The Human Herpesvirus 6 G Protein-Coupled Receptor Homolog U51 Positively Regulates Virus Replication and Enhances Cell-Cell Fusion In Vitro

Zhu Zhen,1† Birgit Bradel-Tretheway,1† Sarah Sumagin,2 Jean M. Bidlack,2 and Stephen Dewhurst1,3*

Departments of Microbiology and Immunology1 and Pharmacology and Physiology,2 and Cancer Center,3 University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

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Human herpesvirus 6 (HHV-6) is a ubiquitous T-lymphotropic betaherpesvirus that encodes two G protein-coupled receptor homologs, U12 and U51. HHV-6A U51 has been reported to bind to CC chemokines including RANTES, but the biological function of U51 remains uncertain. In this report, we stably expressed short interfering RNAs (siRNAs) specific for U51 in human T cells and then infected these cells with HHV-6. Viral DNA replication was reduced 50-fold by the U51 siRNA, and virally induced cytopathic effects were also inhibited. In contrast, viral replication and syncytium formation were unaltered in cells that expressed a scrambled derivative of the siRNA or an irrelevant siRNA and were restored to normal when a human codon-optimized derivative of U51 was introduced into cells containing the U51 siRNA. To examine the mechanism whereby U51 might contribute to viral replication, we explored the signaling characteristics of U51. None of the chemokines and opioids tested was able to induce G protein coupling by U51, and no evidence for opioid ligand binding by U51 was obtained. The effect of U51 on cell-cell fusion was also evaluated; these studies showed that U51 enhanced cell fusion mediated by the G protein of vesicular stomatitis virus. However, a U51-specific antisense had no virus-neutralizing activity, suggesting that U51 may not be involved in the initial interaction between the virus particle and host cell. Overall, these studies suggest that HHV-6 U51 is a positive regulator of virus replication in vitro, perhaps because it may promote membrane fusion and facilitates cell-cell spread of this highly cell-associated virus.
ity to enhance the cell-cell spread of this highly cell-associated human herpesvirus.

**MATERIALS AND METHODS**

**Vector construction.** The U51 wild-type genes (U51nc0) were amplified by standard PCR methods; HHV-6A U51 was cloned from strain U1102. A simian virus 5 (SV5) epitope tag was introduced at the N terminus of U51, and KpnI-EcoRV restriction sites were added to facilitate cloning into the expression vector pcDNA3 (Invitrogen). The primer sets used for adding the SV5 tag was 5'-GGAGTTACCCACATGGGAGGAGCCATCCCAACCCCCCTGCTGGGCTTGGCAAGACCCCGGAG-3' and 5'-GGGCGTGGACAGACCGAGGAGGAGGACGAAGAAGAAGCTTGTGT-3'.

The human codon-optimized (CO) U51 genes were assembled from synthetic oligonucleotides and cloned into pLcPscript (Geneart, Regensburg, Germany), as previously described (9). Note that the amino acid sequences encoded by these CO genes are identical to their wild-type counterparts (9). HHV-6A U51co was then restricted with HindIII and ApaI and cloned into pLcEGFP-N1 retroviral vector (Clontech).

A truncated version of HHV-6A gB without the putative N-terminal signal peptide and C-terminal transmembrane region (nucleotides 23 to 652) was amplified and inserted into the corresponding HHV-6A strain U1102 cosmid DNA clone (36) and then inserted into the Smal-PstI sites of pDisplay plasmid vector (Invitrogen), which contains a signal peptide and a hemagglutinin (HA) epitope tag at the N terminus and a platelet-derived growth factor receptor transmembrane domain at the C terminus. The following primer sets were used for amplification: 5'-TACCCCGGAATATGCCTCATTATATACGACCGGAACT-3' and 5'-TTCGGGACTGCATCTTGGAATTAA-3' (sense) and 5'-TCGGGACTGCATCTTGGAATTAA-3' (antisense). The TaqMan probe used was 5'-TTTCCCAGATGTTTGCTACCAT-3'. GAPDH primers and probes (assay-on-demand reagent) were obtained from a commercial supplier (ABI).

**RNA extraction and real-time PCR.** Total RNA was prepared from SupT1 cells that had been infected with HHV-6 by using High Pure RNA Isolation kits (Roche). Primer extension reactions were performed with Script II First-strand cDNA Synthesis kits (Invitrogen) using oligo(dT) primer, in accordance with the manufacturer's instructions. mRNA expression levels of each gene were quantified by TaqMan real-time reverse transcription-PCR (RT-PCR) using U51specific primers and 5'-UTR specific TaqMan probe. A U51-specific primer set was 5'CCAAGGCGTCGCTGGAAAGGGT-3' (sense) and 5'-TCGACATCTGAGACCGTACGA-3' (antisense). The TaqMan probe used was 5'-TTTTTCCTAGATGTTTGCTACCAT-3'. GAPDH primers and probes (assay-on-demand reagent) were obtained from a commercial supplier (ABI).

