Evidence for a Multiprotein Gamma-2 Herpesvirus Entry Complex[†]

Laurent Gillet and Philip G. Stevenson*

Division of Virology, Department of Pathology, University of Cambridge, Cambridge, United Kingdom

Received 25 May 2007/Accepted 17 September 2007

Herpesviruses use multiple virion glycoproteins to enter cells. How these work together is not well understood: some may act separately or they may form a single complex. Murine gammaherpesvirus 68 (MHV-68) gB, gH, gL, and gp150 all participate in entry. gB and gL are involved in binding, gB and gH are conserved fusion proteins, and gp150 inhibits cell binding until glycosaminoglycans are engaged. Here we show that a gH-specific antibody coprecipitates gB and thus that gH and gB are associated in the virion membrane. A gH/gL-specific antibody also coprecipitated gB, implying a tripartite complex of gL/gH/gB, although the gH/gB association did not require gL. The association was also independent of gp150, and gp150 was not demonstrably bound to gB or gH. However, gp150 incorporation into virions was partly gL dependent, suggesting that it too contributes to a single entry complex. gp150[−] and gL[−] gp150[−] mutants bound better than the wild type to B cells and readily colonized B cells in vivo. Thus, gp150 and gL appear to be epithelial cell-adapted accessories of a core gB/gH entry complex. The cell binding revealed by gp150 disruption did not require gL and therefore seemed most likely to involve gB.

Many viruses devote just one glycoprotein to cell binding and membrane fusion. Herpesviruses devote at least three (35). For example, herpes simplex virus requires gH, gL, gB, and gD for virion infectivity (7) and for transfection-based membrane fusion (41). gH, gL, and gB of Epstein-Barr virus (EBV) or Kaposi's sarcoma-associated herpesvirus suffice for epithelial membrane fusion (15, 17, 29). Although the individual components of herpesvirus entry are well known, how they work together is not. They could act independently, be dispersed on virions and then recruited into a complex by cell binding, or form a complex from the start (16, 30). An added complication is that glycoprotein functions may be cell type specific and different entry complexes made. For example, EBV uses gp350 (40) and gp42 (42) specifically to infect B cells and makes virions with less gp42 that preferentially infect epithelial cells (5).

How virion entry proteins are deployed is important because they are prime neutralization targets. An understanding of their physical form should tell us how neutralization might best be achieved. We are using murine gammaherpesvirus 68 (MHV-68) (3, 33, 38) to define routes to gammaherpesvirus neutralization. Monoclonal antibodies (MAbs) against gH/gL (11) or gB (12) can block MHV-68 infection at a postbinding step, but neither works very well. This may reflect that sensitive neutralization epitopes are protected by associations between virion glycoproteins and revealed only after cell binding. A key initiating event in MHV-68 infection of fibroblasts and epithelial cells involves gp150 and glycosaminoglycans (GAGs) (8). The gp150-GAG interaction does not itself appear to provide significant binding. Instead it relieves a constitutive, gp150-mediated binding inhibition (14). Thus, gp150-deficient virions show little or no deficit in GAG⁺ cell infection and much

enhanced GAG[−] cell infection. EBV gp350, a gp150 homolog, analogously inhibits epithelial infection (32). The major defect of gp150-deficient MHV-68 is poor virion release, presumably because gp150[−] virions bind back to the relatively GAG-deficient surfaces of infected cells, whereas gp150⁺ virions do not (8). Consistent with this model, gp150-deficient virions bind better than the wild type to GAG-deficient CHO cells (14). The implication is that gp150 covers a key cell binding epitope on another virion glycoprotein until it is removed by GAGs and therefore that it is part of a larger entry complex. That GAGs cover a cellular ligand seems less likely, because a cellular GAG deficiency markedly reduces MHV-68 binding (14).

In contrast to some other herpesviruses (31), MHV-68 does not require gL for entry (13). gL-deficient mutants have a cell binding deficit but no obvious deficit in membrane fusion, since cell-cell spread is unimpaired (13). The conformation gH alone adopts is antigenically quite different from that of gH plus gL (11, 13), suggesting that gH/gL is an accessory cell binding module while gH alone is closer to the crucial fusion form. gM is essential (22) and it is possible that gM and gN contribute to entry. But comparison with EBV (19) would suggest that they function mainly in assembly and egress. Thus, the essential (27, 34) entry components are gB and gH. In order to understand better how MHV-68 entry works, we have addressed the following questions: whether gH and gB form a complex and whether this is influenced by gL or gp150, whether gp150 regulates gL-dependent cell binding, and whether MHV-68 remains infectious when it lacks both gL and gp150.

MATERIALS AND METHODS

Mice. Female BALB/c mice were purchased from Harlan U.K. Ltd. (Bicester, United Kingdom), housed in the Cambridge University Department of Pathology, and infected intraperitoneally (5×10^4 PFU/mouse) or intranasally (5×10^3 PFU/mouse) with MHV-68 when 6 to 8 weeks old, under Home Office Project License 80/1992.

Cell lines. BHK-21 cells, CHO-K1 cells, the GAG-deficient CHO-K1 mutant pgs745, NMuMG epithelial cells, L929 cells, 293T cells, and murine embryonic fibroblasts (MEFs) were grown in Dulbecco's modified Eagle medium (Invitrogen, Paisley, United Kingdom) supplemented with 2 mM glutamine, 100 U/ml

* Corresponding author. Mailing address: Division of Virology, Department of Pathology, Tennis Court Road, Cambridge CB2 1QP, United Kingdom. Phone: 44-1223-336921. Fax: 44-1223-336926. E-mail: pgs27@cam.ac.uk.

