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The Herpes Simplex Virus-1 Encoded Glycoprotein B Diverts HLA-DR into the Exosome Pathway

Sebastian Temme,* Anna M. Eis-Hübinger, ‡ Alexander D. McLellan, § and Norbert Koch*

Neutralizing Abs play an important role for immunity against HSV-1 infection. This branch of the immune response is initiated by MHC class II Ag presentation and activation of T cell help. In this study, we show that the HSV-1 encoded glycoprotein B (gB) manipulates the class II processing pathway by perturbing endosomal sorting and trafficking of HLA-DR (DR) molecules. Expression of gB in the human melanoma cell line Mel JuSo results in formation of enlarged DR⁺ intracellular vesicles. Costaining of the vesicles revealed the presence of DR, gB, and the late endosomal marker CD63. The lumen of these late endosomal membranes shows a variable content, containing either gB or CD63, or both CD63 and gB. gB targets DR molecules on their biosynthetic route, after the MHC class II invariant chain is released from the DR heterodimer. gB-DR complexes were detected in a post-Golgi compartment and in exosomes, but not on the cell surface. Interestingly, increasing expression of gB strongly elevated the amount of DR and CD63 released into the exosome pathway. In conclusion, this is a previously undescribed mode of viral immune evasion involving hijacking of DR from its normal transport route to the cell surface, followed by viral-mediated release of DR into the exosome pathway. The Journal of Immunology, 2010, 184: 236–243.

H SV-1, a member of the subfamily Alphaherpesvirinae, is a common human pathogen with a worldwide appearance. Primary infections, which mostly occur during early childhood, are often asymptomatic. About one third of the human population suffers from recurrent infection of skin and other tissues occurring mostly at irregular intervals. Viral reactivation can be induced by certain stimuli, such as UV light or stress, and can cause a recurrent infection. On stimulation, HSV-1 is exported from the ganglia to the epithelium along peripheral nerves leading to infection of epithelial cells (1). Several branches of immune responses are engaged against HSV-1 infection. Neutralizing Abs substantially contribute to immunity against HSV-1 infection (2). This humoral response is complemented by a mixture of cytokines produced by infiltrating CD4⁺ T cells, which, in addition, mediates the stimulation of HSV-1–specific cytotoxic T cells (3, 4).

Despite the production of neutralizing Abs and efficient T cell responses, a substantial proportion of affected hosts shows no lasting protection against a recurrent infection. HSV-1 has evolved under the selection pressure of multiple defense mechanisms occurring in host cells, and complete elimination of HSV-1 is prevented by viral evasion strategies. In particular, the MHC glycoproteins are vulnerable to viral intervention. As an example for a viral evasion strategy, the HIV-encoded Nef protein interferes with the MHC class II (MHCII) processing pathway inducing accumulation of intraluminal vesicles in multivesicular bodies (MVBs). Moreover, surface expression of immature MHCII/invariant chain (Ii) complexes and the presence of these complexes in MVBs is elevated (5). This occurs because of the ability of Nef to impair the adapter protein-2–mediated endocytosis of Ii and fusion of MVBs with lysosomal vesicles (6). Many molecular mechanisms of viral evasion strategies remain elusive.

We recently discovered that during HSV-1 infection of B lymphocytes, the viral glycoprotein B (gB) targets the MHCII processing pathway (7).

In APC, HLA-DR (DR) molecules are intracellularly sorted to MHCII loading compartments, where they encounter processed Ag. These endosomal vesicles gradually accumulate internal vesicles. Coincidently, these endosomes change their content, indicating that they are active sorting organelles. Sorting of DR molecules is accompanied by formation of MVBs, which are composed of a limiting membrane and intraluminal vesicles. In APCs, the purpose of these MVBs is to load MHCII heterodimers with peptide and to deliver peptide-loaded DR molecules to the cell surface. For cell surface expression of MHCII, backfusion of the MVB internal vesicles with the limiting membrane and subsequent amalgation of MVBs with the cell membrane was suggested (8). Moreover, movement of MHCII molecules via tubules that emanate from the MVB toward the plasma membrane was described as a potential mechanism for Ag presentation (9). An additional proportion of the MVBs fuse with lysosomal vesicles that results in degradation of its cargo. Recent data indicate that MVBs can also interact with the plasma membrane and release their luminal content to the extracellular space (10). The molecular composition of the secreted vesicles appears to be similar, if not identical, to that of the internal vesicles of MVBs (11).

