Murine gammaherpesvirus-68 ORF28 encodes a non-essential virion glycoprotein

Janet S. May, Heather M. Coleman, Jessica M. Boname† and Philip G. Stevenson

Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

Murine gammaherpesvirus-68 (MHV-68) ORF28 is a gammaherpesvirus-specific gene of unknown function. Analysis of epitope-tagged ORF28 protein indicated that it was membrane-associated and incorporated into virions in N-glycosylated, O-glycosylated and unglycosylated forms. The extensive glycosylation of the small ORF28 extracellular domain – most forms of the protein appeared to be mainly carbohydrate by weight – suggested that a major function of ORF28 is to attach a variety of glycans to the virion surface. MHV-68 lacking ORF28 showed normal lytic replication in vitro and in vivo and normal latency establishment. MHV-68 ORF28 therefore encodes a small, membrane-bound and extensively glycosylated virion protein, whose function is entirely dispensable for normal, single-cycle host colonization.

INTRODUCTION

Herpesviruses, like all enveloped viruses, encode glycoproteins that mediate the attachment to and entry of virions into host cells. These glycoproteins are generally essential for normal lytic propagation. In contrast to simpler enveloped viruses, herpesviruses also encode accessory glycoproteins, which mediate functions such as immune evasion (Johnson et al., 1988; Lubinski et al., 1999; Bryant et al., 2003). These are generally inessential for virus replication in vitro, but play an important role in host colonization in vivo. Glycoproteins that appear to be inessential for virus replication even in vivo presumably contribute to as yet unknown aspects of the virus life cycle.

Even without knowing the biochemical functions of herpesvirus glycoproteins, it is useful to place them in a hierarchy of importance based on their contributions to virus fitness. Firstly, this tells us something about the basic requirements for host colonization; secondly, such a hierarchy can reveal key targets for therapeutic intervention and infection control. The difficulty with human viruses is that their fitness can be assessed only in vitro or in xenogenic hosts. Thus, the importance for host colonization of accessory glycoproteins, which are often species-specific in function, is not easily determined.

Species specificity is a particular problem with the analysis of gammaherpesviruses, as the known human gammaherpesviruses, Epstein–Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV), function poorly if at all in most experimental animals. We have therefore used a murid gammaherpesvirus related to KSHV, murine gammaherpesvirus-68 (MHV-68; Efstathiou et al., 1990; Virgin et al., 1997), to define a hierarchy of importance for gammaherpesvirus glycoproteins. MHV-68 infects a range of free-living rodents (Blaskovic et al., 1980; Blasdoll et al., 2003) and behaves like a natural pathogen in conventional mouse strains. Thus, it causes an acute, infectious mononucleosis-like illness without long-term sequelae in immunocompetent mice (Doherty et al., 2001), but is lethal to some immunocompromised strains (Cardin et al., 1996; Weck et al., 1997). Significantly, all known MHV-68 immune-evasion functions are preserved in murine cells (Kapadia et al., 1999; Parry et al., 2000; Boname & Stevenson, 2001).

MHV-68 encodes ten predicted membrane-associated glycoproteins. ORF4 protects against complement (Kapadia et al., 2002), ORF74 encodes a G protein-coupled receptor homologue involved in cell signalling (Lee et al., 2003; Moorman et al., 2003) and gp150 promotes virion release from infected fibroblasts (de Lima et al., 2004). These three glycoproteins all contribute to virus lytic propagation, although none is essential. MHV-68 glycoprotein B (gB), gH and gM have homologues in all known mammalian herpesviruses and are essential for infectivity (Moorman et al., 2004; J. S. May & P. G. Stevenson, unpublished data). gB (Herrold et al., 1996) and gH (Molesworth et al., 2000) are similarly essential in EBV. gl and gN are the predicted binding partners for gH and gM, respectively. The MHV-68 ORF27 is involved in intercellular virus spread (J. S. May & P. G. Stevenson, unpublished data). Here, we have addressed the importance of the MHV-68 ORF28 gene
product in virus replication in vitro and in vivo. An ORF28-derived peptide has been identified by mass spectrometry of purified MHV-68 virions (Bortz et al., 2003). However, the protein itself has not been studied, either alone or in the context of virus infection.