**Real-time quantitative DNA-PCR.** The viral DNA load in HHV-6A U1102-infected cells was quantified by TaqMan real-time PCR. The HHV-6A U38 polymerase gene was chosen as a target gene for this purpose, and primer sets used for amplification of U38 were 5'-TGGTCTTGTAACGGTCTTGGGGA-3' (sense) and 5'-TGGTCTTGTAACGGTCTTGGGGA-3' (antisense). A standard curve for U38 DNA quantification was generated by using serially 10-fold diluted plasmid DNA containing the relevant gene sequence. Culture supernatants of virally infected cells were treated with proteinase K. Plasmid DNA was extracted using Wizard DNA extraction kits (Promega). This was used as the template in our experimental assays and was analyzed with a Bio-Rad iCycler. Amplification of standard and sample DNAs was conducted in the same 96-well reaction plate (Bio-Rad) under the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. The detection limit is about 10 copies/reaction. All standards and samples were assayed in triplicate.

**Neutralization assay.** The U51-specific antisera we used was a polyclonal rabbit antiserum directed against HHV-6B U51 (raised against a purified synthetic peptide spanning the third predicted extracellular loop of HHV-6B U51 [CHLPKAA4LSEIESDDK]; there is only a single amino acid difference between HHV-6A and -6B within this region, which is denoted by the underlined residue; note that this same peptide was previously used by Menotti and colleagues to generate a U51-specific antiserum in rabbits [31]). The 15mer peptide was synthesized by SigmaGenosys and injected into rabbits for antibody production. After affinity purification using a peptide-conjugated column, the purified antibody was able to detect both HHV-6A and HHV-6B U51 effectively (down to a dilution of 1:1,000) in an indirect immunofluorescent assay on virus-infected cell cultures (unpublished data). Purified U51 antisera was incubated with 200 μl of HHV-6A U1102 virus supernatant in a total volume of 500 μl at 37°C for 1 h. After that, infection was performed as described above. Note that the antisera was not preincubated and could have been providing immediate complement-directed lysis of virus particles in the event that complement-fixing antibodies were bound to cell-free viruses.