In an earlier study, we have shown that on HSV-1 infection, gB was found in a complex with DR (7). However, this complex was not detected on the cell surface and the impact of this interaction on vesicular transport of DR was not investigated. Here, we provide data revealing novel molecular mechanisms of how gB regulates migration of DR in the endocytic pathway. Because HSV-1 is a lytic virus, infected cells have a short life span. To circumvent the impact...
of the virus on the cell’s physiology, we have established cell clones constitutively expressing gB to explore the interaction of gB with DR molecules. We discovered that gB targets the biosynthetic route of class II molecules and regulates sorting of DR molecules. DR and gB associate in Ag processing compartments, when II is released from DR molecules by degradation. These gB-DR complexes are sorted to endosomes prior to release via exosomes.

Materials and Methods

Abs and biochemicals

The following mouse mAbs 2c2 (anti-gB; IgG2a), 10B7 (anti-gB; IgG1), L243 (anti-DR; IgG2a), ISC3 (anti-DR; IgG2b). TAL-185 (anti-DR); IgG1), LGII-612.14 (anti-HLA–DR, -DP, and -DQ; IgG1), Bu43 (anti-Ii; IgM), clone 37 (BD Bioscience, Heidelberg, Germany; anti-calnexin; IgG1), and FK2 (anti-ubiquitin; IgG1; kindly provided by Dr. J. Höhfeld, Munich, Germany). For immunoprecipitation, lysates from cells or from exosomes were separated by SDS-PAGE. Immunoprecipitated Ags were deglycosylated by treatment with Endo H or with PNGaseF (15). Digestion was performed using the following Abs and biochemicals: anti-mouse Ig Alexa 488 (Invitrogen), anti-mouse Ab (Invitrogen). After addition of the conjugated Abs, the cells were washed twice, and incubated with Alexa 488 or with HRP-conjugated anti-mouse IgG or IgM Abs (Dianova) and ECL (GE Healthcare, Piscataway, NJ) for 1–infected cells containing complex type glycans on their subunits (Fig. 1A, lane 1). Expression of gB, DRα, and DRβ encoding cDNAs. Cells were lysed and the interaction of gB with DR was assessed by immunoprecipitation of gB. The isolated proteins were treated with Endo H (which cleaves high mannosetype N-linked carbohydrates) with PNGaseF (completely cleaves off the high mannose type glycan chains), or left untreated (Fig. 1A, lanes 1–3). Expression of gB, DRα, and DRβ was monitored by Western blotting of cell lysates (right panel). Separation of the digested glycoproteins by SDS-PAGE and subsequent immunoblotting for DRα shows that Endo H digestion (lane 2) shifts the DRα band with the mobility of the untreated glycoprotein in lane 1 to the position of the PNGaseF (lane 3) digested polypeptide. This assay indicates that the DRα-chain contains only high mannose glycan forms, consistent with retention of the glycoproteins in the ER. To examine the specific nature of interaction of gB with DR, we expressed gB with single DRα or DRβ subunits (Fig. 1B). Immunoprecipitation of gB and subsequent immunoblotting for DRα

DNA constructs and transfection

DNA constructs and transfection

The cDNAs encoding HLA-DR α- and β-chains, gB from HSV-1 strain 17, and human IL33 are under the control of the CMV immediate early promoter contained in the pcDNA3.1 vector (Invitrogen) and were described previously (14). COS-7 cells were transfected with jetPEI (Biorol, Hamburg, Germany) according to the manufacturer’s instructions. Briefly, DNA mixture with jetPEI (3 μg/μg DNA) was incubated for 20 min and added to cells. After 48 h, cells were harvested and subjected to Western blotting.

Immunoprecipitation, endoglycosidase treatment, and immunoblotting

Cells or exosomes were lysed with ST buffer, containing 0.5% NP40 and Complete protease inhibitors (Roche, Mannheim, Germany) for 1 h. Cells were incubated with the Ag-specific mAbs. Western blots were developed with HRP-conjugated anti-mouse Ig or IgM Abs (Dianova) and ECL (GE Healthcare, Munich, Germany). For immunoprecipitation, lysates from cells or from exosomes were precleared by preabsorption with CL4B-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). For immunoprecipitation, 1 μg 2c2, L243, or ISC3 Abs and protein G Sepharose, were used. For isolation of gB-His, Ni-NTA–Agarose (Invitrogen) was used. Immunoprecipitates were washed three times with 0.25% NP40 in PBS and subsequently separated by SDS-PAGE. Immunoprecipitated Ags were deglycosylated by treatment with Endo H or with PNGaseF (15). Digestion was performed using the buffers recommended by the manufacturer (New England Biolabs).

