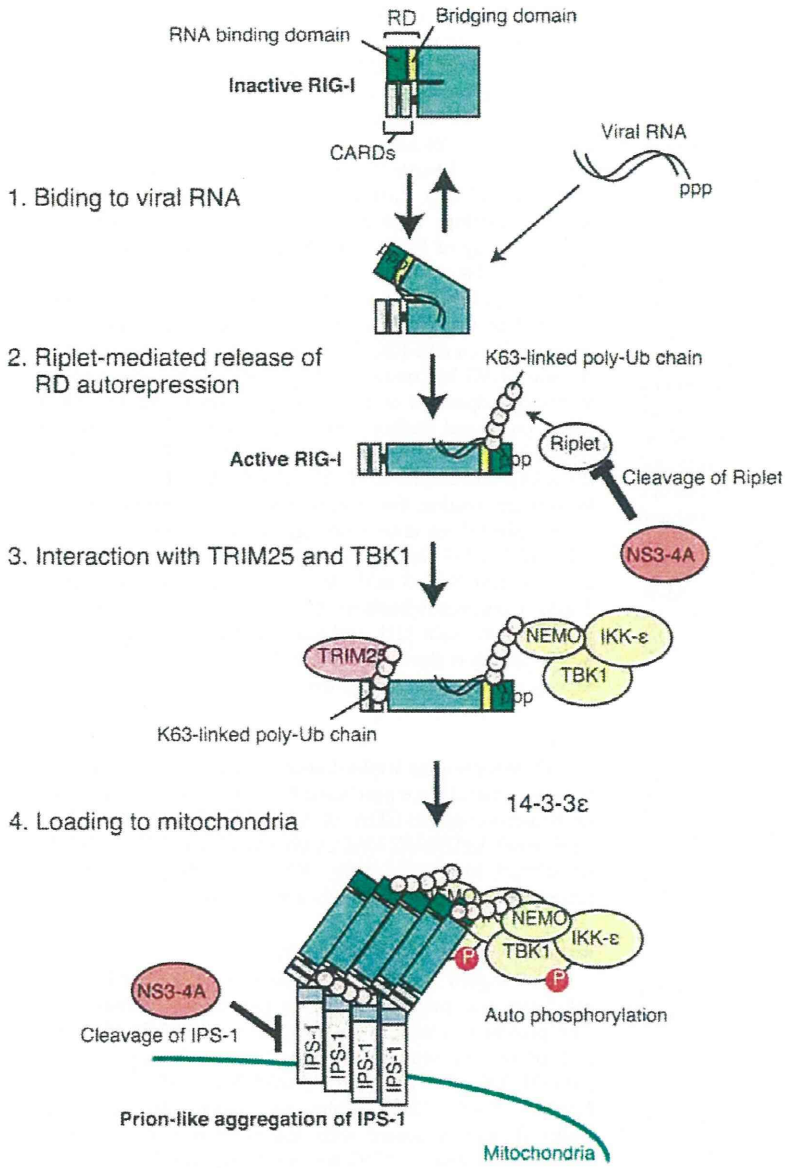

Figure 10. Model for Riplet-mediated RIG-I activation. In resting cell, RIG-I RD represses its CARDs-mediated signaling. When RIG-I CTD associates with viral RNA, Riplet mediates K63-linked polyubiquitination of RIG-I RD, leading to the association with TRIM25 and TBK1. K63-linked polyubiquitin chain mediated by TRIM25 induces RIG-I oligomerization and association with IPS-1 adaptor. TBK1 associated with RIG-I is activated on mitochondria.
doi:10.1371/journal.ppat.1003533.g010

pEF-BOS/FLAG-RIG-I plasmid as a template. The primer sequences are RIG-I 849 851 RR-F: AGT AGA CCA CAT CCC AGG CCA AGG CAG TTT TCA AGT TTT G, RIG-I 849 851 RR-R: CAA AAC TTG AAA ACT GCC TTG GCC TGG GAT GTG GTC TAC T, RIG-I 888R-F: GAC ATT TGA GAT TCC AGT TAT AAG AAT TGA AAG TTT TGT GGT GGA GG, RIG-I 888R: CCT CCA CCA CAA AAC TTT CAA TTC TTA TAA CTG GAA TCT CAA ATG TC, RIG-I 907 909RR-F: GTT CAG ACA CTG TAC TCG AGG TGG AGG GAC TTT CAT TTT GAG AAG, RIG-I 907 909RR-R: CTT CTC AAA ATG AAA GTC CCT CCA CCT CGA GTA CAG TGT CTG AAC. HCV cDNA fragment encoding NS3-4A of JFH1 strain was cloned into pCDNA3.1 (-) vector. The mutation on catalytic site of NS3-4A S139A was constructed by PCR-mediated mutagenesis using primers, NS3-4A S139A-F and NS3-4A S139A-R, and pCDNA3.1 (-)/NS3-4A plasmid as a template. The primer sequences are NS3-4A S139A-F: TTC GAC CTT GAA GGG GTC CGC GGG GGG ACC GGT GCT TTG C and NS3-4A S139A-R: AAG CAC CGG TCC CCC CGC GGA CCC CTT CAA GGT CGA AAG G.