EBV BDLF3 (Kurilla et al., 1995; Nolan & Morgan, 1995) is a positional homologue of ORF28. Both are predicted to be membrane-associated glycoproteins, but otherwise share little sequence similarity. Notably, the MHV-68 and KSHV ORF28 genes encode much shorter polypeptides than BDLF3. EBV BDLF3 is expressed abundantly in oral hairy leukoplaekia (Peñaranda et al., 1997), suggesting that it might be involved in epithelial infection, but EBV lacking BDLF3 shows no obvious in vitro phenotype, apart from enhanced infection of epithelial cells (Borza & Hutton-Fletcher, 1998). Here, we describe a disruption of ORF28 in the MHV-68 genome and analysis of the mutant virus for its replicative fitness in vitro and in vivo. In order to study the protein, we reintroduced it, in an epitope-tagged form, into the genome of the knockout virus. This revealed that ORF28 encodes a small, membrane-associated protein that is heavily glycosylated and incorporated into virions as several different glycoforms.

METHODS

Mice, cells and viruses. Female BALB/c and C57BL/6j mice were purchased from Harlan UK Ltd and housed at the Biological Services Unit of the Cambridge University Department of Pathology. All procedures were carried out in accordance with Home Office Project Licence 80/1579. Mice were infected intranasally at 6–8 weeks of age with 2 x 10^5 p.f.u. virus in a volume of 30 μl. BHK-21 cells, NIH-3T3-CRE cells and murine embryonic fibroblasts (MEFs) were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 100 U penicillin ml^-1, 100 μg streptomycin ml^-1, 50 μM 2-mercaptoethanol and 10% fetal calf serum as described previously (Stevenson et al., 2002). All virus stocks were grown and titrated in BHK-21 cells. Cellular debris was first removed from infected-cell supernatants by pelleting at 600 g for 15 min. Virions were then recovered by high-speed centrifugation (38 000 g, 90 min), resuspended in PBS, sonicated, layered over 15–15% Ficoll gradients and centrifuged again (30 000 g, 90 min). Virions were recovered as a distinct band, resuspended in PBS, pelleted (30 000 g, 90 min), sonicated and stored at −70°C.

Virus titres. Lungs were homogenized in complete medium, frozen, thawed and sonicated. Tissue debris was pelleted by brief centrifugation (1000 g, 1 min) and infectious virus in homogenate supernatants was titrated by plaque assay on MEFs (de Lima et al., 2004). Latent virus was measured by explant culture of single-cell suspensions of spleen cells, overlaid on MEF monolayers (Coleman et al., 2003).