Flow cytometry

Flow cytometry

MJ and MJ-gB cells were detached from the tissue culture dish by incubating cells for 15 min with PBS/2.5 mM EDTA at 37°C. Cells were washed twice in ice-cold PBS containing 2% FCS and 0.05% sodium azide. Primary Ab was added, and cells were incubated for 30 min at 4°C. Washed twice, and incubated with Alexa 488 or with PE-conjugated goat anti-mouse Ab (Invitrogen). After addition of the conjugated Abs, the cells were incubated for 30 min at 4°C. Subsequently, cells were washed three times and analyzed using a BD Biosciences FACScan unit (Mountain View, CA). Exosomes were attached to beads for conducting flow cytometry by incubation with aldehyde-activated microspheres (Invitrogen) overnight at 4°C as previously described (16). Beads were blocked with 50 mg/ml BSA/PBS for 15 min on ice, quenched for 30 min with 100 mM glycine in PBS pH 7.4, and resuspended in FACS buffer (PBS containing 2% FCS and 0.1% sodium azide).

Exosome production and purification

Cells were cultured in serum-free medium (Hybridomed DIF-1000, supplemented with transferrin [10 mg/l], insulin [10 mg/l], serum albumin [1 g/l], and oleic acid derivates [8 mg/l], Biochrom, Berlin, Germany) for 24–48 h to avoid contamination of exosomes with serum-derived products. To remove cellular debris, the cell supernatant was precleared by centrifugation for 30 min at 10 000 g (16). Subsequently, the supernatant was filtered (0.2 μm) and exosomes were pelleted for 2 h at 100 000g. The pellet containing the exosomes was washed twice with 1 ml PBS and resuspended in PBS or in lysis buffer (ST buffer containing 0.5% NP40 and Complete protease inhibitors). For purification, exosomes were subjected to sucrose gradient centrifugation. Fractions were collected from the gradient at the top of the tube. The density of the sucrose gradient fractions was assessed by a refractometer. Gradient fractions were dialyzed with PBS overnight and exosomes were pelleted by ultracentrifugation and resuspended in PBS or lysis buffer.

Fluorescence microscopy

MJ and MJ-gB cells were seeded onto coverslips, cultivated for 24 h, fixed with 4% PFA, and permeabilized with 1% Triton X100. After blocking with Roti-Immunoblock (Carl Roth) the coverslides were incubated with the indicated Abs for 30 min. After washing with PBS, the cells were incubated with Alexa Fluor-coupled secondary Abs (Molecular Probes, Eugene, OR) for 30 min. For double or triple staining, the cells were washed with PBS and incubated with directly coupled primary Abs in the presence of 1% mouse serum. Coverslips were washed with PBS and mounted onto slides using PermaFluor (Innometch, Luminy, France). Cellular staining was visualized by fluorescence microscopy (LSM-510 Meta or Axiopt; Zeiss, Oberkochen, Germany). The size of the optical slice used for confocal microscopy was 1 μm.

Results

HSV-1 encoded gB is not a chaperone for the assembly of DR subunits

A recent study established that DR molecules coalesce with gB from lysates of HSV-1 infected cells (7). The coisolated DR molecules showed resistance to Endo H treatment and were free of II. Endo H resistance indicates transit through Golgi compartments, where the high mannosetype N-linked carbohydrates is trimmed to a complex type glycan. Isolation of gB-DR complexes from HSV-1-infected cells containing complex type glycans on their subunits could suggest that in the endoplasmic reticulum (ER), gB replaces II as a chaperone for folding and subsequent intracellular transport of DR heterodimers (7). To test for a potential chaperone role of gB, we transfected COS cells with gB, DRα, and DRβ encoding cDNAs. Cells were lysed and the interaction of gB with DR was assessed by immunoprecipitation of gB. The isolated proteins were treated with Endo H (which cleaves high mannosetype N-linked carbohydrates), with PNGaseF (completely cleaves off the high mannosetype glycan chains), or left untreated (Fig. 1A, lanes 1–3). Expression of gB, DRα, and DRβ was monitored by Western blotting of cell lysates (right panel). Separation of the digested glycoproteins by SDS-PAGE and subsequent immunoblotting for DRα shows that Endo H digestion (lane 2) shifts the DRα band with the mobility of the untreated glycoprotein in lane 1 to the position of the PNGaseF (lane 3) digested polypeptide. This assay indicates that the DRα-chain contains only high mannose glycan forms, consistent with retention of the glycoproteins in the ER. To examine the specific nature of interaction of gB with DR, we expressed gB with single DRα or DRβ subunits (Fig. 1B). Immunoprecipitation of gB and subsequent immunoblotting for DRα
**FIGURE 1. Interaction of gB with DR molecules in transiently transfected cells.**

