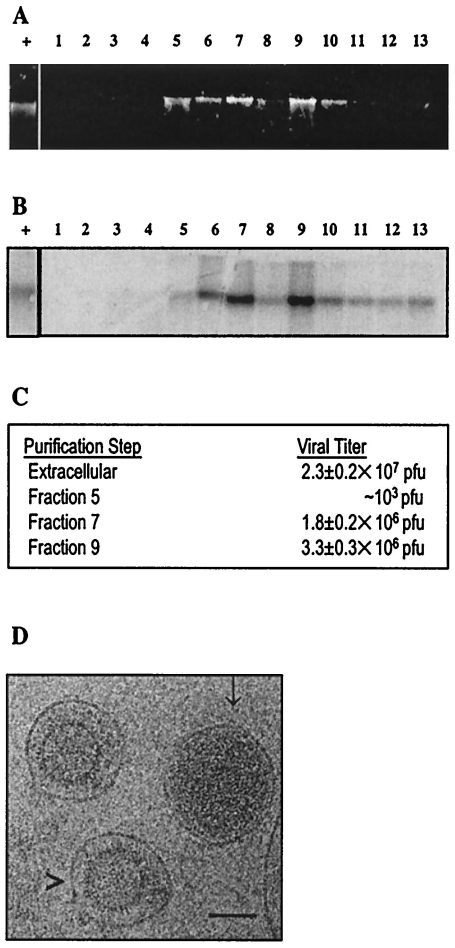

FIG. 1. Purification of extracellular MHV68. (A) Nucleic acids extracted from MHV68 sucrose gradient fractions. Extracellular virus was purified by 5 to 55% sucrose density gradient ultracentrifugation. Numbers shown on top of the panel are fractions collected from top (1) to bottom (13) of the gradient. Nucleic acids were extracted and electrophoretically separated in a 0.75% agarose–Tris-acetate-EDTA gel. (B) Southern blot analysis of extracellular virus. DNA shown in panel A was transferred to a positively charged nylon membrane and probed with random-primed [α^{32}P]dCTP-labeled virus-specific probe (a 760-bp PCR product of ORF67). + is intact viral genomic DNA. (C) Infectivity of sucrose gradient-purified virus. BHK cell monolayers infected with fractions 5, 7, or 9 were incubated in methylcellulose overlay medium at 37°C in 5% CO_2 for 5 days, and numbers of PFU were calculated. Plaque assays were performed twice. (D) Electron cryomicrograph of MHV68 virions and enveloped capsid particles. MHV68 particles from fraction 9 were embedded in vitreous ice and recorded at 100 kV on a JEOL JEM1200 electron cryomicroscope at magnification ×30,000 at a dosage of 6 electrons/angstrom^2. A representative image is shown, with putative virions (with DNA) indicated by an arrow (↓) and noninfectious enveloped particles (no DNA) indicated by an arrowhead (>). Bar, 100 nm.

(65,000 × *g*, 1 h, 4°C). The pellet was resuspended in 50 mM Tris (pH 7.5)–5 mM $MnCl_2$ and digested with 0.03 U of DNase I (Invitrogen)/μl at 37°C for 30 min. Virus was then purified by 5 to 55% discontinuous sucrose density gradient ultracentrifugation (25,000 × *g*, 4.5 h, 4°C) in an SW41Ti rotor (Beckman). Thirteen fractions were isolated from top to bottom of the sucrose gradient. Three fractions (fractions 5, 7, and 9) contained visible bands of material. Nucleic acids extracted from the gradient fractions (Fig. 1A) were tested for viral DNA by Southern blotting with a PCR-generated probe to ORF67 in the MHV-68 genome (Fig. 1B). Fractions 5 through 13 contained elevated signals. Fractions 7 and 9 contained the highest concentrations of viral DNA and were examined for the presence of infectious virus by plaque assay (Fig. 1C). Extracellular virus input of $2.3 \times 10^7 \pm 0.2 \times 10^7$ PFU showed an approximately twofold loss of infectivity during purification. Fraction 7 contained on average $1.8 \times 10^6 \pm 0.2 \times 10^6$ PFU, fraction 9 contained $3.3 \times 10^6 \pm 0.3 \times 10^6$ PFU, while fraction 5 contained approximately 10^3 PFU of infectious virus. An aggregated pellet at the bottom of the ultracentrifuge tube contained $8.0 \times 10^6 \pm 2.4 \times 10^6$ PFU. These results are similar for virus isolated from both NIH 3T3-infected and 293T-infected cell media (data not shown). Infectivity was directly proportional to viral DNA content in fractions 5, 7, and 9. This indicates that extracellular virus is concentrated in fractions 7 and 9. Fractions 5, 7, and 9 were pelleted for further study of virus particle and protein content.