Virus mutagenesis. We mutated ORF28 (genomic co-ordinates 46133–46360) in a bacterial artificial chromosome (BAC)-cloned MHV-68 genome by Rec E/T-mediated recombination (Adler et al., 2000). First, a tetracycline-resistance gene (tet^R) flanked by Flp recombinase target (FRT) sites was PCR-amplified from the plasmid pCP16 by using the primers 5’-TAAAACTACCCACCATAT-TTTAAAATTGTACAAATTTTTTTTGTACGAGAAACAGCTATGACCATGA-3’ and 5’-AGAGGCTGTCGCGGCAACCCCATAT-TATCTGTGGCGCCCTGTTACATAACCCAGGTTTTCCAGTCA-CCAGCT-3’, where the underlined bases match nt 46091–46140 and 46340–46291 of the MHV-68 genome, respectively. The PCR product was electroporated into Escherichia coli JCM879 cells containing the MHV-68 BAC. Rec E/T-mediated recombination between the viral genome and the homologous 50 bp sequences at each end of the PCR product then inserted the tet^R gene into the BAC in place of genomic co-ordinates 46141–46290. DNA was extracted from chloramphenicol/tetracycline-resistant colonies, screened by restriction-enzyme digestion and electroporated into E. coli DH10B. We then removed the tet^R-coding sequence with the Flp recombinase expression plasmid pCP20, leaving a single FRT site plus short flanking plasmid sequences (166 bp in total) in place of the 150 bp genomic deletion (ORF28^-). The 166 bp insert also introduced a premature stop codon into ORF28. The ORF28^- mutant was reverted by repairing the ORF28 locus with an unmutated BamHI-C genomic clone (Efstathiou et al., 1990). The BamHI-C genomic fragment was subcloned into the pS76K-SR shuttle vector. E. coli DH10B harbouring the ORF28^- BAC were then transformed with pS76K-SR-BamHI-C, and RecA-mediated insertion of BamHI-C into the BAC was selected by standard protocols (Adler et al., 2000). After shuttle-plasmid excision, revertant BACs were identified by restriction-enzyme mapping.

An MHV-68 intergenic expression cassette was constructed by first subcloning a bovine growth hormone poly(A) signal from pcDNA3 (Invitrogen) as an SpI1 fragment into the SpI1 site of pSp73 (Promega). A promoter region upstream of the MHV-68 M3 gene (genomic co-ordinates 7281–7780) was amplified by PCR from MHV-68 genomic DNA, including BglI and EcoRI restriction sites in the respective upstream and downstream primers, and cloned into BglI and EcoRII sites of the same vector. We then PCR-cloned the ORF28 coding sequence as an EcoRI-Xhol fragment, including a flag epitope tag (DYKDDDDK) at either its 5’ or 3’ end. Each form of ORF28 was ligated into the EcoRI/XhoI sites of pSp73, between the M3 promoter and the poly(A) signal. Expression cassettes [M3 promoter–ORF28–flag–poly(A) and M3 promoter–flag–ORF28–poly(A)] were excised from pSp73 with BglI and XhoI and blunt-ended with Klenow DNA polymerase (New England Biolabs).

We subcloned a BglI genomic fragment of the MHV-68 genome (genomic co-ordinates 75338–78717) from an EcoRII/HindIII genomic clone (Efstathiou et al., 1990) into the BglI site of otherwise unmodified pSp73. This construct was cut at an MfeI site between the poly(A) signals of ORF57 and ORF58 (genomic co-ordinates 77176–78277), blunt-ended with Klenow DNA polymerase and dephosphorylated. (Pandalus borealis alkaline phosphatase; Roche Diagnostics). We ligated each blunt-ended ORF28 expression construct into the blunted MfeI site, thereby placing it between flanks suitable for recombination into the MHV-68 ORF57/ORF58 intergenic site. Each expression cassette with its recombination flanks was subcloned as a BglI fragment into the BamHI site of pS76K-SR and recombined into the MHV-68 BAC by transient RecA expression as described above. All BACs were reconstituted into infectious virus by transfecting 5 μg BAC DNA into BHK-21 cells using FuGENE 6 (Roche Diagnostics). The loxP-flanked BAC cassette was removed by viral passage through NIH-3T3-CRE cells until green fluorescent protein-positive cells were no longer visible.

Southern blotting. DNA was extracted from virions by alkaline lysis, phenol/chloroform extraction and salt/ethanol precipitation (de Lima et al., 2004). Following restriction-enzyme digestion, samples were electrophoresed on 0–8% agarose gels and transferred to positively charged nylon membranes (Roche Diagnostics). A [32P]dCTP-labelled probe (AP Biotech) was generated by random primer extension (Qbiogene) according to the manufacturer’s instructions. Membranes were hybridized with the probe (65°C, 18 h), washed at a stringency of 0–2% SSC, 0–1% SDS at 65°C and exposed to X-ray film.