RT-PCR and Real-Time PCR

Total RNA was extracted with TRIZOL (Invitrogen), after which the samples were treated with DNaseI to remove DNA

contamination. Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (ABI). Quantitative PCR analysis was performed using Step One software ve2.0. (ABI) with SYBER Green Master Mix (ABI). HCV ss and dsRNA was in vitro synthesized with SP6 and/or T7 RNA polymerase using 3′ UTR of HCV cDNA as template as described previously [46].

Confocal microscopy

Cells were plated onto microscope cover glasses (matsunami) in a 24-well plate. The cells were fixed for 30 min using 3% formaldehyde in PBS and permeabilized with 0.2% Triton X-100 for 15 min. Fixed cells were blocked with 1% bovine serum albumin in PBS for 10 min and labeled with the indicated primary Abs for 60 min at room temperature. Alexa-conjugated secondary Abs were incubated for 30 min at room temperature to visualize staining of the primary Ab staining. Samples were mounted on glass slides using Prolong Gold (Invitrogen). Cells were visualized at a magnification of ×63 with an LSM510 META microscope (Zeiss). Data collected with confocal microscopy were analyzed with ZEISS LSM Image Examiner software. NS3, RIG-I, TBK1, IPS-1, and p-TBK1 were stained with anti-NS3 goat pAb (abcam), anti-RIG-I mouse mAb (Alme-1, ALEXIS BIOCHEMICALS), anti-NAK (TBK1) rabbit mAb (EP611Y, abcam), anti-MAVS (IPS-1) rabbit pAb (Bethyl Laboratories Inc), and anti-p-TBK1 rabbit mAb (Cell Signaling Technology),

Reporter gene analysis

HEK293 cells were transiently transfected in 24-well plates using FuGene HD (Promega) or lipofectamine 2000 (Invitrogen) with expression vectors, reporter plasmids (IFN-β: p125luc), and an internal control plasmid coding *Renilla* luciferase. The total amounts of plasmids were normalized using an empty vector. Cells were lysed in a lysis buffer (Promega), and luciferase and *Renilla* luciferase activities were determined using a dual luciferase assay kit (Promega). Relative luciferase activities were calculated by normalizing the luciferase activity by control. HCV dsRNA (3′ UTR polyU/UC region) was synthesized using T7 and SP6 RNA polymerase as described previously [46].

Pull-down assay

RNA used for the assay was purchased from JBioS. The RNA sequences are as follows: (sense strand) AAA CUG AAA GGG AGA AGU GAA AGU G; and (antisense strand) CAC UUU CAC UUC UCC CUU UCA GUU U. Biotin was conjugated at the U residue at the 3′-end of the antisense strand (underlined). Biotinylated dsRNA was phosphorylated by T4 polynucleotide kinase (TAKARA). dsRNA was incubated for one hour at 25°C with 10 μg of protein from the cytoplasmic fraction of cells that were transfected with Flag-tagged RIG-I, Riplet, and/or HA-tagged ubiquitin expressing vectors. This mixture was added into 400 μl of lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% Glycerol, 1% NP-40, 30 mM NaF, 5 mM Na_3VO_4, 20 mM iodoacetamide, and 2 mM PMSF) containing 25 μl of streptavidine Sepharose beads, rocked at 4°C for two hours, harvested by centrifugation, washed three times with lysis buffer, and resuspended in SDS sample buffer.