[†] Published ahead of print on 26 September 2007.

penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (PAA Laboratories, Linz, Austria). Medium for MEFs was further supplemented with 50 µM 2-mercaptoethanol. L929-gp150 cells were made by retroviral transduction (4). The gp150 coding sequence (genomic coordinates 69466 to 70917) was amplified by PCR (Hi Fidelity PCR kit; Roche Diagnostics Ltd., Lewes, United Kingdom) using 5' and 3' primers that incorporated EcoRI and XhoI restriction sites, respectively. The PCR product was cloned into the EcoRI and XhoI sites of pMSCV-IRES-ZEO (4) and transfected with the pEQPAM3 packaging plasmid (28) into 293T cells by use of Fugene-6 (Roche Diagnostics). Supernatants were collected 48 h and 72 h posttransfection, supplemented with 6 µg/ml Polybrene, and added to L929 cells. Transduced cells were selected with 500 µg/ml Zeocin (Invitrogen Corporation, Paisley, United Kingdom).

Viruses. Disruptions of gL (13), gp150 (8), and enhanced green fluorescent protein (eGFP)-tagged glycoprotein M (12) were combined by shuttle vector-based bacterial artificial chromosome (BAC) mutagenesis (1) and analyzed by restriction enzyme digestion and ethidium bromide staining of electrophoresed DNA fragments. Viruses were reconstituted by transfecting BAC DNA into BHK-21 cells with Fugene-6. For in vivo infections, the loxP-flanked BAC cassette was removed by virus passage through NIH-3T3-CRE cells (37). Virus stocks were grown and virus titers determined by plaque assay in BHK-21 cells (8). Infected cells and supernatants were sonicated after harvesting, cell debris was pelleted by low-speed centrifugation ($1,000 \times g$, 3 min), and virions were recovered from supernatants by high-speed centrifugation ($38,000 \times g$, 90 min). Latent virus in spleens was measured by infectious center assay (8) on MEF monolayers, which were fixed and stained for plaque counting after 5 days. Preformed infectious virus—that recovered from freeze-thawed spleen homogenates—contributed <1% of the total recoverable infectivity (data not shown), so the infectious center assay essentially measured reactivating latent virus.

Antibodies. All MHV-68-specific MAbs were derived from MHV-68-infected BALB/c mouse spleens by fusion with NS0 cells. These were as follows: for gp150, T1A1, T7F5 (14), and T4G2 (8); for gp70, 58-16D2 and T3B8 (14); for gH/gL, T2C12 and T7G7 (11); for gH only, MG-9B10, MG-4A12, MG-2E6, and MG-1A2 (13); for pan-gH, 8C1 (11); for gB, T1F7, T7H9 (21), MG-2C10, and MG-4D11 (12); for gp48, T8A11 (25) and 6D10 (23); for gN, 3F7 (22); and for ORF17, 150-7D1 (13).

Flow cytometry. Cells exposed to eGFP⁺ viruses were trypsinized, washed in phosphate-buffered saline (PBS), and analyzed directly for green fluorescence. For surface staining, cells were incubated (1 h, 4°C) with MHV-68 glycoprotein-specific MAbs followed by fluorescein-conjugated rabbit anti-mouse immunoglobulin G (IgG) polyclonal antibody (PAb) (Dako Cytomation, Ely, United Kingdom) or Alexa 633-conjugated goat anti-mouse PAb (Invitrogen) (1 h, 4°C). B cells were stained with a phycoerythrin-conjugated, CD19-specific rat MAb (BD Biosciences, Oxford, United Kingdom). The cells were washed two times in PBS and analyzed on a FACSCalibur using Cellquest software (BD Biosciences).

Immunoprecipitation and immunoblotting. MHV-68 virions were lysed (45 min, 4°C) in 1% digitonin, 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride plus Complete protease inhibitors (Roche Diagnostics). Insoluble debris was removed by centrifugation ($13,000 \times g$, 15 min). Viral glycoproteins were then precipitated with glycoprotein-specific MAbs plus protein A-Sepharose. The beads were washed five times in lysis buffer and the precipitated proteins eluted by heating (95°C, 5 min) in Laemmli's buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. The membranes were then probed with MHV-68-specific MAbs plus horseradish peroxidase-conjugated goat anti-mouse IgG2a or IgM PABs (Southern Biotech, Birmingham, AL), followed by enhanced chemiluminescence substrate development (APBiotech). Virion lysates were denatured and immunoblotted directly. In general, the amount of lysate loaded on gels was 1/20 of the amount used for immunoprecipitations.

RESULTS

Glycoproteins B and H associate in the MHV-68 virion membrane. We first tested whether gB and gH—the core components of MHV-68 entry—were associated in virions. Such an association would make functional sense, as gB and gH both play key roles in herpesvirus membrane fusion (41) but has not been clearly established for any herpesvirus. Wild-type MHV-68 virions were recovered from infected cell supernatants and lysed in 1% digitonin. gH was then immunoprecipitated

with MAb 8C1, which recognizes gH regardless of whether gL is present (13). The *H2-K^b*-specific MAb Y3 provided an isotype-matched control. The precipitates were immunoblotted for gB, gp70, gN, gp48, and gp150 (Fig. 1). gN associates with gM (22) and gp48 with ORF58 (23), so apart from ORF28 (24), these immunoblots covered the main candidate glycoprotein partners for gH and gH/gL. gH precipitation clearly enriched for gB in comparison with gp70, gN, gp150, or gp48. The abundance of coprecipitating gB contrasted with the very limited amounts of coprecipitating gp70, gN, and gp150, which probably reflected some incomplete virion lysis and were felt not to be significant.