J. S. May and others
Immunoblotting and immunoprecipitation. Flag epitope-tagged ORF28 was identified by using the mAb M2 (Sigma), mAbs specific for the MHV-68 gp150 (de Lima et al., 2004) and gB (Lopes et al., 2004) have been described previously. The gH/gL-specific mAb T4C5 immunoprecipitates an 85 kDa band from MHV-68-infected cells, identified as gH by mass spectrometry (data not shown). For immune precipitation, MHV-68-infected cells (5 p.f.u. per cell, 18 h) were labelled for 5–30 min with [35S]cysteine/methionine (Perkin-Elmer Life Sciences) and chased for 30–300 min with 1 mM unlabelled cysteine/methionine (Boname & Stevenson, 2001). Labelled cells were lysed on ice for 30 min in 1% Triton X-100, 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, with complete protease inhibitors (Roche Diagnostics). Insoluble debris was removed by centrifugation (13000 g, 15 min). Flag-tagged proteins were then immunoprecipitated with agarose-coupled mAb M2 (Sigma). The agarose beads were washed five times in lysis buffer and heated (95 °C, 5 min) in Laemmli’s buffer. Dissociated proteins were then resolved by Tris/Tricine SDS-PAGE (Schaegger & von Jagow, 1987) and exposed to X-ray film. In some experiments, proteins were immunoprecipitated from unlabelled virions with either agarose-coupled mAb M2 or agarose-coupled jacalin, a lectin specific for O-linked glycans (Vector Laboratories), and then immunoblotted. For immunoblotting, virus stocks or purified virions were lysed directly in Laemmli’s buffer, heated (95 °C, 5 min, or 50 °C, 10 min) with or without 2-mercaptoethanol, resolved by Tris/Tricine SDS-PAGE, transferred to PVDF membranes (Perbio Science) and immunoblotted with either the flag-specific mAb M2 or the gp150-specific mAb T4G2, each followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako). Development was with ECL reagents (AP Biotech).

Immunofluorescence. BHK-21 cells were infected overnight with MHV-68 (2 p.f.u. per cell) and then either stained directly or first fixed in 4% paraformaldehyde (20 min, room temperature) for flag epitope staining, or in methanol (5 min, −20 °C) for other mAbs. Unless stated otherwise, cells were permeabilized after fixation with 0.1% Tween 20 in PBS. Samples were blocked for 30 min in PBS/10% fetal calf serum, with or without 0.1% Tween 20. Specific staining was with the anti-flag mAb M2, the gH/gL-specific mAb T4C5, the gB-specific mAb T7H9 (Lopes et al., 2004) or the gp150-specific mAb T4G2 (de Lima et al., 2004). Detection was with Alexa 488- or Alexa 594-coupled goat anti-mouse IgG (1 h, 4 °C). Cells were washed three times in PBS/0.1% Tween 20 after each antibody incubation. Nuclear counterstaining was with DRAQ5. Fluorescence was visualized with an Olympus microscope plus Hamamatsu digital camera or with a Leica confocal microscope.
RESULTS

ORF28 disruption in the viral genome

We disrupted the MHV-68 ORF28 by replacing its central two-thirds with a 166 bp non-coding insert (Fig. 1a). This deleted the ORF28 transmembrane domain and terminated normal translation after 3 aa (Fig. 1b). In order to track the fate of the ORF28 protein in the context of viral infection, we reintroduced the ORF28 coding sequence into the ORF28-deficient BAC, but with either an N-terminal (flag-ORF28) or a C-terminal (ORF28-flag) epitope tag (Fig. 1b). Tagged ORF28 was transcribed from an ectopic MHV-68 M3 promoter (van Berkel et al., 1999), placed between the poly(A) sites of ORF57 and ORF58 (Fig. 1a). Southern blots (Fig. 1c) confirmed the predicted genomic structure of each virus.