Immunoprecipitation

Splenocytes (1×10^7) were infected with or without VSV at MOI = 10 for eight hours, after which cell extracts were prepared with lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 30 mM NaF, 5 mM Na_3VO_4, 20 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride). Immunoprecipitation used an anti-RIG-I Rabbit monoclonal antibody (D14G6, Cell Signaling Technology). To detect endogenous K63-linked polyubiquitin chain that is ligated to RIG-I, 6×10^7 of mouse splenocyte were infected with SeV at MOI = 0.2 for 24 hours. Immunoprecipitation was performed with anti-RIG-I mAb (D14G6). Anti-K63-linkage specific polyubiquitin (D7A11) Rabbit mAb (Cell Signaling) was used for western blotting. HEK293FT cells were transfected with or without 0.8 μg of HCV dsRNA in a 6-well plate. HCV dsRNA (HCV 3′ UTR polyU/UC region) was synthesized using T7 and SP6 RNA polymerase as previously described [46]. Cell lysates were prepared at the indicated times. Immunoprecipitation was performed with an anti-RIG-I mouse monoclonal antibody (Alme-1). An anti-FLAG M2 monoclonal antibody (Sigma) was used for the immunoprecipitation of FLAG-tagged protein. An anti-TRIM25 rabbit polyclonal antibody (abcam), an anti-p-TBK1 rabbit mAb (Cell Signaling Technology), an anti-NAK (TBK1) rabbit mAb (EP611Y), and an anti-RNF135 (Riplet) pAb (SIGMA), were used for western blotting. For ubiquitination assay, immunoprecipitates were washed three times with high salt lysis buffer ((20 mM Tris-HCl pH 7.5, 1M NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 30 mM NaF, 5 mM Na_3VO_4, 20 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride) to dissociate unanchored polyubiquitin chain [21], and then washed once with normal lysis buffer described above for SDS-PAG analysis. Band intensity was semi-quantified using Photoshop software.

RNAi

siRNAs for human Riplet (Silencer Select Validated siRNA) and negative control were purchased from Ambion. siRNA sequences for Riplet are: (sense) GGA ACA UCU UGU AGA CAU Utt and (anti-sense) AAU GUC UAC AAG AUG UUC CCac. siRNA was transfected into cells using RNAiMax Reagent (Invitrogen) according to the manufacture's instructions.

In vitro NS3/4A cleavage assay

FLAG-tagged Riplet was expressed in HEK293FT cells, and cell lysate was prepared with the lysis buffer described above. The protein was immunoprecipitated with anti-FLAG antibody and protein G sepharose beads, and washed with Buffer B (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40). The samples were suspended in 50 μl of Buffer B, and incubated with 400 ng of recombinant NS3-4A (rNS3-4A) protein at 37°C for one hour, and then subjected to SDS-PAGE analysis. The NS3-4A protein was purchased from AnaSpec Inc (CA). N-terminal GST-fused Riplet (1–210 aa) (rRiplet) was purchased from Abnova. 500 ng of rRiplet was incubated with or without 500 ng of rNS3-4A in 10 μl of reaction buffer (20 mM Tris-HCl (7.5), 4% Glycerol, 5 mM DTT, 150 mM NaCl, 0.1% of Triton-X100, 0.9% polyvinyl alcohol) at 37°C for 30 min.

Accession numbers

The accession numbers are Riplet (BAG84604), TRIM25 (NP_005073), TBK1 (NP_037386), IKK-ε (AAF45307), IPS-1 (BAE79738), RIG-I (NP_055129), and G3BP (CAG38772).

Supporting Information

Figure S1 K63-linked polyubiquitination of RIG-I RD. HA-tagged ubiquitin and FLAG-tagged RIG-I RD expression vectors were transfected into HEK293FT cells. 24 hours after transfection, the cells were infected with VSV at MOI = 1 for six

hours. Then, cell lysate was prepared. Immunoprecipitation was carried out using anti-FLAG antibody. The samples were subjected to SDS-PAGE, and the proteins were detected by western blotting using anti-HA, FLAG, and K63-linked poly-ubiquitin specific antibodies.
(TIF)

Figure S2 Intracellular localization of RIG-I, NEMO, and p-TBK1 proteins. (A) HeLa cells were transfected with HCV dsRNA using lipofectamine 2000 reagent. The cells were fixed six hours after transfection. The microscopic analysis was performed using anti-RIG-I mAb (Alme-1) and anti-NEMO pAb. (B) HeLa cells were transfected with HCV dsRNA using lipofectamine 2000 reagent (Invitrogen). The cells were fixed at indicated hour. The microscopic analysis was performed using anti-RIG-I mAb (Alme-1). (C) HepG2 cells were transfected with HCV dsRNA using lipofectamine 200 reagent. The cells were fixed six hours after the transfection. The microscopic analysis was performed using anti-RIG-I (Alme-1) mAb and anti-p-TBK1 mAb.
(TIF)