The association between gB and gH is independent of gL, gp150, and gp70. To date we have identified two MHV-68 gH conformations: one defined by MAbs such as T7G7, which recognize gH plus gL, and one defined by MAbs such as MG-9B10, which recognize gH alone in preference to gH/gL (13). MAb 8C1 does not distinguish between these forms. Most of the gH in wild-type virions is bound to gL and therefore is 8C1 reactive and T7G7 reactive (13). Both 8C1 and T7G7 coprecipitated gB (Fig. 2A). Thus, the major form of gH on wild-type virions was associated with gB.

The paucity of MG-9B10-reactive gH on wild-type virions (13) perhaps explains why this MAb failed to coprecipitate gB. However, MG-9B10 also failed to coprecipitate gB from gL-deficient virions (Fig. 2A), which contain more MG-9B10-reactive gH (13). MAbs MG-4A12, MG-2E6, and MG-1A2, which like MG-9B10 recognize and immunoprecipitate gH alone in preference to gH/gL (13), also failed to coprecipitate gB from either wild-type or gL-deficient virions (Fig. 2B). The association between gB and gH did not depend on gL, as gB was readily coprecipitated from gL-deficient virions by MAb 8C1 (Fig. 2A). Instead, it appeared that gL-deficient virions may contain another form of gH associated with gB and recognized by MAb 8C1 but not recognized by MG-4A12, MG-2E6, MG-1A2, or MG-9B10. Although not all gH was necessarily associated with gB, there was a wild-type entry complex that included gH, gB, gL, and a gL-deficient entry complex that included gH and gB.

The gH/gB association was also independent of gp150 (gp150[−]) and gp70 (gp70[−]) (Fig. 2C). MAb 8C1 even coprecipitated gB from MHV-68 lacking both gL and gp150. For each virus, gN was abundant in lysates and sparse in precipitates, whereas gB was abundant in both. Independence from gL suggested that the gH extracellular domain made little contribution to the gB/gH association, since it is antigenically quite different between wild-type and gL-deficient virions (11, 13). And the association was much weaker in 1% Triton X-100 than in 1% digitonin (data not shown), implying that intramembrane interactions were important. The simplest interpretation would be a direct contact between gB and gH within the virion membrane.

Glycoprotein L promotes the incorporation of gp150 into virions. Although we could find no evidence by immunoprecipitation that gp150 associates with gH/gL or gB either in virions or in infected cells, immunoblots showed that virions lacking gL contained less gp150 than the wild type (Fig. 3A). gL disruption also reduced gp150 staining on infected cell surfaces (Fig. 3B). BHK-21 cells infected with gL-deficient MHV-68 displayed less surface gH, gB, and gp70 than the wild

FIG. 1. The MHV-68 gB coprecipitates with gH. MHV-68 virions were lysed in 1% digitonin. gH was immunoprecipitated (IP) with MAb 8C1 (IgG2b). The H2-K^b-specific MAb Y3 provided an isotype-matched control. The precipitates were then immunoblotted with glycoprotein-specific IgG2a MAbs plus an IgG2a-specific secondary antibody. The original lysate (lys) was immunoblotted in parallel to show the relative levels of input glycoproteins. The positions of the immunoprecipitating immunoglobulin heavy and light chains are shown (Ig-H and Ig-L, respectively). gp70 also has lower-molecular-mass forms due to posttranslational cleavage and differential O-glycosylation (14). Virion gB is predominantly cleaved from its 120-kDa full-length form into 65-kDa N-terminal (gB-N) and 55-kDa C-terminal (gB-C) fragments. The data are from one of five equivalent experiments.

type, probably due to gL⁻ virions binding poorly (13), but gp150 was reduced much more. This reduction was surprising, since transfected gp150 reaches the cell surface without other viral proteins (Fig. 3C). However, gp150 may be targeted to the trans-Golgi network in infected cells for secondary envelopment, such that it mostly reaches the cell surface on virions.

Cells infected with gp150-deficient MHV-68 showed no corresponding lack of gH/gL (Fig. 3D). Indeed, more cell surface gH/gL and gB were detected by flow cytometry, consistent with more gp150-deficient virions accumulating on the plasma membrane than seen with the wild type (8). Thus, while gp150 recruitment depended on gL, gH/gL recruitment did not depend on gp150. It is possible that gL interacted with gp150 independently of gH, but as gL binds to gH, the simplest explanation would be that gH/gL recruits gp150 to virions. This would make gp150 a fourth component of the MHV-68 entry complex, consistent with its function of regulating cell binding.

The gp150 knockout phenotype is independent of gL. The gL⁻ MHV-68 phenotype of poor binding to BHK-21 cells (13) is very different from the gp150⁻ phenotype of poor release from BHK-21 cells and normal binding (8). The reduction in gp150 content of gL-deficient virions was therefore incidental to their main phenotype. But did the gp150 knockout phenotype require gL? gp150 appears to cover a cell binding epitope

(8, 14); gL contributes to cell binding, probably as gH/gL (13); and gL appeared to recruit gp150 to virions (Fig. 3), again probably as gH/gL. It therefore seemed plausible that gp150 might cover a cell binding epitope on gH/gL. We tested this by measuring the binding to GAG-deficient cells of an MHV-68 mutant lacking both gL and gp150. In order to assay binding, the mutants were constructed on a genetic background of eGFP-tagged gM (12). gM is an abundant virion protein, so this nonattenuating modification of the endogenous gM makes virions strongly fluorescent. Glycoprotein expression by each eGFP-tagged knockout virus (Fig. 4A) was equivalent to that of the corresponding untagged knockout. Specifically, each virus expressed gp70, gL disruption precluded recognition by the gH/gL-specific MAb T2C12, gp150 disruption precluded recognition by the gp150-specific MAb T1A1, gL disruption reduced gp150 staining, and gp150 disruption increased gH/gL staining.