The ORF28-deficient mutant showed no in vitro growth deficit with either high- or low-multiplicity infection (Fig. 2). Thus, ORF28 is, by implication, non-essential for binding to and entry into fibroblasts and for virion morphogenesis and release.

Identification of flag epitope-tagged ORF28 by immunofluorescence

We used the viruses expressing flag-tagged ORF28 to localize ORF28 in infected cells (Fig. 3). Both flag-ORF28 and ORF28-flag were seen predominantly on intracellular membranes (Fig. 3a) in a perinuclear distribution (Fig. 3b). A similar distribution was seen with gB and gp150 (Fig. 3b), both known components of MHV-68 virions. The epitope tag of flag-ORF28 was also detectable on intact infected

---

Fig. 2. Growth of ORF28-deficient MHV-68 *in vitro*. BHK-21 cells were infected with wild-type (WT, ■), ORF28-deficient (28−, ○) or revertant (REV, ●) viruses at a concentration of 5 or 0.01 p.f.u. per cell. Virus replication over time was measured by plaque assay on BHK-21 cells.

Fig. 3. Localization of ORF28 in infected cells. (a) BHK-21 cells were infected (2 p.f.u. per cell, 18 h) with ORF28-flag or flag-ORF28 virus or left uninfected. Cells were fixed, permeabilized and stained with the anti-flag M2 mAb. (b) BHK-21 cells were infected (0.5 p.f.u. per cell, 18 h) with ORF28-flag or flag-ORF28 virus or left uninfected. Cells were fixed, permeabilized and stained for the flag epitope (green), gB or gp150 (red). Nuclei were counterstained with DRAQ-5 (blue). (c) BHK-21 cells were infected (2 p.f.u. per cell, 18 h) with ORF28-flag (28F), flag-ORF28 (F28) or wild-type (WT) MHV-68. They were then either fixed and permeabilized (perm) or left intact (non-perm) and stained for the flag epitope. Cells were stained with secondary antibody only as a negative control and with the gH/gL-specific mAb T4C5 as a positive control.
cells (Fig. 3c), whereas that of ORF28-flag was not. ORF28 therefore reached the cell surface, presumably as a component of virions, and, despite its lack of a canonical, N-terminal signal sequence, was a type I transmembrane protein. The predicted extracellular domain of ORF28 is only 29 aa in length and is therefore likely to present only a very small target for antibody. Consistent with this prediction, we failed to identify mAbs specific for ORF28 that recognized unfixed, MHV-68-infected cells in >1500 MHV-68-specific hybridomas. We therefore used flag-tagged ORF28 for all further analyses.

Identification of ORF28 in virions by immunoblotting

Immunoblots established that both ORF28-flag and flag-ORF28 were incorporated into virions (Fig. 4a). Surprisingly, purified virions contained multiple forms of each protein: two major forms of flag-ORF28 and four major forms of ORF28-flag. RT-PCR analysis of mRNA from infected cells showed no evidence for ORF28 splicing (data not shown). Thus, the different forms of ORF28 were likely to reflect post-translational modifications. ORF28 has one consensus N-linked glycosylation site and 12 consensus O-linked glycosylation sites, so one possibility was differential glycan attachment to the ORF28 peptide backbone.

Of the four forms of ORF28-flag, the smallest (band 1) was not affected by endoglycosidase H (EH) or by protein N-glycanase F (PF) (Fig. 4b), indicating that it lacked N-linked glycans. This band was detected in virions, so flag-ORF28 was incorporated into virions independent of N-linked glycosylation. Band 2 was digested by EH and therefore had an immature N-linked glycan (Fig. 4b). The more diffuse band 3 was digested only by PF, indicating a complex N-linked glycan (Fig. 4b). The size of band 3 implied that glycan accounted for approximately 75 % of its total mass. This was not entirely N-linked glycan, as PF digestion reduced it only to the size of band 2. Note that comparison with EH digestion indicated that band 2 was digested to the level of band 1 by PF. Some of band 3 was therefore likely to be O-linked glycan.