MHV68 virion morphology. Extracellular MHV68 particle morphology was studied by electron cryomicroscopy, which reveals the intact forms of the viral particles by transmission projection without staining or dehydration (14, 63, 75). Two predominant morphologies of particles are present in fractions 7 and 9 (Fig. 1D). Enveloped icosahedral capsids devoid of visible viral DNA and containing only a low-density tegument region (i.e., noninfectious enveloped particles) were present with denser enveloped, tegumented nucleocapsids with characteristic herpesvirus virion morphology (14, 50, 75), including the "fingerprint" pattern of close-packed double-stranded DNA (9, 74). While these particles were present in approximately equal ratios in fraction 7, fraction 9 contained predominantly virions. A small number of heterogeneous particles were also present, including naked capsids, which most likely resulted from the loss of the viral envelope from virions or noninfectious enveloped particles during purification. The existence of two or more kinds of enveloped extracellular particles has been documented for other herpesviruses, including human cytomegalovirus, herpes simplex virus type 1 (HSV-1), and pseudorabies virus (5, 29, 37).

Virion-associated fractions contain MHV68 virion proteins. Proteins in fractions 7 and 9 were examined for the presence of MHV-68 structural protein homologues and envelope glycoproteins. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting, using polyclonal antisera raised in rabbits against bacterially expressed ORF26 (triplex-2/capsid protein homologue) (66), ORF65/M9 (small capsid protein homologue) (42, 66), or virion-associated glycoprotein-150, a product of the M7 gene (59). ORF26, ORF65, and gp150 antigens were found in both fractions (Fig. 2A) at molecular masses of 39, 26, and 130 to 150 kDa, respectively. The presence of MHV68 capsid antigens and virion-associated glycoprotein is in accordance with the electron cryomicroscopy observation revealing enveloped viral particles in both fractions 7 and 9 (Fig. 1D). In contrast, fraction 5 showed only minimal levels of the three antigens (data not shown). No signal was detected upon reprobing the blot with monoclonal antibody against actin (Sigma), excluding copurification of the abundant 42-kDa

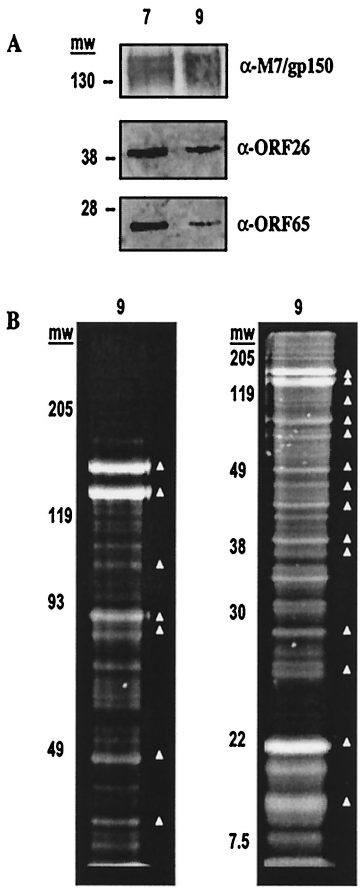

FIG. 2. Fractions 7 and 9 contain virion antigens. (A) Western blot using polyclonal antiserum raised in rabbits against bacterially expressed and purified viral capsid proteins (ORF26, middle panel, and ORF65, lower panel) and envelope protein (gp150, upper panel). (B) Proteins from fraction 9 are separated on SDS–8% (left) or 15% (right) PAGE and stained with SYPRO-Ruby (Molecular Probes). Bands were excised, digested in sequence-grade modified trypsin, and analyzed by liquid chromatography with tandem mass spectrometry. Proteins matching the MHV68 proteome (△) correspond to Table 2 from high to low apparent molecular weight (mw); cellular protein matches and unidentified bands are not marked.

cellular form of this protein with the virion-associated fractions (not shown).