Figure S3 NS3-4A of HCV cleaves IPS-1 and Riplet but not IKK-ε. (A) HA-tagged Riplet was transfected into HEK293 cells together with NS3-4A. 24 hours after transfection, cell lysate was prepared and subjected to SDS-PAGE. The proteins were detected by western blotting and CBB staining. (B, C) HA-tagged IKK-ε (B) or IPS-1 (C) expression vectors were transfected into HEK293FT cells with or without NS3-4A of HCV expression vector. 24 hours after the transfection, the cell lysate was prepared, and analyzed by SDS-PAGE. The proteins were detected by western blotting using anti-HA or anti-β actin antibodies. (D) HA-tagged IPS-1 or HA-tagged Riplet expression vector was transfected into HEK293FT cells with or without NS3-4A expression vectors. 24 hours after transfection, cell lysate was prepared and subjected to SDS-PAGE. The proteins were detected by western blotting using anti-HA antibody. (E, F) N-terminal FLAG-tagged Riplet (E) or C-terminal HA-tagged Riplet (F) expression vector was transfected into HEK293FT cells with NS3-4A or NS3-4A*. 24 hours after the transfection, cell lysates were analyzed by SDS-PAGE. (G) HA-tagged wild-type Riplet or mutant Riplet-C21A expression vector were transfected into HEK293FT cells with NS3-4A or NS3-4A*. 24 hours after the transfection, the cell lysate was prepared, and analyzed by SDS-PAGE. The proteins were detected by western blotting using anti-HA or anti-β actin antibodies. (H, I) RIG-I, Riplet, Riplet-3A (H), and/or Riplet C21A (I) mutant expression vectors were transfected into HEK293 cells together with p125luc reporter and *Renilla* luciferase. 24 hours after transfection, luciferase activity was measured.
(TIF)

Figure S4 siRNA for Riplet or control was transfected into HeLa cells in 24-well plate using RNAi MAX (Invitrogen) according to manufacture's protocol. 48 hours after transfection, the cells were transfected with 100 ng of HCV dsRNA. Six hours after transfection, the cells were fixed and stained with anti-RIG-I mAb (Alme-1) and anti-mouse Alexa-488 Ab.
(TIF)

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Author Contributions

Conceived and designed the experiments: HO MMi MMa TS. Performed the experiments: HO MMi. Analyzed the data: HO MMi. Contributed reagents/materials/analysis tools: HO MMi. Wrote the paper: HO MMi TS.

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Journal of Virology

Herpesvirus 6 Glycoproteins B (gB), gH, gL, and gQ Are Necessary and Sufficient for Cell-to-Cell Fusion

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Herpesvirus 6 Glycoproteins B (gB), gH, gL, and gQ Are Necessary and Sufficient for Cell-to-Cell Fusion

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The human herpesvirus 6 (HHV-6) envelope glycoprotein gH/gL/gQ1/gQ2 complex associates with host cell CD46 as its cellular receptor. Although gB has been suggested to be involved in HHV-6 infection, its function in membrane fusion has remained unclear. Here, we have developed an HHV-6A (strain GS)and HHV-6B (strain Z29) virus-free cell-to-cell fusion assay and demonstrate that gB and the gH/gL/gQ1/gQ2 complex are the minimum components required for membrane fusion by HHV-6.

Human herpesvirus 6 (HHV-6), betaherpesvirus subfamily (1), includes two species, A (HHV-6A) and B (HHV-6B) (2–4). HHV-6B mainly infects immune cells, such as $CD4^+$ T-lymphocytes, monocytes, and dendritic cells, and also causes exanthema subitum during primary infection in children (5). HHV-6B can reactivate from latency in immunocompromised patients and cause pneumonitis, hepatitis, and encephalitis (6, 7). However, the molecular basis of HHV-6A pathogenicity is unclear.