Cells were lysed with 0.5% NP40, and gB was immunoprecipitated (mAb Bu43). (A) Lysates of cells transfected with DRα, DRβ, and gB encoding cDNAs were digested with Endo H (Fig. 1B). Western blots demonstrating expression of gB, DRα, and DRβ are shown on the right. (B) gB was coexpressed with DRα or with DRβ in the presence or absence of Ii. MW (kDa) shows colocalization of gB and DR in a proportion of the displayed vesicles. Fig. 2E, upper panel, demonstrates costaining of gB (left) with DR (middle). An overlay of the fluorescence staining shows colocalization of gB and DR in a proportion of the displayed vesicles (Fig. 2E, upper panel, right), indicating that the viral glycoprotein and MHCII molecules are contained in some of the enlarged vacuolar structures. To characterize the vesicles stained for DR or for gB, cells were intracellularly labeled with a CD63 Ab specific for a member of the tetraspanin family that is present in late endosomes. Fig. 2E, lower panel. Similar to costaining with gB and DR, gB (left) and CD63 (middle) were detected in enlarged vesicles, of which some yellow staining after overlay of the images. An enlargement of costaining of gB with CD63 is shown in Fig. 2F. The display of gB (left) shows most vesicles abundantly stained for gB, but some exhibit a circular staining, which may define the limiting outer membrane. In contrast, CD63 (middle) was specifically detected in the lumen of the stained vesicles (compare merged images on the right). Triple staining was conducted to demonstrate colocalization of CD63 and gB with DR. In Fig. 2G, red (DR)-, green (gB)-, and blue (CD63)-labeled Abs identify large vesicular structures. The merged picture (lower left) shows coexpression of gB, DR, and CD63 in enlarged vesicular structures. We conclude that DR and gB are contained in morphologically altered endocytic vesicles, which are defined by the presence of CD63.

**Interaction of gB and DR molecules in the MHCII processing pathway**

In a previous paper (7), we reported that gB and MHCII molecules were coisolated from HSV-1-infected cells. It is difficult, however, to study the impact of gB on the MHCII pathway in infected cells, because the level of gB depends on the time of infection. We investigated physical interaction of gB with DR in stable transfected MJ-gB cells. gB was immunoprecipitated from MJ-gB lo and from MJ cell lysates as a control. Coisolated DRα was visualized by Western blotting (Supplemental Fig. 1A). An immunoprecipitate of gB showed a band for coisolated DRα (lane 1), which confirms previous data obtained with HSV-1–infected cells (7). To distinguish ER-exported from ER-retained glycoproteins, we examined carbohydrate maturation of gB–associated DRα–chain monitored by Western blotting. The DRα glycoprotein contains two N-linked sugars; whereas in ER, they are one and two sugars, respectively. 

**Stable expression of gB in MJ cells**

To establish a role of gB on the processing pathway of MHCII molecules, we examined the impact of the viral glycoprotein on the intracellular route of DR in a stably gB-transfected cell clone. The human melanoma cell line MJ, which constitutively produces DR and Ii, was transfected with a cDNA encoding gB. The selected MJ cell clones (MJ-gB) were monitored by flow cytometry for gB surface expression. Mean fluorescence values of two MJ-gB cell clones are shown in comparison with nontransfected MJ cells (Fig. 2A). We established two MJ cell clones with different levels of gB (MJ-gB hi and MJ-gB lo). Immunoblotting of cell lysates from MJ-gB hi and lo cell clones for gB confirms the different levels of gB expression (Fig. 2B, upper panel). Blotting for actin indicates that similar amounts of cellular extracts were separated (lower panel). A comparison of gB-transfected cells with HSV-1–infected MJ cells indicated that similar amounts of gB were detected by Western blotting (Fig. 2C). Labeling for actin showed that lanes contained equivalent cell numbers. By HSV-1 infection experiments it had been demonstrated that gB localizes to late endosomal membranes (18). In APCs, this intracellular compartment harbors DR molecules. To examine a potential impact of gB on intracellular MHCII localization, DR molecules were stained in MJ, in MJ-gB lo, and in MJ-gB hi cells (Fig. 2D). Although MJ cells show typical staining of DR on microvesicles in the cytoplasm, the transfected MJ cells contain large intracellular vacuoles stained for DR. In MJ cells, larger vesicles only occasionally were detected, which suggests that expression of gB appears to induce structural changes by formation of enlarged vesicles. The size of the DR-stained vesicles increases from MJ-gB lo to MJ-gB hi cells. Transient transfection of other cell types, such as the lung fibroblast cell line IMRS and Raji B lymphoma cells, revealed that in these cells gB is also contained in large intracellular vesicles (not shown).
glycans, one of which acquires Endo H resistance on export from the ER to the Golgi, whereas the second glycan chain remains Endo H sensitive. Supplemental Fig. 1A, lane 2, shows resistance of one DRα-linked carbohydrate after Endo H digestion. The mobility shift in lane 2 compared with the undigested DRα-chain band in lane 1 indicates that the second glycan chain was Endo H sensitive. A complete cleavage of both carbohydrate chains was achieved by PNGaseF digestion (lane 3). Moreover, we tested whether the gB-DR immunocomplexes contain Ii. Immunoprecipitates of gB were immunoblotted for Ii (Supplemental Fig. 1B). From the stably gB-transfected MJ cell clone or as a control from untransfected MJ cells, no band corresponding to Ii was coisolated with gB (lanes 2 and 1). Immunoblotting of gB indicated the presence of gB in immunoprecipitates from MJ-gB cell lysates (lane 6). No gB band was detected in the MJ cell lysate (lane 5) demonstrating the specificity of the immunoprecipitation. The presence of Ii in cell lysates is shown in lanes 3 and 4. We conclude from the data in Supplemental Fig. 1 that the gB coisolated DR molecules contain no Ii. Carbohydrate maturation detected on gB-coisolated DRα-chain and the absence of Ii in these complexes suggests that the viral glycoprotein and the α-chain encounter in a post-Golgi compartment, presumably, when Ii is released from the DR heterodimer by degradation.