The gL⁻ mutant bound less well than the wild type to GAG⁺ CHO cells (Fig. 4B), consistent with published data (13). However, the gL⁻ gp150⁻ mutant showed no binding deficit. gp150 disruption therefore compensated for the binding deficit associated with gL disruption. This suggested that the reduced levels of gp150 on gL⁻ virions (Fig. 3A) might mask somewhat the gL-associated binding deficit and could

FIG. 2. The gB/gH association does not extend to all forms of gH but is independent of gL, gp150, and gp70. (A) gH was immunoprecipitated (IP) from lysates of wild-type (WT) or gL-deficient (gL⁻) virions (the gL⁻ DEL-STOP mutant) with MAbs specific for gH only (MG-9B10 [9B10]), gH/gL (T7G7), or all forms of gH (8C1). The precipitates and the original lysates were immunoblotted for gB with the IgM MAb MG-2C10, which recognizes an epitope close to its N terminus, and an IgM-specific secondary antibody. The data are from one of three equivalent experiments. (B) gH was precipitated from lysates equivalent to those shown in panel A but using a different gL knockout (gL-DEL) and three different gH-only-specific MAbs: 4A12 (MG-4A12, IgG2a), 2E6 (MG-2E6, IgG2b), and 1A2 (MG-1A2, IgG1). MAb 8C1 provided a positive control. The precipitates were immunoblotted for gB with MAb MG-2C10 plus an IgM-specific secondary antibody as in panel A. Some cross-reactivity with the precipitating immunoglobulin heavy (Ig-H) and light (Ig-L) chains is evident. The data are from one of two equivalent experiments. (C) gH was immunoprecipitated from lysates of wild-type (WT), gp70-deficient (gp70⁻), gp150-deficient (gp150⁻), and double knockout (gL⁻ gp150⁻) virions. For the last of these, two independently derived mutants are shown (mut1, mut2). The precipitates and the original lysates were then immunoblotted for the C-terminal half of gB with MAb MG-4D11 and for gN with MAb 3F7 as a control of immunoprecipitation specificity. The data are from one of two equivalent experiments.

explain our previous observation that the deficit is quite variable between virus stocks (13). The gL⁻ gp150⁻ mutant also bound almost as well as the gp150⁻ mutant to GAG⁻ CHO cells and much better than did the wild type. Thus, in this context the increase in binding from gp150 disruption was more important than the decrease in binding from gL disruption. The gp150 knockout phenotype clearly did not depend on gL.

Similar results were obtained using human cytomegalovirus IE1 promoter-driven viral eGFP expression as a readout of infection (Fig. 4C). Although MHV-68 does not form plaques on CHO cells, eGFP expression provides a sensitive measure of infection, since eGFP⁺ virus titers on CHO cells are at least equal to plaque titers on BHK-21 cells or MEFs. In this assay, viruses and cells were left together overnight. Thus, the gL knockout had time to infect GAG⁺ CHO cells at a level equivalent to wild-type virus. But the wild type was still unable to infect GAG⁻ CHO cells at a level approaching that of the gp150⁻ gL⁻ mutant. These data were consistent with gL-dependent binding being less important for CHO cell infection than gp150/GAG-revealed binding. Indeed, even the gL knockout infected GAG⁻ CHO cells better than the wild-type virus did, consistent with its lower gp150 content (Fig. 3A). The gL knockout did not show increased binding to the GAG⁻ CHO cells (Fig. 4B), possibly because this was a less sensitive assay: infection with a single virion should give eGFP expression, whereas detectable binding requires multiple virions. Also, less time was available for binding than for infection. Alternatively, the modest gp150 deficiency of the gL knockout could have enhanced infection after binding. Increased GAG-deficient cell infection by gp150-deficient MHV-68 correlates with increased binding, but gp150 could also inhibit the infection of GAG-deficient cells downstream of binding.

gp150 and gL are both dispensable for binding to B cells. MHV-68 lacking gL binds normally to NS0 myeloma cells (13), and MHV-68 lacking gp150 infects NS0 cells better than the wild type (8). Both single-gene knockouts also establish normal latency in splenic B cells. However, this could reflect some functional redundancy between gL and gp150. We therefore tested whether a lack of both might compromise MHV-68 binding to CD19⁺ splenic B cells (Fig. 5). BHK-21 fibroblasts and NMuMG epithelial cells provided binding controls. The gL⁻ mutant generally bound less well than the wild type to BHK-21 cells, although the difference in this assay was discernible only at a multiplicity of infection of 3. The gp150⁻ and gL⁻ gp150⁻ mutants both bound better than the wild type, confirming that in this setting gp150 disruption more than compensates for gL disruption. NMuMG cells showed little difference between any of the viruses. Wild-type and gL⁻ viri-

ons bound much the same to B cells, whereas gp150⁻ and gL⁻ gp150⁻ virions both bound much better. Thus, gL was not important for B-cell binding, gp150 was inhibitory, and there was no evidence of a deficit with one being masked by the other.