Identification of abundant virion-associated proteins by mass spectrometry. Abundant MHV68 proteins were further analyzed in the virion-associated fractions by mass spectrometry. Fraction 9, which contains high levels of viral DNA, virion antigens, and infectious virus, was selected for proteomic analysis. Bands containing proteins were excised individually from a denaturing SDS-polyacrylamide gel (Fig. 2B) and digested in-gel with sequence-grade modified trypsin (Promega), and peptides were extracted for analysis by micro-liquid chromatography with tandem mass spectrometry (LCMSMS) using an ion-trap mass spectrometer (LCQ-DECA; ThermoFinnegan, San Jose, Calif.) (54, 70). Fragment ionization was performed on abundant peptides in each sample. LCMSMS-generated peptide mass and sequence tag data were collected using Excalibur software and then matched to the predicted MHV68 proteome using the program Sequest (Table 1). Of 23 prominent protein bands excised from the gel (Fig. 2B), 14 contained peptides positively identifying proteins in the viral genome (Table 2). Proteins predicted to be structural components of the gammaherpesvirus capsid, tegument, and envelope as well as several putative novel virion-associated proteins were identified.

Homologues of capsid proteins. Five protein homologues to the capsid proteins of other gammaherpesviruses were identified (1, 42, 66), including ORF26 (triplex-2/capsid), ORF62 (triplex-1/DNA maturation/capsid), ORF25 (major capsid protein), ORF65/M9 (small capsid protein), and ORF29 (DNA packaging protein). Capsid proteins were detected in protein bands approximately corresponding to the predicted molecular masses of the polypeptides. For example, the major capsid protein encoded by ORF25 (predicted mass, 153.2 kDa) was found at approximately 160 kDa. Detection of peptides matching the major gammaherpesvirus capsid protein homologues by LCMSMS and detection of capsid proteins by Western blotting at similar molecular masses, confirmed for ORF26 (detected at 39 kDa) and ORF65 (detected at 26 kDa) in Fig. 2A, validated the efficacy of mass spectrometry for identifying other proteins associated with the MHV68 virion.

Tegument protein homologue. One tegument protein homologue was identified in the virion-associated fraction. Peptides matching ORF75c, one of three KSHV ORF75/FGARAT homologues in the MHV68 genome (51, 66), were detected in a band close to the predicted mass of the full-length protein (145.7 kDa). ORF75c possibly represents the most abundantly packaged full-length ORF75 homologue in the MHV68 virion, since ORF75c is the most highly expressed of the three ORF75 homologues in MHV68 (20). A herpesvirus saimiri ORF75 homologue, the gene 75/EILF1 protein, is a virion protein (12).

Homologues of envelope proteins. The MHV68 genome also encodes a number of genes highly homologous to conserved gammaherpesvirus glycoprotein genes thought to be associated with the virion envelope (1, 6, 41, 43, 58, 66). These include ORF8 (glycoprotein B) and ORF22 (glycoprotein H). A band excised from the SDS-polyacrylamide gel at approximately 88 kDa showed peptide matches to ORF8. KSHV gB is a virion envelope-associated protein (6) implicated in integrin-receptor-mediated signaling during virus entry (4). Previous study of MHV68 virions did not detect glycoprotein B as a virion-associated protein (58), although notably, the purification protocols used in this study and the previous study contain significant differences. Peptides matching ORF22 (glycoprotein H) were detected at a molecular mass of 105 kDa. Glycoprotein H is a virion-associated glycoprotein present in HSV-1 virions and is essential for infectivity (24, 26). The identification of putative MHV68 tegument and envelope homologues in the virion-associated fraction demonstrates that some proteins localized to these virion compartments exist in sufficient quantity to detect and identify by LCMSMS.