**Opioid receptor binding assay.** To determine if HHV-6B U51 bound opioids, CHO-CAR cells were infected with a recombinant adenovirus that expressed the
human codon-optimized HHV-6B U51 open reading frame (HHV6BCOwt) using methods previously described (59). Membranes from these cells were then prepared and incubated with opioids that were selective for the µ (\(^{[3]}\)H)DAMGO, 5 nM), δ (\(^{[3]}\)H)naltrindole, 1 nM, \(^{[3]}\)H)DPDPE, 10 nM), and κ (\(^{[3]}\)H)U69-593, 5 nM; \(^{[3]}\)H)remazolium) receptors. Also, the nonselective antagonist \(^{[3]}\)H)diprenorphine was tested to determine if HHV-6B U51 would bind this nonselective high-sensitivity opioid. Nonselective binding was measured by the inclusion of either 10 µM naloxone or 10 µM of the unlabeled compound. After a 60-min incubation, binding was terminated by filtering the samples through Schleicher & Schuell no. 32 glass fiber filters (Keene, NH) using a Brandel 48-well cell harvester. Filters were soaked for at least 60 min in 0.25% polyethyleneimine for \(^{[3]}\)H)naltrindole and \(^{[3]}\)H)U69-593 binding experiments. After filtration, filters were washed three times with 3 ml of cold 50 mM Tris-HCl, pH 7.5, and were counted in 3 ml of scintillation fluid using bovine serum albumin as the standard. The membranes were frozen at -80°C until use. HEK293 cell membranes as described above (15) were incubated with 11 different ligands (ICI, 10 nM; DAMGO, 100 nM; Met-enkephalin, 1 µM; the selective peptide [DPDPE, 1 µM; the selective antagonist naltrindole, 1 µM; and the selective peptide DAMGO, 1 µM) in assay buffer for 60 min at 30°C in a final volume of 0.5 ml. The reaction mixture consisted of 50 mM Tris-HCl, 3 mM MgCl2, 100 mM NaCl, and 0.2 mM EGTA, pH 7.4. The membranes were vortexed, followed by centrifugation at 40,000 x g for 30 min at 4°C. The membrane pellet was resuspended in membrane buffer, and the centrifugation step was repeated. The membranes were then resuspended in assay buffer, which consisted of 50 mM Tris-HCl, 3 mM MgCl2, 100 mM NaCl, and 0.2 mM EGTA, pH 7.4. Assay incubation was performed in membrane buffer (10 µg of membrane protein) for 10 min at 4°C, followed by three washes with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.5. Samples were counted in 3 ml of scintillation fluid. Data represent the percent binding and are presented as relative light units. The selected siRNA targeting sequences were then subjected to a BLAST search against the entire nonredundant nucleotide sequence database in order to ensure that only the intended viral gene would be recognized. These siRNAs were then cloned into a linearized pSuppressorRetro (pSR) vector downstream of the U6 promoter. To screen the functional activity of these siRNA constructs, we cotransfected HEK293 cells with a plasmid expression vector encoding an SV5 epitope-tagged derivative of HHV-6A U51 plus the various siRNA-carrying pSR vectors (as well as constructs carrying an irrelevant control siRNA). The U51 protein expression level was then examined by Western blot analysis using a monoclonal antibody directed against the SV5 epitope tag. As shown in Fig. 1A, the expression of U51 protein (around 30 kDa) was markedly down-regulated by both si6U51-812 and si6U51-130 (over 80%) but not by the irrelevant siRNA (sINeg.Contrl.) or the empty vector alone. These results demonstrate that siRNAs can specifically and efficiently inhibit U51 protein expression in mammalian cells in a transient transfection system. Since the viral envelope glycoprotein B (gB) is known to be essential for replication of herpes simplex virus type 1, CMV, and other herpesviruses, siRNAs directed against HHV-6 gB were designed for use as a positive control in experiments aimed at testing the effect of U51-specific siRNAs on viral replication. The gB-specific siRNAs were tested using a similar approach to that described for the U51 siRNAs. As shown in Fig. 1B, transient expression of HHV-6 gB was efficiently blocked (over 90%) by both of the gB siRNAs that were tested. We therefore selected the HHV-6A gB-specific siRNA (si6gB-A861) for use in our subsequent experiments.
Cell lines stably expressing siRNA-U51 suppressed U51 expression upon virus infection. In order to examine the role of U51 in HHV-6A replication, we set out to derive stable cell lines that expressed one of our U51 siRNAs (si6U51-812 and si6U51-130). For these experiments, we elected to use a cell line that would be highly susceptible to HHV-6A infection; we therefore chose to work with SupT1 cells. These lymphoid suspension cells are difficult to transfect by standard means (electroporation or lipid-mediated DNA transfer), and we therefore created retroviral vectors that expressed a short hairpin RNA which would be expected to direct the generation of U51-specific short interfering RNA. SupT1 cells were then transduced with recombinant retrovirus particles and subjected to G418-mediated selection, and single colonies were picked and expanded. To confirm the specific gene silencing effect of siRNA-U51 in SupT1 cells, we then infected the cells with HHV-6A, and U51 mRNA levels were quantified 24 h postinfection (Fig. 2). After normalization of U51 expression data (using GAPDH mRNA levels as an internal control), we determined that U51 mRNA was decreased by over 90% in cells stably expressing si6U51-812 or si6U51-130 relative to unmodified SupT1 cells or SupT1-siNeg.Ctrl. cells that were infected with HHV-6A. Moreover, the growth properties of the clonal, siU51-expressing SupT1 sublines were found to be indistinguishable from parental SupT1 cells (data not shown).

U51-specific siRNA inhibited HHV-6A replication and virally induced syncytium formation. To test whether expression of a U51-specific siRNA would have any effect on virus replication in vitro, a panel of siRNA-expressing SupT1 sublines was infected with HHV-6A strain U1102 at an MOI of 0.1 TCID₅₀/cell. These experiments were performed using several independent clonal SupT1 cell lines, each of which stably expressed a U51-specific siRNA (si6U51-812 or si6U51-130), as well as cells stably expressing siNeg.B and cells that expressed an irrelevant control siRNA (siNeg.Ctrl.). Six days later, when these cultures were examined under the light microscope, a significant reduction was detected in virally induced cytopathic effects (syncytium formation) in those cultures which expressed either the U51-specific siRNA or the gB-specific siRNA; no change in virally induced syncytium formation was detected in cells that expressed the irrelevant control siRNA (Fig. 3A to D).