All the viruses bound more rapidly to splenic B cells than to BHK-21 or NMuMG cells, as noted before for wild-type and gL⁻ MHV-68 binding to NS0 cells (13). Thus, BHK-21 and NMuMG cell fluorescence increased from 30 min to 300 min, whereas B-cell fluorescence tended to decrease. (The gM-eGFP signal is gradually lost from BHK-21 cells after fusion, presumably because the input gM becomes degraded [data not shown]. The same may also be true of B cells.) The absolute levels of B-cell binding were not necessarily higher: primary B cells are less autofluorescent than transformed cells, so low levels of additional fluorescence are more readily discerned. But what binding there was occurred more rapidly.

gp150 and gL are both dispensable for B-cell infection in vivo. We next tested whether gp150⁻ gL⁻ MHV-68 could infect B cells in vivo. This was a necessary test since MHV-68 may not infect B cells in vivo as cell-free virions; direct cell-cell viral spread is also possible. BALB/c mice were infected intranasally with wild-type or knockout viruses, and 13 days later, at the peak latent load, virus-infected spleen cells were enumerated by infectious center assay (Fig. 6A). There was no significant difference between any of the viruses. We also tested spleen cell colonization at 12 days postintraperitoneal infection (Fig. 6B). Here, the gL knockout showed a deficit, although latency was still established. The gL⁻ gp150⁻ double knockout showed no deficit. Thus, in this setting as in vitro (Fig. 4B), gp150 disruption may be able to compensate for a binding deficit associated with gL disruption.

DISCUSSION

Herpesvirus entry is complicated. The example of EBV suggests that gammaherpesviruses infect different cells in different ways: gp350/gp42/gB/gH/gL for B cells and gH/gL/gB for epithelial cells, with gp42 and gp350 being inhibitory (5, 32). MHV-68 offers an interesting comparison. gB and gH are also likely to be involved in membrane fusion (15, 29, 41), and their association in the virion membrane was consistent with an integrated function. The association was independent of gL and gp150. These accessory components appeared to modify the core entry complex to promote epithelial cell/fibroblast infection: gL by improving cell-type-specific binding and gp150 by making binding GAG dependent. The effects reinforced each other, in that gL promoted gp150 incorporation into

FIG. 3. Viral gp150 expression depends in part on gL. (A) gL⁺ virions—the wild type (WT), the gL⁻ DEL revertant (rev), and the gL⁻ STOP revertant—were compared with three independent gL mutants, i.e., gL⁻ DEL, gL⁻ DEL-STOP, and gL⁻ STOP, for gp150 content by immunoblotting with MA b T1A1. MA b 150-7D1, which recognizes the ORF17 cleaved capsid component, was used as a loading control. The data are from one of three equivalent experiments. (B) BHK-21 cells were left uninfected (UI) or were infected (2 PFU/cell, 18 h) with wild-type (WT) or gL⁻ DEL-STOP (gL⁻) MHV-68 and then immunostained for surface glycoprotein expression as indicated and analyzed by flow cytometry. The data are from one of three equivalent experiments. (C) L929 cells were stably transduced with the MHV-68 M7 ORF, which encodes gp150, and then stained with naive mouse serum, WT MHV-68-immune mouse serum, gp150⁻ STOP mutant-immune (gp150⁻) mouse serum, or the gp150-specific MA b T4G2. The cells were not fixed or permeabilized, so positive staining reflects cell surface gp150 expression. The data are from one of five equivalent experiments. (D) BHK-21 cells were infected (2 PFU/cell, 18 h) with WT or gp150-FRT mutant (gp150⁻) MHV-68 and then stained for cell surface viral glycoprotein expression as indicated. The data are from one of five equivalent experiments.

FIG. 4. The gp150 disruption and gL disruption phenotypes are distinct. (A) The gL⁻ DEL-STOP (gL⁻) and gp150⁻ STOP (gp150⁻) mutations were transferred individually and together onto a gM-eGFP background, in which virions are fluorescent by virtue of a C-terminal eGFP tag on gM. BHK-21 cells were infected (2 PFU/cell, 18 h) and then stained for cell surface glycoproteins as shown to ensure that the gM-eGFP mutation had no effect on gL or gp150. WT, wild type (gM-eGFP only); UI, uninfected. Each histogram shows gated eGFP⁺ cells stained with MAb as shown plus Alexa633-conjugated anti-mouse IgG. (B) GAG⁺ and GAG⁻ CHO cells were exposed to gM-eGFP virions as shown for different times to allow binding and then washed three times in PBS and analyzed for fluorescence transfer by flow cytometry. Abbreviations: WT, gM-eGFP only; gL⁻, gL-deficient gM-eGFP; gp150⁻, gp150-deficient gM-eGFP; gL⁻gp150⁻, gM-eGFP double gL gp150 knockout. Cells were exposed to 3 PFU/cell for each virus. Each stock contained equivalent amounts of protein per PFU as measured by immunoblotting for gN. At 300 min postinfection, new eGFP-gM expression is undetectable, as cycloheximide treatment makes no difference to fluorescence (data not shown). The data are from one of three equivalent experiments. (C) BAC⁺ rather than gM-eGFP⁺ versions of the gL and gp150 mutants were used to infect GAG⁺ and GAG⁻ CHO cells (18 h) at the multiplicities shown. Infection was quantitated by flow cytometric assay of viral eGFP expression. The data are from one of two equivalent experiments.

virions. MHV-68 has no obvious gp42 homolog, but the modification of gH by gL (11, 13) is perhaps analogous to the modification of the EBV gH/gL by gp42 (20).

MHV-68 does not seem to show the producer cell-dependent tropism switch of EBV (5): most gH in fibroblast-derived MHV-68 virions is complexed with gL (13), and there is enough gp150 to maintain GAG dependence. Thus, fibroblast-derived viruses infect fibroblasts—the basis of plaque assays.