Novel virion-associated proteins. Several predicted MHV68 gene products identified as virion-associated proteins have not been previously identified in the virions of gammaherpesviruses. These include ORF20, ORF24, ORF28, and ORF48 (Table 2). In addition, two unannotated proteins whose homologues have been suggested to be virion-associated proteins in other gammaherpesviruses, ORF45 (77) and ORF52 (53), were identified. ORF20, containing a predicted N-terminal

TABLE 1. Peptide identification by mass spectrometry[a]

Accession no.	Gene product	Predicted function	Size (aa)	Distinct peptides identified[b]
NP_044848	ORF8	gB	849	69–80/VCGVAATGETFR 188–198/PVDGLTGNIQR 267–276/RADMRVREVK 488–497/DTLMWYELSK 638–661/TVELYSSTERKLASSVFDIESMFR
NP_044858	ORF20	Fusion protein	254	1–16/MNELGAKQLLNKLPKR VTAHLIFK 111–144/VEGSNQLRDSAKALAVLAPVGTDPCR
NP_044860	ORF22	gH	730	95–118/DSIFVTNDTIHIDSDSLFVCPVGR 374–382/FLSGVQIER 508–514/FDFSANK
NP_044862	ORF24	Unknown	717	104–133/IDTCTYVPVIYSFEQTDAHYDGMGPGKLR 253–262/LMSVCEIQLR 347–365/PSNGEMLMNLYRRIDYLPK 680–688/NSNYLSFNK
NP_044863	ORF25	Major capsid	1,373	185–203/QAPPTFILQSINDPSAGR 239–259/DYVLAVLSDAVTAVNSESVFK 325–332/NFDSFLSR 374–382/VFAIESLQR 738–752/VSFYIGDELYDNQER 1041–1054/TDELLTENILYSNR 1073–1101/ADAVGFDVSNEIASLDTAMGYSSTLIPAR
NP_044864	ORF26	Capsid	298	127–151/LDSNDVNLVFPSVVPAGLAQMGIQK 152–166/ILMYNLYSNLLAAER
NP_597855	ORF28	Unknown	75	50–58/CFFWVMYKR 50–72/CFFWVMYKRAQIMGLPAQALLSR 59–72/AQIMGLPAQALLSR
NP_044866	ORF29	DNA package	348	29–51/DANEKMVNVVSYVCSEHMEDFNK 300–339/NTKCIYHKNKTTTFQSKTHTMSDDVLIACV MTCYVMTTNK
NP_044882	ORF45	IRF7-b. hom.	206	1–9/MDPFKKPVR 1–14/MDPFKKPVRMLPIK 90–100/VSESSTSEDSD 160–177/ETQSDSSSDSSGNSHKKR 177–199/RRVQEEESSRILKTPAPISGNGK 190–199/TPAPISGNGK 190–206/TPAPISGNGKYNWPWLD
NP_044885	ORF48	Unknown	333	5–29/MDLETREGGGCQQVSILMVTSDREK 159–174/RPHTDTCVSLKTLQR 177–210/TAMLGNPETLSIWTLDDLVEDPVVFK GYESAIRR 287–306/SHDQEFYTEAFKEDLHIGLK 307–332/RLVAMVLGQNHSWLDGFLTDTIVTGK
NP_044889	ORF52	Unknown	135	5–19/KPDKTYEEMVKEVER 9–19/TYEEMVKEVER 33–48/SSGAVSSDDSILTAAK
NP_044900	ORF62	Capsid	380	49–58/RESIIVSSSR 49–66/RESIIVSSRALGAVAMR 50–58/ESIIVSSSR 59–66/ALGAVAMR 79–95/AVTEQELTSLLQSLTLR 96–120/VDVSMEETTVGASGGIGPSSQTETK 96–121/VDVSMEETTVGASGGIGPSSQTETKK 49–66/LTRVVIAMDRYGGLLGGFLR 154–162/LLTPISLTR 202–215/NLHEGLVFVGPLIK 231–246/GDTVLNESLSHGLVLK 247–266/LPVEQFMDFETTNTFHYTGR 298–307/TPIEGPEFTR 359–374/GGGLSLIEIPDFTVSR
NP_044903	ORF65	Small capsid	186	8–18/APAFHPEPHNK 30–51/DSIGKDPEEAPVPLLLHTCAVR 52–61/FYEEYKEKTR 62–71/DNTLPTLVNK
NP_044915	ORF75c	Tegument	1,310	4–30/HFAFIYFGDSQYNETEKELIEDTEAGR 21–30/ELIEDTEAGR 79–88/LPVTATPIDK 831–843/LTLAGTIFQQISK 1011–1021/TDLGLMGPGMR 1240–1248/NLEAAHYPR