gB association excludes peptide binding and surface expression of DR

DR heterodimers were immunoisolated from MJ-gB cell lysates with mAb L243. This Ab detects a conformation-sensitive epitope and binds the DR heterodimer only, when it is free of Ii (19). Fig. 3A shows...
L243 immunoprecipitates from MJ (lane 1) and from MJ-gB (lane 2) cell lysates separated by SDS-PAGE and subsequent immunoblotting for gB. Lane 2 demonstrates that the L243 mAb precipitates gB from MJ-gB cell lysates. As expected, no band corresponding to gB was coisolated from MJ cells. This result indicates that gB-bound DR carries the L243 epitope, which is consistent with the finding in Supplemental Fig. 1B, that the gB-DR complex is free of Ii. We next examined whether gB-bound DR molecules contain peptides. A proportion of peptide-loaded DR molecules from MJ-gB cells exhibits resistance to heating and SDS treatment and can be visualized as a 50-kDa band (peptide-loaded MHCIIN [pMHCIIN]) (Fig. 3B, lane 1). The pMHCIIN heterodimer dissociates after boiling into free MHCIIN subunits (DRα, lane 2). Lanes 3–6 show immunoprecipitates of gB from lysates of MJ-gB cells using NiNTA Agarose (binds to His-tag of gB), subsequently blotted for DRα (lane 3, nonboiled; lane 4, boiled) and for gB (lane 5, nonboiled; lane 6, boiled). The immunoprecipitate of gB (lane 3) blotted for DRα exhibits a band for monomeric DRα, but no pMHCIIN band, regardless of whether the sample was boiled or nonboiled (lanes 3 and 4). This result demonstrates that gB-DR complexes contain no peptides, which mediates resistance of the DR heterodimers to SDS treatment at room temperature. Subsequent blotting for gB (lanes 5 and 6) demonstrates that gB was immunoprecipitated and DR was coisolated in lanes 3 and 4. Presumably, binding of gB to the DR heterodimer occurs after degradation of Ii. In addition, binding of gB to DR may inhibit subsequent uptake of peptide.

To confirm that gB-DR complexes are not expressed on the cell surface, MJ and MJ-gB cells were incubated at 4°C with the mAb 2c/2 against gB. Cells were lysed and gB-immunoprecipitates were Western blotted for DRα and lanes 4 and 5 for gB. The positions of gB, DRα, and of IγH and L chains are indicated. D, Confocal analysis of MJ-gB (upper panel) and of MJ (lower panel) cells costained for ubiquitin (left) and for CD63 (middle) (original magnification ×1000). Cells were fixed, permeabilized, and stained with mAb FK2 (ubiquitin) and Alexa 488-coupled anti-CD63 mAb (MEM-259). The images were merged on the right. Yellow staining displays colocalization. Arrows indicate three costained vesicles in MJ-gB cells. N indicates the location of the nucleus.