DNA sequence analysis suggests the intriguing possibility that gp150 could be spliced to reduce its stalk length and hence its inhibitory effect. However, we have found no evidence for this by reverse transcription-PCR from MHV-68-infected epithelial cells, fibroblasts, or macrophages (data not shown). Only limited conclusions are possible at present, because how MHV-68 normally infects B cells remains unclear. Transient activation (36) and abortive infection (9) have been reported in

FIG. 5. gp150 inhibits and gL does not affect MHV-68 binding to splenic B cells. BHK-21 fibroblasts, NMuMG epithelial cells, and CD19⁺ splenic B cells were exposed to gM-eGFP⁺ versions of the gL and gp150 mutants for different times and at different multiplicities as in Fig. 4B. The cells were washed two times in PBS and analyzed for fluorescent virus uptake by flow cytometry. Abbreviations: UI, uninfected (no virus added); WT, gM-eGFP⁺ gL⁺ gp150⁺ (wild type); gL⁻, gM-eGFP⁺ gL⁻ gp150⁺; gp150⁻, gM-eGFP⁺ gL⁺ gp150⁻; gL gp150⁻, gM-eGFP⁺ gL⁻ gp150⁻. Splenic cells were costained with a phycoerythrin-conjugated anti-CD19 MAb to identify B cells. The data are from one of two equivalent experiments.

two previous studies, but neither study addressed the efficiency of infection. By use of eGFP linked to the ORF73 episome maintenance protein (10, 26) as a readout, A20 B-cell infection was markedly improved by syndecan-1 expression (2), implying that their infection by cell-free virions is normally poor. The main alternative is infection by cell-cell spread. A B-cell-orientated gB/gH-type entry complex could potentially be derived from the epithelial cell-orientated gp150/gH/gL/gB entry com-

FIG. 6. gp150 and gL are both dispensable for latency establishment in vivo. (A) BALB/c mice were infected intranasally with eGFP⁻ MHV-68 mutants as shown. Thirteen days later, the viral load in spleens was measured by infectious center assay. Each point corresponds to one mouse. (B) BALB/c mice were infected intraperitoneally with eGFP⁻ MHV-68 mutants as described for panel A. Twelve days later, the viral load in spleens was measured by infectious center assay. Each point corresponds to one mouse. The gL⁻ mutant titers were significantly below the wild-type titers ($P < 0.01$; Student's *t* test), but the gp150⁻ and gL⁻ gp150⁻ titers were not significantly different from that for the wild type.

plex if gp150 and gH/gL were engaged by epithelial cells, as in EBV transfer infection (32) by a mechanism wherein virion engagement by one cell type relieves entry inhibition for another.

Normal latency establishment by gp150-deficient MHV-68 (Fig. 5) (8) contradicts the data of Stewart et al., which showed a deficit (39). They also found less B-cell binding by a gp150 knockout recovered from mouse lungs. We find their data unconvincing. The large deletion they used to disrupt gp150 could easily have disrupted another viral function, as the latency transcripts of MHV-68 remain largely unmapped. Furthermore, they generated just one mutant. BAC-based revertants do not control for problems after virus reconstitution, so an independent mutant is usually required to establish a phenotype. With regard to binding, (i) virus from lungs tends to be heavily contaminated with cell debris, making accurate binding estimates difficult; (ii) ex vivo virus may have antibody and

complement attached; and (iii) the difference observed in viral genome copy numbers was marginal at best, i.e., less than twofold by real-time PCR. Thus, we feel that no good evidence exists for gp150 promoting B-cell infection. Our data would argue the converse.

Why should gammaherpesviruses limit infection by adding inhibitory glycoproteins to the entry complex? A possible explanation is antibody evasion. If gp150 hides a cell binding epitope from its ligand, it should also hide it from antibody. How would this work? gH/gL was evidently not the main cell binding ligand protected by gp150, since gp150 disruption enhanced cell binding even without gL. However, gH/gL might be protected as well. A need for GAG interactions to reveal gH/gL for binding could explain why gL-dependent binding was not evident for GAG-deficient CHO cells (Fig. 4B). (gH/gL itself does not appear to bind to GAGs [14].) The most likely main candidate for gp150-regulated binding is gB: N-terminal gB binds to cells (14) and an association of gp150 with gH/gL (inferred from gL-dependent gp150 incorporation into virions) would also bring it close to gB, since gB and gH were linked. The rescue of gL-deficient BHK-21 cell binding to above wild-type levels by gp150 disruption (Fig. 6) indicated that the binding regulated by gp150 was at least as strong as that dependent on gL. But if gL-dependent binding is weak, why did the gL knockout bind noticeably less well than the wild type to GAG⁺ CHO cells (Fig. 4B)? It is possible that the contribution of gL to cell binding is partly indirect. For example, gH/gL binding could help to displace gp150 from gB. Thus, without gH/gL the displacement of gp150 by GAGs may be inefficient.