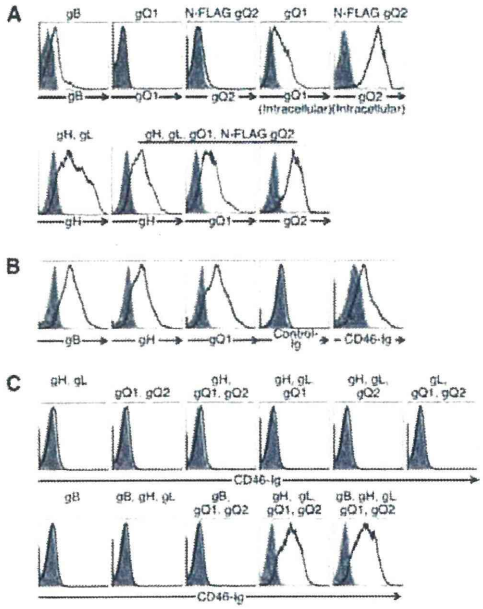

FIG 1 Flow cytometric analyses of cell surface expression of viral glycoproteins in cells transfected with plasmids expressing the glycoproteins. The transfected glycoprotein(s) is shown at the top of each figure panel. gQ2 was FLAG tagged. (A) Expression of HHV-6B glycoprotein(s) in 293T cells transfected with plasmids expressing HHV-6B glycoprotein(s) (black lines) or mock transfected (gray-shaded areas). Cells were stained with anti-gB (H-AR-2; Bioworld Consulting Laboratories), anti-gH, anti-gQ1 (2D6; NIH, AIDS Reagent Program), or FLAG (L5; Biolegend) MAb followed by staining with anti-mouse IgG antibody. (B) Cell surface expresson of HHV-6B glycoproteins in virus-infected cells and association of CD46 with HHV-6B-infected cells. HHV-6B-infected (black lines) or mock-infected (gray-shaded areas) Molt-3 cells were stained with anti-gB, anti-gH, or anti-gQ1 MAb followed by staining with anti-mouse IgG antibody and either CD46-Ig or control Ig (VZV gB-Ig) followed by staining with anti-human IgG Fc portion antibody. (C) Association of CD46 with HHV-6B glycoproteins. 293T cells that were transfected with plasmids expressing HHV-6B glycoprotein(s) (black lines) or mock transfected (gray-shaded areas) were stained with CD46-Ig.

The association of several viral glycoproteins with their respective cellular receptors induces virus envelope-cell membrane fusion during viral entry. It has been reported that HHV-6 gH/gL forms a complex with gQ1 and gQ2 and that this complex binds to CD46, which has been reported to function as a cellular receptor for HHV-6 (8–11). gB and a gH/gL complex are conserved in all herpesviruses and thought to play a pivotal role in membrane fusion and herpesvirus infection (12–17). Studies of gBs and gHs of other herpesviruses have elucidated the molecular mechanisms of virus envelope-cell membrane fusion (18–21). Although some antibodies against HHV-6 gB have been reported to block HHV-6B infection (22, 23), the function of HHV-6 gB during viral infection remains unclear.

To identify the requirement of HHV-6 glycoproteins for virus-induced membrane fusion during the virus infection, each of the glycoproteins was amplified and expressed from HHV-6B (Z29). Briefly, the genomic sequences of gH, gL, gO, gQ1, and gQ2 were amplified from total DNA of HHV-6B-infected Molt3 cells (Riken BRC, Tsukuba, Japan) and cloned into pCAGGS-MCS expression vector (24). For detection purposes, the FLAG epitope was inserted in frame at the N termini of gO and gQ2 genes. The full-length gB gene containing a promoter and poly(A) tail sequences was amplified by recombinant PCR using plasmids containing partial gB sequences (nucleotides [nt] +1 to +1718 and +1713 to +2493). The purified PCR product was used for transient transfection of 293T cells. Expression of transfected genes was analyzed by flow cytometry. gB and the gH/gL complex were detected on the cell surface using anti-gB monoclonal antibody (MAb) and gHA2 antibody, respectively (Fig. 1A) (25). Cells transfected with plasmid encoding gQ1 or N-terminal FLAG-tagged gQ2 ex-

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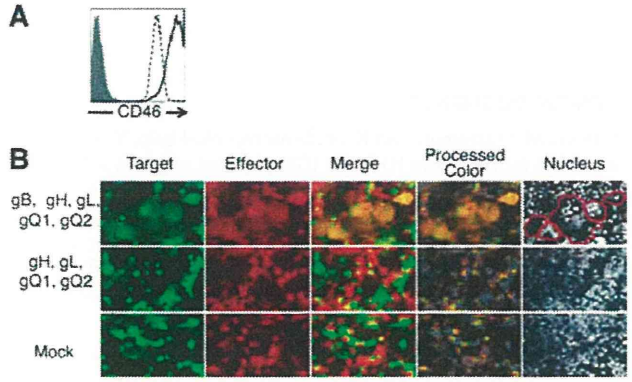

FIG 2 Fluorescence microscopy of fusion of 293T effector and target cells. (A) To quantify CD46 expression on the surface of 293T cells, 293T cells were stained with anti-CD46 MAb (J4.48; Coulter) (dotted line) or with isotype control antibody (gray-shaded area), and CD46-transfected 293T cells were stained with anti-CD46 MAb (solid line) and analyzed by flow cytometry. (B) 293T effector cells were transfected with plasmids expressing HHV-6B glycoproteins or mock transfected with a plasmid expressing DsRed. 293T target cells were transfected with a plasmid expressing CD46 and a plasmid expressing GFP. After 72 h coculture, cells were analyzed by fluorescence microscopy. Cell nuclei were stained with Hoechst 33258 fluorescence dye; blue fluorescence from nuclei appears gray. Fused cells are delineated by red lines.