Band 4 was affected little by either EH or PF digestion (Fig. 4b), suggesting that it had only O-linked glycans. Its very large apparent molecular mass – at least

---

**Fig. 4.** Identification of the ORF28 gene product. (a) Wild-type (WT), flag-ORF28 and ORF28-flag virions were purified on Ficoll density gradients, lysed in Laemmli’s buffer and immunoblotted for flag-tagged ORF28 with mAb M2 or for gB with mAb T7H9 as a loading control. The major flag-tagged proteins are indicated by arrowheads. (b) Virion proteins were digested with protein N-glycanase F (PF), endoglycosidase H (EH) or left undigested (nil) and then immunoblotted for the flag epitope tag. Bands corresponding to those in (a) are indicated. gB was used as a control virion protein. (c) Equivalent samples to those in (a) were electrophoresed on a 10 % Tris-buffered gel rather than a 12 % Tris/Tricine-buffered gel, primarily to resolve the ORF28-flag band 4. This now appeared as three separate PF-resistant bands, suggesting that its very high apparent molecular mass was due mainly to extensive O-linked glycosylation.
200 kDa – seemed unlikely. More likely was the possibility that a large amount of O-linked glycan made it migrate anomalously slowly during Tris/Tricine SDS-PAGE. Analysis of equivalent samples by Tris-buffered SDS-PAGE revealed several components to band 4 (Fig. 4c), with molecular masses of approximately 40, 80 and 200 kDa. These presumably corresponded to different degrees of O-linked glycosylation. None of these bands showed evidence of N-linked glycans (i.e. none shifted in size following PF digestion).

The lower band of flag-ORF28 (Fig. 4b, band 5) was not digested by EH or PF, indicating that it had no N-linked glycan. Again, this did not preclude its incorporation into virions. The upper band of flag-ORF28 (Fig. 4b, band 6) was digested by PF but not by EH, indicating a mature N-linked glycan, albeit a smaller one than was attached to ORF28-flag band 3. In Fig. 4(c), a very minor flag-ORF28 band at 40 kDa was seen to be partially digested by PF, but not by EH.

Flag-ORF28 had one more methionine residue than ORF28-flag (Fig. 1b), but this did not explain the size difference between ORF28-flag band 1 and flag-ORF28 band 5 in Fig. 4(b). Differences in post-translational processing seemed more likely. One possibility was that a small N-terminal fragment of ORF28 was normally cleaved off during protein maturation, but not in the presence of an N-terminal flag epitope tag. Alternatively, band 5 could have had an O-linked glycan that was not present in band 1. As N-glycan addition was not required for ORF28 to exit from the endoplasmic reticulum – ORF28-flag band 1 and flag-ORF28

![Fig. 5. Maturation of the ORF28 protein.](image)

(a) BHK-21 cells were infected (2 p.f.u. per cell, 18 h) with flag-ORF28, wild-type or ORF28-flag virus as indicated and then pulse-labelled for 30 min with [35S]cysteine/methionine (t=0). The cells were then chased for 5 h in the presence of excess unlabelled cysteine/methionine (t=300). Flag-tagged protein was immunoprecipitated with agarose-coupled M2 mAb. Immunoprecipitates were digested (+) or not (−) with endoglycosidase H (EH) as indicated. Bands corresponding to those in Fig. 4(a) are indicated by arrowheads. (b) Cells were infected as above with flag-ORF28 or ORF28-flag virus. They were then pulse-labelled for 15 min with [35S]cysteine/methionine and chased for 15 or 30 min as indicated before anti-flag immunoprecipitation and EH digestion. (c) Infected cells were pulse-labelled for 5 min and chased for 15 min before immunoprecipitation and EH digestion as in (b). (d) In order to test whether ORF28-flag (28F) band 4 might be aggregated protein, virions were solubilized in Laemmli’s buffer at 50°C for 10 min rather than 95°C for 5 min. This made no difference to the distribution of ORF28-flag protein. Flag-ORF28 (F28) and wild-type (WT) virions are shown for comparison. Arrowheads mark the various ORF28 gene products, as in Fig. 4(a).
band 5 were incorporated into virions (Fig. 4a, b) – the lack of an N-linked glycan on band 5 would not have precluded its acquisition of an O-linked glycan.