Cell binding by virions is evidently very different from that by recombinant glycoproteins. For example, the MHV-68 gp70 is abundant in virions and binds strongly to GAG⁺ cells (14), yet gL-deficient MHV-68 binds poorly to GAG⁺ cells despite expressing gp70, while gp70-deficient MHV-68 shows no deficit (14). Two considerations stand out. First, while secreted glycoproteins (which include gp70) may need a high affinity to bind, the very high avidity of virions should allow good binding even at low affinity. Second, glycoproteins on virions may be constrained by their interactions to operate in series rather than in parallel. There is crystallographic evidence for conformation changes in the human immunodeficiency virus gp120 (6) and the herpes simplex virus gD (18). These examples are hardly likely to be unique. The associations and functional interactions reported here suggest that the MHV-68 entry glycoproteins form a single complex that sequentially engages host ligands. A possible sequence would be gp150 reversibly engaging GAGs to reveal gH/gL, gH/gL engagement promoting further gp150 displacement and revealing gB, and then gB engagement triggering entry. If the whole cascade is dependent on gp150 engaging GAGs, can antibody block infection at this step? We have found no evidence for this: neither gp150-specific sera nor >100 gp150-specific MAbs neutralize infection to a significant degree (L. Gillet and P. G. Stevenson, unpublished data). The simple electrostatic interactions typical of GAG binding are widespread in mammalian physiology, so antibodies against GAG-binding patches may be counterselected by the need to avoid autoreactivity. The prevalence of GAG interactions in persistent virus infections suggests that

they provide a relatively protected key with which to unlock viral entry.

ACKNOWLEDGMENTS

We thank Janet May and Susanna Colaco for outstanding technical support.

Laurent Gillet is a Postdoctoral Researcher of the Fonds National Belge de la Recherche Scientifique (FNRS). Philip G. Stevenson is a Wellcome Trust Senior Clinical Fellow. This work was also supported by Medical Research Council grants G0400427 and G9800903 and CR-UK grant C19612/A6189. Philip G. Stevenson was supported by GR076956MA.