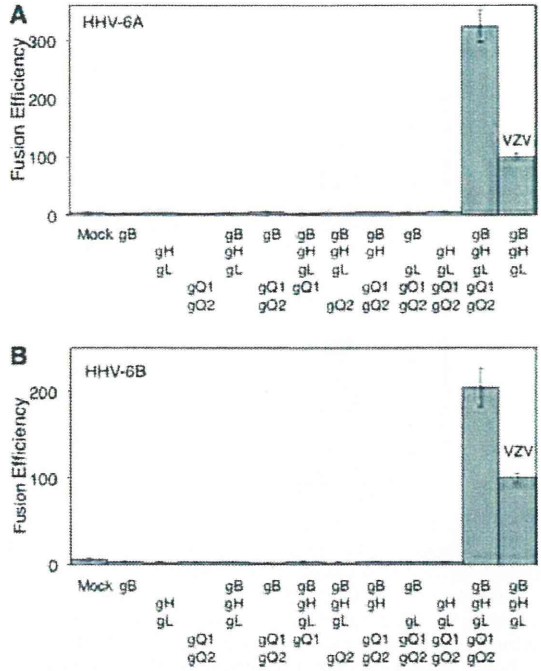

FIG 3 Quantification of cell-to-cell fusion mediated by HHV-6 glycoproteins. (A) 293T effector cells transfected with plasmids expressing HHV-6A glycoproteins, T7 polymerase, and *Renilla* luciferase were cocultured with 293T target cells transfected with plasmids expressing CD46 and firefly luciferase. After 72 h coculture, both luciferase signals were measured. The relative fusion efficiency was calculated as follows: [(HHV-6 firefly luciferase activity/HHV-6 *Renilla* luciferase activity) × 100]/(VZV firefly luciferase activity/VZV *Renilla* luciferase activity). (B) Quantification of cell-to-cell fusion efficiency mediated by HHV-6B glycoproteins was performed as described for HHV-6A in the panel A legend. Error bars show the means ± standard deviations (SD) of the results determined with quadruplicated samples. Data are representative of at least three independent experiments.

pressed the corresponding proteins. gQ1 and gQ2 were detected intracellularly but not on the cell surface, although they were detected on the surface of cells cotransfected with gH and gL. N-terminal FLAG-tagged gO was also expressed only on the surface of cells cotransfected with gH and gL (data not shown). The level of gB expression on HHV-6B-infected cells was higher than on gB-transfected cells. However, the levels of gH and gQ1 expression on transfected cells were higher than on infected cells (Fig. 1A and B).

We then generated a flow cytometry analysis that used CD46-Ig fusion protein to analyze HHV-6B glycoproteins that bind to CD46 (26). CD46-Ig specifically associated with HHV-6B-infected Molt-3 cells but not mock-infected cells (Fig. 1B). The 293T cells which were transfected with HHV-6 glycoprotein(s) and stained with CD46-Ig showed that CD46-Ig did not bind to cells expressing gH and gL, gB alone, or gH, gL, and gB but did bind to cells transfected with gH, gL, gQ1, and gQ2 (Fig. 1C). Expression of gB did not affect CD46-Ig binding to cells expressing gH, gL, gQ1, and gQ2. These results suggested that CD46 associated with a gH/gL/gQ1/gQ2 complex on the cell surface.

To identify HHV-6 glycoproteins that mediate membrane fusion, we developed a HHV-6 virus-free cell-to-cell fusion assay. 293T effector cells were cotransfected with the plasmids expressing HHV-6B glycoproteins and a plasmid expressing DsRed or were mock transfected. 293T target cells were cotransfected with plasmid expressing CD46 and green fluorescent protein (GFP) (Fig. 2A). Effector cells were cocultured with target cells 24 h after transfection. After coculture for 72 h, the cells were analyzed by fluorescence microscopy. As shown in Fig. 2B, yellow, giant, fused cells were observed when effector cells were cotransfected with plasmids expressing HHV-6B gB, gH, gL, gQ1, and gQ2 and cocultured with CD46-transfected target cells. However, no fused cells were found in the absence of gB.