**ORF28 N-linked glycan maturation**

We followed the process of ORF28 glycan maturation by pulse–chase metabolic labelling and anti-flag immunoprecipitation (Fig. 5). The flag-ORF28 band 6 showed progressive acquisition of EH resistance (Fig. 5a, b), changing from mostly EH-sensitive after a 15 min pulse to mostly EH-resistant after a 30 min chase (Fig. 5b) and completely EH-resistant after a 5 h chase (Fig. 5a).

ORF28-flag showed little or no change in glycosylation from a 30 min pulse to a 5 h chase (Fig. 5a). After a 15 min pulse, there was some increase in the band 3 and band 4 forms of ORF28-flag over the next 15 min, but not after that (Fig. 5b). Thus, the glycan maturation of ORF28-flag appeared to be rapid, but limited to only a subset of the total protein. The exception to this was band 2, which was almost entirely EH-sensitive in virions (Fig. 4b), but retained a significant EH-resistant component after a 300 min chase in infected cells (Fig. 5a). The EH-resistant protein in band 2 presumably matured to the EH-resistant band 3 form prior to virion release.

The band 4 form of ORF28-flag was evident even after a 5 min label (Fig. 5c). This very rapid appearance suggested that band 4 might simply be protein that was aggregated by heating prior to SDS-PAGE. However, band 4 was not reduced in amount when ORF28-flag virions were solubilized at 50 °C rather than 95 °C prior to SDS-PAGE (Fig. 5d). Also, an equal amount of aggregation would have been expected with the flag-ORF28 protein. More likely was the possibility that it acquired O-linked glycans very rapidly.

**ORF28 O-linked glycans**

In order to identify O-linked glycans on ORF28, we immunoprecipitated virion proteins with the lectin jacalin, which binds to galactose-β1-3-N-acetylgalactosamine (Sastry et al., 1986) (Fig. 6). Whereas anti-flag mAb precipitated all of the ORF28 bands, jacalin precipitated bands 3 and 4, but not other bands of ORF28-flag, and band 5 but not band 6 of flag-ORF28 (Fig. 6). Jacalin has been reported to bind high-mannose N-linked glycans (Bourne et al., 2002), but under the immunoprecipitation conditions used, it notably failed to bind to the EH-sensitive ORF28-flag band 2. O-linked glycan on ORF28-flag band 3 provided an explanation for its partial size reduction with PF digestion (Fig. 4b). ORF28-flag band 4, which was resistant to digestion by PF (Fig. 4c), was presumably O-glycosylated to a much greater degree than band 3.

In the control samples, some anti-flag mAb was evidently carried over from the flag immunoprecipitation and detected in anti-gp150 and anti-flag immunoblots (Fig. 6). Nevertheless, it was clear that anti-flag did not precipitate gp150, whereas jacalin did, consistent with the serine/threonine-rich stalk of gp150 being O-glycosylated.

**Replication of ORF28-deficient MHV-68 in vivo**

Although ORF28 disruption did not compromise MHV-68 lytic replication in vitro, many viral functions are...
redundant in this setting. We therefore tested the capacity of ORF28-deficient MHV-68 to replicate in vivo. After intranasal infection of BALB/c mice (Fig. 7a), there was at most a small delay in lytic virus replication in the lungs. The kinetics of latency establishment and amplification were normal (Fig. 7b). In C57BL/6 mice, there was again a slight reduction in lytic replication in infected lungs in the absence of ORF28 (Fig. 7c). However, there was no deficit compared with the revertant virus. Thus, pathogenesis assays showed no evidence of either a lytic or a latent replication deficit associated with the disruption of ORF28.