REFERENCES

- Adler, H., M. Messerle, M. Wagner, and U. H. Koszinowski. 2000. Cloning and mutagenesis of the murine gammaherpesvirus 68 genome as an infectious bacterial artificial chromosome. *J. Virol.* **74**:6964–6974.
- Bennett, N. J., J. S. May, and P. G. Stevenson. 2005. Gamma-herpesvirus latency requires T cell evasion during episome maintenance. *PLoS Biol.* **3**:e120.
- Blaskovic, D., M. Stancekova, J. Svobodova, and J. Mistrikova. 1980. Isolation of five strains of herpesviruses from two species of free living small rodents. *Acta Virol.* **24**:468.
- Boname, J. M., and P. G. Stevenson. 2001. MHC class I ubiquitination by a viral PHD/LAP finger protein. *Immunity* **15**:627–636.
- Borza, C. M., and L. M. Hutt-Fletcher. 2002. Alternate replication in B cells and epithelial cells switches tropism of Epstein-Barr virus. *Nat. Med.* **8**:594–599.
- Chen, B., E. M. Vogan, H. Gong, J. J. Skehel, D. C. Wiley, and S. C. Harrison. 2005. Structure of an unliganded simian immunodeficiency virus gp120 core. *Nature* **433**:834–841.
- Davis-Poynter, N., S. Bell, T. Minson, and H. Browne. 1994. Analysis of the contributions of herpes simplex virus type 1 membrane proteins to the induction of cell-cell fusion. *J. Virol.* **68**:7586–7590.
- de Lima, B. D., J. S. May, and P. G. Stevenson. 2004. Murine gammaherpesvirus 68 lacking gp150 shows defective virion release but establishes normal latency in vivo. *J. Virol.* **78**:5103–5112.
- Dutia, B. M., J. P. Stewart, R. A. Clayton, H. Dyson, and A. A. Nash. 1999. Kinetic and phenotypic changes in murine lymphocytes infected with murine gammaherpesvirus-68 in vitro. *J. Gen. Virol.* **80**:2729–2736.
- Fowler, P., S. Marques, J. P. Simas, and S. Efstathiou. 2003. ORF73 of murine herpesvirus-68 is critical for the establishment and maintenance of latency. *J. Gen. Virol.* **84**:3405–3416.
- Gill, M. B., L. Gillet, S. Colaco, J. S. May, B. D. de Lima, and P. G. Stevenson. 2006. Murine gammaherpesvirus-68 glycoprotein H-glycoprotein L complex is a major target for neutralizing monoclonal antibodies. *J. Gen. Virol.* **87**:1465–1475.
- Gillet, L., M. B. Gill, S. Colaco, C. M. Smith, and P. G. Stevenson. 2006. The murine gammaherpesvirus-68 glycoprotein B presents a difficult neutralization target to monoclonal antibodies derived from infected mice. *J. Gen. Virol.* **87**:3515–3527.
- Gillet, L., J. S. May, S. Colaco, and P. G. Stevenson. 2007. Glycoprotein L disruption reveals two functional forms of the murine gammaherpesvirus 68 glycoprotein H. *J. Virol.* **81**:280–291.
- Gillet, L., H. Adler, and P. G. Stevenson. 2007. Glycosaminoglycan interactions in murine gammaherpesvirus-68 infection. *PLoS ONE* **2**:e347.
- Haan, K. M., S. K. Lee, and R. Longnecker. 2001. Different functional domains in the cytoplasmic tail of glycoprotein B are involved in Epstein-Barr virus-induced membrane fusion. *Virology* **290**:106–114.
- Handler, C. G., R. J. Eisenberg, and G. H. Cohen. 1996. Oligomeric structure of glycoproteins in herpes simplex virus type 1. *J. Virol.* **70**:6067–6075.
- Hutt-Fletcher, L. M. 2007. Epstein-Barr virus entry. *J. Virol.* **81**:7825–7832.
- Krummenacher, C., V. M. Supekar, J. C. Whitbeck, E. Lazear, S. A. Connolly, R. J. Eisenberg, G. H. Cohen, D. C. Wiley, and A. Carfi. 2005. Structure of unliganded HSV gD reveals a mechanism for receptor-mediated activation of virus entry. *EMBO J.* **24**:4144–4153.
- Lake, C. M., and L. M. Hutt-Fletcher. 2000. Epstein-Barr virus that lacks glycoprotein gN is impaired in assembly and infection. *J. Virol.* **74**:11162–11172.
- Li, Q., S. M. Turk, and L. M. Hutt-Fletcher. 1995. The Epstein-Barr virus (EBV) BZLF2 gene product associates with the gH and gL homologs of EBV and carries an epitope critical to infection of B cells but not of epithelial cells. *J. Virol.* **69**:3987–3994.
- Lopes, F. B., S. Colaco, J. S. May, and P. G. Stevenson. 2004. Characterization of the murine gammaherpesvirus 68 glycoprotein B. *J. Virol.* **78**:13370–13375.
- May, J. S., S. Colaco, and P. G. Stevenson. 2005. Glycoprotein M is an essential lytic replication protein of the murine gammaherpesvirus 68. *J. Virol.* **79**:3459–3467.
- May, J. S., B. D. de Lima, S. Colaco, and P. G. Stevenson. 2005. Inter-cellular gamma-herpesvirus dissemination involves co-ordinated intracellular membrane protein transport. *Traffic* **6**:780–793.
- May, J. S., H. M. Coleman, J. M. Boname, and P. G. Stevenson. 2005. Murine gammaherpesvirus-68 ORF28 encodes a non-essential virion glycoprotein. *J. Gen. Virol.* **86**:919–928.
- May, J. S., J. Walker, S. Colaco, and P. G. Stevenson. 2005. The murine gammaherpesvirus 68 ORF27 gene product contributes to intercellular viral spread. *J. Virol.* **79**:5059–5068.
- Moorman, N. J., D. O. Willer, and S. H. Speck. 2003. The gammaherpesvirus 68 latency-associated nuclear antigen homolog is critical for the establishment of splenic latency. *J. Virol.* **77**:10282–10303.
- Moorman, N. J., C. Y. Lin, and S. H. Speck. 2004. Identification of candidate gammaherpesvirus 68 genes required for virus replication by signature-tagged transposon mutagenesis. *J. Virol.* **78**:10282–10290.
- Persons, D. A., M. G. Mehaffey, M. Kaleko, A. W. Nienhuis, and E. F. Vanin. 1998. An improved method for generating retroviral producer clones for vectors lacking a selectable marker gene. *Blood Cells Mol. Dis.* **24**:167–182.
- Pertel, P. E. 2002. Human herpesvirus 8 glycoprotein B (gB), gH, and gL can mediate cell fusion. *J. Virol.* **76**:4390–4400.
- Rodger, G., J. Boname, S. Bell, and T. Minson. 2001. Assembly and organization of glycoproteins B, C, D, and H in herpes simplex virus type 1 particles lacking individual glycoproteins: no evidence for the formation of a complex of these molecules. *J. Virol.* **75**:710–716.
- Roop, C., L. Hutchinson, and D. C. Johnson. 1993. A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. *J. Virol.* **67**:2285–2297.
- Shannon-Lowe, C. D., B. Neuhierl, G. Baldwin, A. B. Rickinson, and H. J. Delecluse. 2006. Resting B cells as a transfer vehicle for Epstein-Barr virus infection of epithelial cells. *Proc. Natl. Acad. Sci. USA* **103**:7065–7070.
- Simas, J. P., and S. Efstathiou. 1998. Murine gammaherpesvirus 68: a model for the study of gammaherpesvirus pathogenesis. *Trends Microbiol.* **6**:276–282.
- Song, M. J., S. Hwang, W. H. Wong, T. T. Wu, S. Lee, H. I. Liao, and R. Sun. 2005. Identification of viral genes essential for replication of murine gammaherpesvirus 68 using signature-tagged mutagenesis. *Proc. Natl. Acad. Sci. USA* **102**:3805–3810.
- Spear, P. G., and R. Longnecker. 2003. Herpesvirus entry: an update. *J. Virol.* **77**:10179–10185.
- Stevenson, P. G., and P. C. Doherty. 1999. Non-antigen-specific B-cell activation following murine gammaherpesvirus infection is CD4 independent in vitro but CD4 dependent in vivo. *J. Virol.* **73**:1075–1079.
- Stevenson, P. G., J. S. May, X. G. Smith, S. Marques, H. Adler, U. H. Koszinowski, J. P. Simas, and S. Efstathiou. 2002. K3-mediated evasion of CD8(+) T cells aids amplification of a latent gamma-herpesvirus. *Nat. Immunol.* **3**:733–740.
- Stevenson, P. G., and S. Efstathiou. 2005. Immune mechanisms in murine gammaherpesvirus-68 infection. *Viral Immunol.* **18**:445–456.
- Stewart, J. P., O. J. Silvia, I. M. Atkin, D. J. Hughes, B. Ebrahimi, and H. Adler. 2004. In vivo function of a gammaherpesvirus virion glycoprotein: influence on B-cell infection and mononucleosis. *J. Virol.* **78**:10449–10459.
- Tanner, J., J. Weis, D. Fearon, Y. Whang, and E. Kieff. 1987. Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. *Cell* **50**:203–213.
- Turner, A., B. Bruun, T. Minson, and H. Browne. 1998. Glycoproteins gB, gD, and gH/gL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system. *J. Virol.* **72**:873–875.
- Wang, X., and L. M. Hutt-Fletcher. 1998. Epstein-Barr virus lacking glycoprotein gp42 can bind to B cells but is not able to infect. *J. Virol.* **72**:158–163.