[a] Supplementary table of LCMSMS peptide matches: NCBI accession number, predicted MHV68 gene product, function, and size (amino acids [aa]). Distinct matching peptides were identified in mass spectrometry experiments using Sequest. More than 1,200 peptides were detected in experiments, the top 2.5% of which matched to the predicted MHV68 proteome with X-correlation factors, $1.4 < X < 6.3$, under conditions of static modification of cysteine (+57 Da) and differential modification of methionine (+16 Da). All gene products reported include at least one match with an X value of > 1.4. Match data was curated using Excel.

[b] Position/sequence.

TABLE 2. Proteomic identification of MHV-68 virion-associated proteins[a]

Mass excised (kDa)	Accession no.	Gene product	Predicted function	Predicted mass (kDa)	No. of peptides	Most confident peptide[b]
160	NP_044863	ORF25	Major capsid protein	153.2	7	738–752/VSFYIGDELYDNQER
130	NP_044915	ORF75c	Tegument/FGARAT	145.7	6	831–843/LTLAGTIFQQISK
105	NP_044860	ORF22	Glycoprotein H	82.9	3	374–382/FLSGVQIER
88	NP_044848	ORF8	Glycoprotein B	96.6	4	638–661/TVELYSSTERKLASSVFDIESMFR
80	NP_044866	ORF29	DNA packaging	73.9	3	300–339/NTKCIYHKNKTITFQSKTHTMSDDVLIACVMTCYVMTTNK
48	NP_044882	ORF45	IRF7-binding homologue	22.4	4	1–9/MDPFKKPVR
45	NP_044858	ORF20	Fusion protein	28.3	2	111–144/VEGSNQLRDSAKALAVLAPVGTDPCRVTAHLIFK
42	NP_044900	ORF62	Triplex-1 (capsid)	42.7	8	247–266/LPVEQFMDFETTNTFHYTGR
39	NP_044864	ORF26	Triplex-2 (capsid)	33.4	2	152–166/ILMYNLYSNLLAAER
39	NP_044885	ORF48	Unknown	37.9	6	307–332/RLVAMVLGQNHSWLDGFLTDTIVTGK
38	NP_044862	ORF24	Unknown	82.9	4	104–133/IDTCTYVPVIYSFEQTDAHYDGMGPGKLR
28	NP_044882	ORF45	IRF7-binding homologue	22.4	3	190–199/TPAPISGNGK
26	NP_044889	ORF52	Unknown	14.8	2	79–95/AVTEQELTSLLQSLTLR
26	NP_044903	ORF65	Small capsid protein	19.9	4	30–51/DSIGKDPEEAPVPLLLHTCAVR
22	NP_044889	ORF52	Unknown	14.8	16	79–95/AVTEQELTSLLQSLTLR
22	NP_044903	ORF65	Small capsid protein	19.9	4	30–51/DSIGKDPEEAPVPLLLHTCAVR
15	NP_597855	ORF28	Unknown	8.5	3	59–72/AQIMGLPAQALLSR

[a] For each band excised (at approximately the molecular mass listed) and digested, peptide masses and sequence data are matched to the predicted MHV68 proteome using Sequest. Summary table of matches: NCBI accession number, predicted MHV68 gene product, function, and molecular mass. The number of matching peptides and sequence of best matching peptide to predicted gene product are shown.