DISCUSSION

Because EBV and KSHV can only be analysed readily in vitro, the importance of accessory glycoproteins in the gammaherpesvirus life cycle has remained largely speculative. MHV-68 has allowed some progress in this area, as it can reveal deficits in host colonization. Nevertheless, the MHV-68 model as it stands is far from perfect; as yet, we have no handle on in vivo virus reactivation or transmission. These are important considerations, as the best way to control persistent viral infection in a population is probably to limit the transmission from infected carriers. ORF28 was found to be completely non-essential for a single cycle of host colonization. Its function may therefore lie in the largely unexplored area of virus transmission.

ORF28 encoded a type I transmembrane glycoprotein. Its most notable feature, conserved in the EBV BDLF3 (Kurilla et al., 1995; Nolan & Morgan, 1995), was extensive glycosylation. There appeared to be interference between O-linked and N-linked ORF28 glycosylation, probably due to steric hindrance: glycosylating enzymes would have very limited access to the small extracellular domain of ORF28 once some glycan had been added. An N-terminal flag epitope tag exacerbated this interference, such that flag-ORF28 was largely either N- or O-glycosylated. Thus, jacalin precipitated flag-ORF28 band 5, but not band 6 (Fig. 6), whereas PF digested band 6, but not band 5 (Fig. 4b); jacalin also precipitated minor, higher-molecular-mass forms of flag-ORF28 (Fig. 6), which showed no evidence of N-glycosylation (Fig. 4b). There was also evidence for interference between O-linked and N-linked glycosylation of ORF28-flag: band 3 had both O-linked and N-linked glycans, but the most heavily O-glycosylated band 4 lacked N-linked glycan (Fig. 4b, c). One result of this interference was considerable heterogeneity in ORF28 glycosylation. Classically, N-linked precedes O-linked glycosylation. However, N-linked ORF28 glycosylation was clearly far from 100% efficient (Fig. 4b), leading to forms of flag-ORF28 and ORF28-flag with solely O-linked glycans. The very rapid appearance of ORF28-flag band 4 (Fig. 5c) further suggested that O-glycosylation could occur at a point very close to the time of protein synthesis. The absence of flag-ORF28 forms smaller than the apparently O-glycosylated band 5, even with a very short labelling.
window (Fig. 5c), suggested that O-linked glycan might even be added co-translationally. However, no such mechanism has yet been described, so this remains speculative. An alternative explanation is that some forms of ORF28 associated better with another O-glycosylated protein.

The ORF28 cytoplasmic tail probably interacts with the virion tegument. The 29 aa extracellular domain of ORF28 must be close to a minimal polypeptide for membrane insertion, trafficking and extensive glycosylation. It is probably too small for significant secondary structure, consistent with very rapid maturation and no requirement (Helenius & Aebi, 2001) for N-linked glycan in protein folding. The implication is that ORF28 glycosylation relates more to its function in the virus life cycle. Adding carbohydrate to the virion surface could protect the virion membrane against attack by host defences such as complement, for which the inherent diversity of ORF28 glycosylation might be important. O-linked glycans could also improve the adhesion of MHV-68 virions to some cell types. Finally, carbohydrate may help virions to resist environmental insults such as dehydration; depending on the route of natural transmission, virions may need to be physically robust.

ACKNOWLEDGEMENTS

We thank Susanna Colaco for deriving MHV-68 glycoprotein-specific mAbs. This work was funded by project grant G9901295 of MRC co-operative group G9800903 and by project grant 059601 from the Wellcome Trust. P. G. S. is an MRC/Academy of Medical Sciences Clinician Scientist (G108/462).

REFERENCES