FIGURE 3. gB inhibits peptide binding and cell surface expression of associated DR heterodimers. A, Cell lysates from MJ and from MJ-gB cells were immunoprecipitated with mAb L243 for DR, SDS-PAGE separated and immunoblotted for gB. The position of gB in lane 2 is indicated on the right. The bands below the gB band are degradation products of gB. B, gB was immunoprecipitated from MJ-gB cell lysates. Samples were boiled or nonboiled and separated in lanes 3–6 by SDS-PAGE. Cell lysates were separated in lanes 1 and 2. Subsequently, the gel was immunoblotted for DRα (lanes 1–4). Lanes 3 and 4 were reprobed for gB (lanes 5 and 6). The positions of peptide-loaded DR (pMHCIIN), of DRα and gB and of m.w. markers are indicated. C, MJ and MJ-gB cells were incubated for 30 min at 4°C with mAb 2c/2 against gB. Cells were washed to remove unbound mAb and lysed with 0.5% NP40. Abound surface gB was isolated with protein G Sepharose. Samples were separated by SDS-PAGE in lanes 3–6. Lanes 1–3 were Western blotted for DRα and lanes 4 and 5 for gB. The positions of gB, DRα, and of IγH and L chains are indicated. D, Confocal analysis of MJ-gB (upper panel) and of MJ (lower panel) cells costained for ubiquitin (left) and for CD63 (middle) (original magnification ×1000). Cells were fixed, permeabilized, and stained with mAb FK2 (ubiquitin) and Alexa 488-coupled anti-CD63 mAb (MEM-259). The images were merged on the right. Yellow staining displays colocalization. Arrows indicate three costained vesicles in MJ-gB cells. N indicates the location of the nucleus.
Release of gB-DR complexes via exosomes

Recently, it was shown that some cell types release small vesicles with a size of 40–100 nm, designated as exosomes (reviewed in (20)). These exosomes are derived from MVBSs. The route of exosomal release may also be targeted by viral proteins (21). A release of gB and of gB-DR complexes by exosomes could demonstrate a novel route for the HSV-1-encoded gB and of gB-associated DR. To elucidate some details of trafficking in the endocytic pathway, we investigated whether MJ cells secrete exosomes. We isolated exosomes by high-speed centrifugation from the supernatant of cultured MJ cells (see Materials and Methods). Western blotting of lysates from the exosomal preparation in comparison with cell lysates indicated the presence of DRα (Fig. 4A). In this experiment, the amount of separated lysates was standardized to yield equal intensities for the DRα band from cells and from exosomes. In contrast to cell lysates, Ii was not detected in lysates of exosomes. Calnexin, as an ER marker, and CD63, as an endosomal marker, were only detected in the cell lysate or in the exosomal preparation, respectively. Application of increased amounts of cell lysate led to detection of CD63 (not shown). Subsequently, MJ-gB derived exosomes were examined on density separation for the presence of gB and of DRα. Isolated exosomes were separated by ultracentrifugation on a sucrose gradient. Western blotting of the sucrose gradient fractions for gB and DRα revealed the presence of both the viral and the DR subunit at a sucrose density of 1.15–1.20 g/ml (Fig. 4B). This result suggests that the gB delivered to endosomes is subsequently released by exosomes.

To examine the amount of material released by MJ and by MJ cells expressing gB, exosomal preparations from these cells were coupled to beads and analyzed by flow cytometry (see Materials and Methods). This method has been reported to yield quantitative and reproducible results (22). Equal numbers of MJ and of MJ-gB cells were cultured in the same volume for the same time to produce exosomes. Fig. 4C demonstrates the detection of gB on secreted material from MJ-gB lo with increasing amounts received from MJ-gB hi cells, but not from MJ cells as a control. Exosomal material from gB-transfected MJ clones contained a far greater amount of CD63 and of DR, compared with exosomes secreted by MJ cells with gB hi cells showing the highest CD63 and DR levels. A control Ab showed no fluorescence staining for any of the exosomal material. This result indicates that expression of gB elevates the amount of DR and CD63 released by exosomes.

To confirm this result, we produced exosomes as described previously and analyzed their DR content by Western blotting (Fig. 4D). Two preparations of exosomes were obtained from supernatant after 24 or 48 h of cell culture and separated by SDS-PAGE (middle and lower panel). Blotting for DRα shows that the amount of the MHCII heavy chain obtained after 24 or 48 h was substantially increased in exosomal preparations from MJ-gB hi cells compared with MJ cells. gB was detected in exosomes from MJ-gB hi cells (upper panel, right lane), but not in exosomes from MJ cells (upper panel, left lane).