[b] Position/sequence.

domain homologous to HSV UL24 gene products (1), was detected at approximately 45 kDa by SDS-PAGE. Mutation of the HSV-1 UL24 gene impairs viral replication in tissue culture and in the mouse eye (31), and the HSV-2 UL24 protein is packaged into the HSV-2 virion (28). ORF24 encodes an uncharacterized gene product, detected in the virion-associated fraction at 38 kDa. ORF24 encodes a protein with a C-terminal domain with significant homology (36% identity) to the human cytomegalovirus (HCMV) UL87 protein family, whose function is unknown (1). ORF28 encodes a predicted 8.5-kDa gene product containing a transmembrane domain, which was detected at approximately 15 kDa in the virion-associated fraction. The ORF28 protein is uncharacterized. A transcript in the intergenic region between ORF27 and ORF29 of the MHV68 genome is reported to be expressed with late kinetics and is suggested to encode ORF28 (3). Peptides matching ORF45 were found at approximately 48 and 28 kDa in fraction 9 virions. The ORF45 gene is expressed as an early-late viral transcript, and the ORF45 protein is observed as a doublet at approximately 48 and 51 kDa in infected cells (32). Peptides at approximately 39 kDa matched to ORF48, encoding a 37.9-kDa polypeptide of unknown function. KSHV ORF48 is expressed as an immediate-early transcript (79), encoding a protein with 23% sequence identity to the predicted MHV68 ORF48 protein (66) and 19% identity to the hypothetical EBV BRRF2 protein (51). Peptides matching the primary gene product encoded by ORF52 were found at 26 and 22 kDa. ORF52 is a highly expressed late transcript suggested to encode a virion protein (3, 20). ORF52 encodes a protein with unknown function, homologous to KSHV ORF52 (28% identity) and EBV BLRF2 (40% identity) (66). BLRF2 putatively encodes the p21 protein component of the EBV viral capsid antigen complex and is detected in EBV virions at 21 and 23 kDa (53). The identification of predicted viral proteins associated with the MHV68 virion indicates that these proteins may be involved in virion morphogenesis, structure, or function during initial infection of the cell.

In order to examine the possibility of cellular proteins associating with MHV68 virion fractions, LCMSMS peptide data not positively identified by matching against the predicted MHV68 proteome was used to search a database of mammalian proteins using the program Sonar MS/MS (22). Six peptides matched five cellular protein sequences with expectation values (*e*) of <0.035, and in two cases they were consistent with work on other herpesviruses. One peptide from approximately 40 kDa matched annexin I, and one matched annexin II. Annexin II has been reported to be associated with purified HCMV particles and to bind glycoprotein B (46, 47, 71). Two peptides matching a cytoplasmic β-actin homologue (CAA27369) were identified at 28 kDa in fraction 9 virions. It has been suggested that an immunologically distinct form of cytoplasmic actin is packaged into the HCMV virion (7). However, similar to the case with the HCMV-associated actin, commercially available monoclonal antibody to cytoplasmic actin (Sigma) does not recognize a protein at 28 kDa in MHV68 virions (not shown). Two more cellular proteins were identified by one match apiece: the hypothetical protein similar to BR-1 (NP_062810) at 44 kDa and the endomembrane protein MP70 (NP_542123) at 40 kDa. Mass spectrometry data for the remaining bands was of insufficient quality for positive identification. This does not preclude the existence of other viral or cellular components of the MHV68 virion. The presence of host cell proteins associated with MHV68 virions may provide insight into the pathway of virion egress, though the functional roles of these proteins, if any, are speculative.

ORF45 is a virion-associated protein. The gene product of MHV68 ORF45 was selected for further analysis as a putative virion-associated protein. We have previously identified MHV68 ORF45 as a gene important for viral replication. Inhibition of MHV68 ORF45 by RNA interference leads to a drastic reduction in the expression of lytic viral proteins and reduced production of virus progeny (32). We sought to study the association of ORF45 with MHV68 virions. Polyclonal antisera raised in rabbits against the full-length ORF45 gene product expressed in *Escherichia coli* detected a polypeptide in the virion-associated fractions at approximately 48 kDa on a

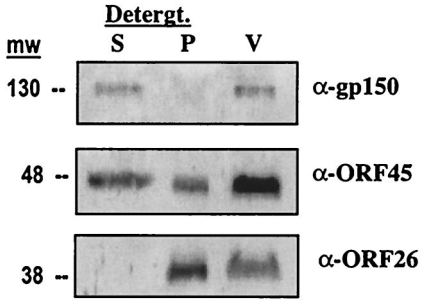

FIG. 3. ORF45 is a novel virion-associated protein partly resistant to detergent (Detergt.) treatment. Virions (lane V, approximately 2,000 PFU, fraction 9) were incubated with Triton X-100 (2%) and SDS (0.1%) for 30 min at 37°C and pelleted in a tabletop centrifuge at 23°C, 20,000 × *g* for 25 min. The supernatant (S) and pellet (P) were removed, denatured in Laemmli buffer, and separated on an SDS–15% polyacrylamide gel. Western blots were incubated with polyclonal antisera to recombinant viral proteins: glycoprotein 150 (upper panel), ORF45 (middle panel), and ORF26 (lower panel). mw, molecular weight.