To quantify fusion efficiency, a dual-luciferase reporter assay was used as previously reported (15). 293T effector cells were cotransfected with plasmid expressing HHV-6B glycoproteins, T7 polymerase (pCAGT7), and *Renilla* luciferase (as an internal control) and cocultured with 293T target cells transfected with CD46 and T7 promoter-driven firefly luciferase (pT7EMCluc) for 72 h. Firefly and *Renilla* luciferase activities were then measured, and fusion efficiency was calculated as described in the Fig. 3 legend. The fusion efficiency of varicella-zoster virus (VZV) envelope glycoproteins was measured as a control (15). Cell-to-cell fusion was 10.2-fold more efficient with gB-, gH-, gL-, gQ1-, and gQ2-transfected effector cells than with mock-transfected effector cells (Fig. 3B). In the absence of gB, gH, gL, gQ1, or gQ2, no significant fusion activity was observed. gO of human cytomegalovirus (HCMV) and HHV-6 have been suggested to form a complex with gH and gL, with the complex being involved in HCMV entry (9, 27). However, transfection with HHV-6B gO did not affect cell-to-cell fusion induced by HHV-6B gB, gH, gL, gQ1, and gQ2 (data not shown). This is in agreement with the previous report that gO is not essential for HCMV cell-to-cell fusion (27). CD46-Ig also bound to HHV-6A (strain GS) gH, gL, gQ1, and gQ2 transfectants (data not shown), and cell-to-cell fusion was observed using HHV-6A envelope glycoproteins (Fig. 3A) (28). Furthermore, cell-to-cell fusion using either HHV-6A or -6B glycoproteins was inhibited by both anti-CD46 and anti-HHV-6A gB MAbs (clone 87-y-13), similar to reports in which syncytium formation by HHV-6A was abrogated by these MAbs (Fig. 4) (29, 30). These

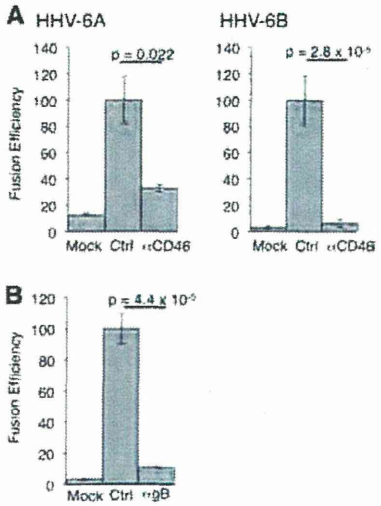

FIG 4 Effect of anti-CD46 and anti-gB MAbs on HHV-6-glycoprotein-mediated cell-to-cell fusion. (A) Cell-to-cell fusion efficiency mediated by HHV-6A and HHV-6B glycoproteins was measured in the presence of anti-CD46 MAb (M75), in the absence of anti-CD46 MAb (Ctrl), and in mock-transfected cells as described in the Fig. 3 legend. Fusion efficiency was calculated as follows: [(firefly luciferase activity/*Renilla* luciferase activity) × 100]/[(firefly luciferase activity/*Renilla* luciferase activity) in control cells]. (B) Cell-to-cell fusion efficiency mediated by HHV-6A glycoproteins was measured in the presence of anti-HHV-6A gB MAb (clone 87-y-13) and in the absence of anti-gB MAb (Ctrl) and in mock-transfected cells as described in the panel A legend. Error bars show the means ± SD of the results determined with quadruplicated samples. The statistical difference was determined by the Student's *t* test. A difference with $P < 0.05$ was considered statistically significant. Data are representative of at least three independent experiments.

results suggested that both HHV-6A and HHV-6B require gB, gH, gL, gQ1, and gQ2 for cell-to-cell fusion.

Cell-to-cell fusion assays were also done *in trans*; i.e., some cells were transfected only with plasmid(s) gB, gH/gL, and/or gQ1/gQ2 and other cells were transfected with plasmids expressing all the other glycoproteins. Little cell-to-cell fusion was observed in *in trans* fusion assays (data not shown). These results suggested that *cis* expression of HHV-6 gB, gH, gL, gQ1, and gQ2 is required for cell-to-cell fusion, unlike that of herpes simplex virus (HSV) and HCMV, in which all the envelope glycoproteins do not need to be expressed on the same cell (17, 31).

This is the first report showing that the HHV-6A and HHV-6B envelope glycoproteins gB, gH, gL, gQ1, and gQ2 are required for cell-to-cell fusion. Herpesviruses enter via two different pathways: (i) direct fusion of the viral envelope with the host cell membrane or (ii) endocytosis followed by fusion between the viral envelope and endosomal membranes (32). Since membrane fusion is needed for herpesvirus entry, our results are consistent with previous reports that anti-gB, -gH, and -gQ1 antibodies block HHV-6 infection (22–24, 33–36). Moreover, our results are also supported by an earlier report that gB and gH are required for polykaryocyte formation after virus infection of permissive cells in cell culture (29). Considering that gBs and gHs of other herpesviruses associate with their respective cellular receptors during viral entry and cell-to-cell fusion (15, 26, 37–41), HHV-6 gB may also mediate viral entry and cell-to-cell fusion by interaction with cellular receptors that are currently unknown in addition to the binding of the gH, gL, gQ1, and gQ2 complex to its receptor CD46. The virus-free HHV-6 fusion assay system developed in this study should help elucidate the HHV-6 entry mechanism.