We next examined whether gB-associated DR could be detected in these exosomes released from MJ-gB cells. Exosomes derived from MJ-gB and as a control from MJ cells were lysed and immunoprecipitated for gB, SDS-PAGE separated and Western blotted for DRα (Fig. 4D). In the presence of gB, a DRα band was detected, which is not present in a control immunoprecipitate in the absence of gB (lanes 1 and 2). This result indicates that gB associated DR molecules are released from MJ-gB cells by exosomes.

Discussion

HSV-1 infection shows multiple effects on APCs, which manipulates the immune responsiveness of the host (23). A substantial part of the invasive activity of the virus is concentrated in the extranuclear space of the infected cells, where assembly of the viral envelope and the tegument precedes viral propagation (24). Because a significant proportion of immunity to HSV-1 infection is Ab-mediated, the virus interferes with T cell help for B cells, by preventing MHCII Ag presentation by infected cells (25). Inhibition of Ag presentation can be achieved by targeting the MHCII processing pathway through interaction with viral proteins.

We show here that HSV-1 uses its envelope protein gB to alter MHCII trafficking in the endocytic pathway. The primary function of gB is to mediate contact of HSV-1 to the cell membrane and to permit entry of the virus into the cell. An additional role of gB in APCs is to interact with DR molecules (7, 14).

Because viral infection is accompanied by cellular reorganization and cell lysis, which hides some of the molecular effects of gB, we established cell clones stably transfected with gB. In this study, we discovered that gB targets DR heterodimers after the DR subunits have acquired complex type carbohydrates on their N-linked glycans in Golgi compartments. This gB-DR complex is free of Ii. We suggest that gB targets DR molecules from the biosynthetic route, after Ii is degraded. Subsequently, gB binds to the DR groove and inhibits association of peptide to the DR heterodimer. Costaining of gB, DR, and CD63 suggests that these markers are contained in enlarged vesicular structures of endosomal origin. Intervention of gB in this route appears to induce formation of enlarged vesicles. Several altered cellular states have been described that result in enlarged endocytic vacuoles. Wortmannin, a PI3-kinase inhibitor, induces swollen endosomes, in fibroblast cells that accumulate intraluminal vesicles (26). Rab7-depleted cells also revealed enlarged MVBSs packed with a dense content of intraluminal vesicles (27). In contrast to treatment of fibroblast cells, the effect of wortmannin on MJ cells appears to reduce the number of intraluminal vesicles (28). The phenotype of swollen endosomes induced by sucrose uptake yields vacuoles largely depleted of vesicles (26). A modification of endocytic vesicles was also observed by overexpression of Ii in COS cells (29). In stably transfected cells these enlarged endocytic compartments were identified as early as well as late endosomes and lysosomes (30). Formation of these macromosomes was found to delay transport of luminal cargo from endosomes to lysosomes retarding degradation (29, 30).

These published data indicate that reorganization of endocytic vesicles to enlarged structures may affect biogenesis of MVBSs and trafficking of cargo to lysosomal compartments. The enlarged vesicles observed in cells expressing gB are smaller than the vacuolar structures discussed previously and therefore may represent a variant entity.

Interestingly, the human herpes virus 6 (HHV-6) induces formation of MVBSs (21). In intraluminal vesicles contained in these MVBSs, HHV-6-derived gycoproteins B and M were detected. HHV-6 uses the cellular exosomal pathway of the host cell. The biogenesis of exosomes is a result of invagination of the endocytic membrane that leads to formation of luminal vesicles, yielding MVBSs. Fusion of MVBSs with the plasma membrane results in release of exosomes. Vesicles released by MJ-gB cells contain gB, DR, and other markers, such as CD63, which are characteristic of exosomes. This result indicates that gB gains access to luminal vesicles, followed by release of exosomes. Because gB-DR complexes are not found on the cell surface, but in exosomes, it was not clear how gB manipulates DR trafficking in the MJ-gB cells. An attractive target for viruses is the ubiquitin system, which regulates intracellular sorting of proteins and their degradation (31). Ubiquitination was reported to be a signal for sorting of intraluminal bodies in MVBSs (32).

In fact, we found ubiquitination of gB in immunoprecipitates of the viral proteins from MJ-gB cells (Supplemental Fig. 2) that confirms previously published data (18). Ubiquitination of gB could
FIGURE 4. Exosomal release by MJ cells. Cells were cultivated for 24 h in serum-free medium. Exosomes isolated by centrifugation, filtration, and ultracentrifugation were subsequently resuspended in PBS. A, Lysates from MJ cells and from MJ-derived exosomes were separated by 12% SDS-PAGE, blotted onto nitrocellulose, and probed for DRα (mAb TAL-1B5), for Ii (mAb Bu43), for Calnexin (clone 37), and for CD63 (MEM-259) (To resolve a CD63 signal, the lysate was nonboiled and nonreduced). The amount of cell lysate and exosome lysate separated by SDS-PAGE was adjusted to the expression of the DRα-chain.