Western blot (Fig. 3, lane V). This molecular weight range corresponds to one molecular weight (48,000) at which ORF45 is found by mass spectrometry analysis. Next, the sensitivity of virion-associated ORF45 to detergent was examined. Virions (2,000 PFU) from fraction 9 were treated with 2% Triton X-100, 0.01% SDS, and 22.5 mM EDTA at 37°C for 30 min, followed by 10 s of sonication and centrifugation (21,000 × *g*, 25 min, 23°C). Supernatant (detergent-sensitive) and pellet (detergent-resistant) phases were collected for analysis by Western blotting (Fig. 3, lanes S and P). The efficacy of this technique was demonstrated by solubilization of envelope protein (gp150) but not capsid protein (ORF26). ORF45 protein is partially solubilized, appearing in both detergent-sensitive and detergent-resistant phases. This observation can be reconciled by the hypothesis that an ORF45 gene product is packaged into the virion tegument. Partial sensitivity of the gene product to detergent implies that the ORF45 protein is bound to the capsid but less strongly than an integral capsid protein like ORF26. ORF45 is not as sensitive to detergent as envelope glycoprotein (gp150), which is almost completely removed by the detergent treatment. Thus, ORF45 may be associated with both the capsid and envelope as a component of the tegument. It has been recently suggested that KSHV ORF45 protein is a tegument protein (77). KSHV ORF45 protein has 33% sequence identity to MHV68 ORF45 protein and has been reported to interfere with interferon regulatory factor 7-mediated signaling (78), suggesting that the protein plays a role in modulation of innate immunity in infected cells. Experiments are under way to study the functional role of MHV68 ORF45 as a component of the virion.

The apparent molecular mass distribution and relative abundances of virion-associated polypeptides found in MHV68 (Fig. 2B) resembled that characterized in other gammaherpesviruses, including EBV (18, 19), herpesvirus saimiri (33), KSHV (42, 68), alcelaphine herpesviruses 1 and 2 (52), and murine herpesvirus 72 (49). The existence of glycoproteins with a high apparent mass and capsid proteins, particularly the major capsid protein, were observed in a number of these studies (6, 18, 19, 33, 42, 49, 68). However, the amino acid sequences of most of the virion proteins were not defined for these gammaherpesviruses. Using LCMSMS, we have identified a number of these proteins in MHV68, including homologues of capsid, tegument, and envelope proteins encoded in other gammaherpesvirus genomes (1, 6, 12, 42, 45, 51, 66). These include the structural components of the nucleocapsid identified in KSHV, the ORF29 packaging protein homologue, the putative tegument protein encoded by ORF75c, and glycoproteins B and H. We have also identified four proteins not previously predicted to be associated with gammaherpesvirus virions (ORF20, ORF24, ORF28, and ORF48), three of which have not been annotated. We also detected ORF52 protein, a homologue of a putative EBV virion protein of unknown function, and ORF45 protein in association with MHV68 virions. The identification of conserved MHV68 proteins previously not reported to be associated with the gammaherpesvirus virion, as well as cellular proteins, indicates possible functions of these proteins during the early stages of infection or in virion maturation and egress. In addition, the determinants of tissue tropism and the decision of whether to pursue a latent or lytic course of infection are not well understood. MHV68 is capable of infecting a number of tissues in the mouse, including lymphocytes, lung and intestinal epithelial cells, vascular endothelial cells, and several cell types in the brain (17, 44, 48, 60, 61, 65). Identification of virion components, particularly tegument and envelope proteins which may modulate the intracellular environment early in infection, is an essential step for understanding gammaherpesvirus pathogenesis in vivo. Determining the functional roles of these proteins in MHV68 infection awaits future studies.

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