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Hepatitis C Virus Infection Induces Inflammatory Cytokines and Chemokines Mediated by the Cross Talk between Hepatocytes and Stellate Cells

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Inflammatory cytokines and chemokines play important roles in inflammation during viral infection. Hepatitis C virus (HCV) is a hepatotropic RNA virus that is closely associated with chronic liver inflammation, fibrosis, and hepatocellular carcinoma. During the progression of HCV-related diseases, hepatic stellate cells (HSCs) contribute to the inflammatory response triggered by HCV infection. However, the underlying molecular mechanisms that mediate HSC-induced chronic inflammation during HCV infection are not fully understood. By coculturing HSCs with HCV-infected hepatocytes *in vitro*, we found that HSCs stimulated HCV-infected hepatocytes, leading to the expression of proinflammatory cytokines and chemokines such as interleukin-6 (IL-6), IL-8, macrophage inflammatory protein 1α (MIP-1α), and MIP-1β. Moreover, we found that this effect was mediated by IL-1α, which was secreted by HSCs. HCV infection enhanced production of CCAAT/enhancer binding protein (C/EBP) β mRNA, and HSC-dependent IL-1α production contributed to the stimulation of C/EBPβ target cytokines and chemokines in HCV-infected hepatocytes. Consistent with this result, knockdown of mRNA for C/EBPβ in HCV-infected hepatocytes resulted in decreased production of cytokines and chemokines after the addition of HSC conditioned medium. Induction of cytokines and chemokines in hepatocytes by the HSC conditioned medium required a yet to be identified postentry event during productive HCV infection. The cross talk between HSCs and HCV-infected hepatocytes is a key feature of inflammation-mediated, HCV-related diseases.

Hepatitis C virus (HCV) can cause chronic liver disease, which can progress to fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (1). Clearance of HCV during the acute phase of infection is associated with a robust CD4 and CD8 T-cell response to multiple viral epitopes (2). However, clearance of HCV infection often fails because of an intermediate cytotoxic T-cell response that is unable to eliminate the infection but causes hepatocyte destruction. T-cell-mediated hepatocytotoxicity poses a high risk for progression to chronic liver inflammation and damage (3). During chronic HCV infection, chemokine-chemokine receptor interactions are particularly important for the recruitment of T cells to sites of inflammation in the liver. Liver-infiltrating lymphocytes in HCV patients exhibit increased expression of CXCR3 and CCR5 (4). Moreover, intrahepatic chemokines, such as RANTES, macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and IP-10, are elevated in HCV patients (5), and intrahepatic proinflammatory cytokine levels are correlated with the severity of inflammation and liver fibrosis (6).

The induction of proinflammatory cytokines and chemokines is triggered by viral proteins and double-stranded RNA (dsRNA) from HCV. The HCV core protein induces inflammatory cytokines through the STAT3 signaling pathway (7). Retinoic acid-inducible gene I (RIG-I) and Toll-like receptor 3 (TLR-3) are cellular sensors that recognize HCV dsRNA, resulting in production of chemokines such as interleukin-8 (IL-8), RANTES, MIP-1α, and MIP-1β (8, 9). Recently, an alternative mechanism for HCV-induced inflammation was reported. It was demonstrated that NS5B, the viral RNA-dependent RNA polymerase (RdRp), catalyzes production of small RNA species that trigger an innate immune response, leading to the production of both interferon (IFN) and inflammatory cytokines (10).

Hepatic stellate cells (HSCs) represent 5 to 8% of the total human liver cells and reside in the Disse space (11). Activation or transdifferentiation of HSCs is regulated by growth factors, including transforming growth factor β (TGF-β), which are associated with pathological conditions such as liver injury, cirrhosis, and cancer (11, 12). During liver injury, quiescent HSCs become activated and convert into highly proliferative, myofibroblast-like cells, which produce inflammatory and fibrogenic mediators (13). In a human hepatoma model, the cross talk between tumor hepatocytes and activated HSCs induced an inflammatory response, and the amounts of cytokines and chemokines associated with hepatocyte-HSC cross talk correlated to HCC progression (14).

Although direct induction of liver inflammation by HCV infection through cellular sensors or HCV proteins is well documented, little is known about the mechanisms governing the proinflammatory cytokines and chemokines that are produced during the interactions between HCV-infected hepatocytes and HSCs. Here, we show that HSCs can act as an inflammatory mediator to HCV-infected cells. Infection of hepatocytes with HCV

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