(B) Exosomal preparations from MJ cells were separated by ultracentrifugation on a sucrose gradient and separated into 12 fractions. Fractions 6–10 were separated by SDS-PAGE and immunoblotted for DRα and gB. Density of the sucrose fractions is shown on the top. M.w. markers are indicated on the left and the position of gB and DRα is shown on the right. C, Exosomes obtained from equal numbers of MJ and from MJ-gB cells were coated to aldehyde/sulfate latex beads and probed for gB (2c/2), for DR (ISCR3), or for CD63 (MEM-259), followed by incubation with anti-mouse IgG R-PE secondary Ab. Finally, exosomes were analyzed by flow cytometry. The diagram shows mean fluorescence intensity subtracted of values for beads without exosomes. MJ exosomes (unfilled column) MJ-gB lo (dark gray column) and MJ-gB hi (light gray column). For control of background staining an isotype mAb was used for labeling. D, MJ and MJ-gB hi cells were cultured for 24 or 48 h. Exosomes were isolated from the supernatant (see Materials and Methods), lysed and separated by SDS-PAGE. SDS gels were immunoblotted for gB (upper panel) and for DRα (middle and lower panels). E, Exosomes derived from MJ and MJ-gB cells were lysed with 0.5% NP-40. Lysates were precipitated against gB using the mAb 2c/2. Immunoprecipitates were separated by 12% SDS-PAGE and Western blotted with mAb TAL-1B5 against DRα-chain. The arrows indicate the positions of the Ig H and L chains and of DRα-chain.
regulate trafficking in the endosomal pathway (33). In dendritic and in transfected cells, ubiquitin modification of DRα and DRβ sub-units was reported to drive sorting to the luminal vesicles of MVBS (34, 19). However, in MJ-gB cells we could not detect significant ubiquitination of the DR subunits (not shown). In contrast, gB showed extensive ubiquitination that is likely to play a role in hijacking DR into the exosome pathway.

Several stimuli were demonstrated to induce or increase the release of exosomes from primary or from tumor cell lines (35). In this study, we have demonstrated that overexpression of a viral protein enforces transfected cells to increase disposal of cellular material. Our data suggest that cellular ubiquitination of gB correlates with increased release of exosomal material.

Sorting of proteins into MVBS, which gives rise to formation of exosomes was shown to transfer MHCII and cosstimulatory molecules to other cell types, indicating that exosomes can transfer MHCII/peptide complexes to other cell types (10, 36, 37). The recipient cells then acquire the ability to present Ag. Many other reports indicate that exosomes mediate intercellular communication during the immune response (reviewed in Refs. 10 and 11). It is conceivable that a transfer of gB or of gB/DR complexes by exosomes from HSV-1-infected cells to other cells may also modulate immune responses to the viral Ag.

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Disclosures
The authors have no financial conflict of interest.

References
Supplementary Figures

Fig. S1. Intracellular trafficking of gB and DR glycoproteins

A) gB was immunoprecipitated from cell lysates of MJ-gB cells using mAb 2c/2. IPs were treated with endoglycosidase H (Endo H) or with PNGaseF overnight. Undigested (lane 1), Endo H (lane 2), and PNGaseF (lane 3) digested gB immunoprecipitates were separated by SDS PAGE and immunoblotted for DRα (mAb TAL-1B5). Arrows indicate the positions of Ig heavy (H) and light (L) chains and of glycosylated and deglycosylated DRα chains. B) gB-immunoprecipitates of MJ (lanes 1 and 5) and of MJ-gB (lanes 2 and 6) cell lysates were immunoblotted for Ii (mAb Bu43, lanes 1 and 2) or for gB (mAb 10B7, lanes 5 and 6). Lanes 3 and 4 show lysates immunoblotted for Ii. The position of Ii is shown on the left. gB and Ig heavy (H) and light (L) chains were indicated on the right.

Fig. S2. Ubiquitination of gB. MJ and MJ-gB cells were lysed and immunoprecipitated for gB. Immunoprecipitates were SDS PAGE separated and western blotted with mAb FK2 for gB (lanes 1 and 2) or for ubiquitin (lanes 3 and 4). The position of gB and of Ab subunits H and L are indicated on the left. The gB immunoprecipitate contains some degradation product of gB (lane 2).
Figure S1