Virus replication in these cultures was also examined by performing a quantitative real-time DNA PCR assay using...
Expression of a codon-optimized form of U51 can restore virus replication in SupT1 cells that express a U51-specific siRNA. To determine whether the inhibitory effect of the U51 siRNA on virus replication was indeed due to a specific effect on U51 gene expression, we performed an "add-back" experiment. For this purpose, we took advantage of an available, human codon-optimized (CO) version of the U51 ORF. This synthetic ORF encodes the authentic U51 protein but does so using altered codons relative to the wild-type U51 gene (9). As a result, the expression of the codon-optimized U51 ORF should be resistant to inhibition by our U51 siRNA. We verified this by performing transient transfection experiments analogous to those shown in Fig. 1A; these studies revealed that the expression of the CO-U51 gene was indeed unaffected by the si6U51-812 siRNA (data not shown).

A recombinant retrovirus expressing the U51-CO gene was then constructed and used to transduce SupT1 cells that expressed the si6U51-812 siRNA, at an MOI of 10. This construct has previously been shown to result in high levels of U51 expression, both intracellularly and on the surface of all cell types that we have analyzed (9).

Twenty-four hours after retroviral transduction, the cells were then infected with HHV-6A U1102 at an MOI of 0.1 TCID50/cell. Virally induced cytopathic effects, virus load, and cell growth properties were then measured 6 days later. The results, which are presented in Fig. 4, show that coexpression of the codon-optimized U51 ORF restored virally induced cytopathic effects and viral replication in the SupT1(si6U51-812) cell line.

Virus infectivity was not affected by a U51-specific antibody. Previous studies have shown that 7-transmembrane receptors encoded by the human and mouse cytomegaloviruses (UL33, M28) may be incorporated into enveloped virus particles (30, 37). This suggested to us the possibility that HHV-6 U51 might play a role in virion attachment or entry to target cells. We therefore designed an experiment to test this hypothesis.

Briefly, we mixed HHV-6A virions with an affinity-purified polyclonal antiserum directed against U51 and then tested whether this had any neutralizing effect on virus infectivity. As controls, we used an irrelevant antiserum (directed against a nonconserved peptide from HHV-7 U51) as well as a human antiserum known to contain high levels of virus-neutralizing antibodies. After incubation with these various antisera for 1 h at 37°C, the HHV-6A inoculum was then added to SupT1 cells, and viral load in culture supernatants was then measured 5 days thereafter by quantitative DNA PCR analysis (Fig. 5). As expected, viral replication was essentially abolished in the cultures that received virions premixed with the positive control human serum. In contrast, there was no significant difference in the level of viral replication in cultures that received untreated virus inocula, inocula preincubated with the HHV-6 U51-specific antiserum, or inocula that were treated with the irrelevant antiserum. It is important to note that the U51-specific antiserum was not heat inactivated and thus would have been expected to be capable of mediating complement-directed lysis of virus particles had it bound to cell-free virions. Thus, these data suggest that U51 is most likely not involved in the initial interaction between HHV-6 virions and their target cells. However, this does not rule out the possibility that U51 may be involved either in modulating host cell signaling, so as to favor more efficient virus replication, or in the cell-cell spread of virus, perhaps by promoting fusion of virus-infected cells with virus-negative targets, as has been previously suggested for HCMV US28 (39).

U51-mediated cell signaling. In order to examine whether U51 might contribute to cell signaling events, we performed a series of experiments to examine both ligand binding and G protein coupling. For this set of experiments, we paid particular attention to the possibility that U51 might interact with opioid ligands in light of the previously noted similarity between U51 and human opioid receptors (23). For our initial ligand binding experiments, we transfected cells with recombinant adenovirus vectors that encoded a human codon-optimized form of U51, because it has been shown previously in our lab that codon optimization will enhance U51 expression 10- to 100-fold in mammalian cells (9). As noted previously, use of the codon-optimized constructs permits cell surface expression of U51, even in cell lines that are not of T-cell lineage (9); this contrasts with results reported by Menotti and
colleagues, using a non-codon-optimized expression system that probably resulted in lower total levels of protein expression (31).

Briefly, our ligand binding studies revealed that membranes from cells which expressed the HHV-6B U51 protein did not specifically bind the μ-selective opioid, [3H]DAMGO, the δ-selective opioid, [3H]naltrindole or [3H]DPDPE, or the κ agonist, [3H]U69,593 or [3H]bremazocine. Also, HHV-6B U51 did not specifically bind the nonselective opioid receptor antagonist [3H]diprenorphine (data not shown).

FIG. 3. Effect of U51 knockdown on virus replication and syncytium formation. SupT1 cells stably expressing siRNA targeting U51, gB, or an irrelevant sequence (siNeg.Ctrl.) were infected with HHV-6A strain U1102 at an MOI of 0.1 TCID_{50}/cell. Virally induced cytopathic effects were then examined in the cultures at 6 days postinfection. The photomicrographs shown were taken on an Olympus IX81 microscope under bright-field illumination; final magnification is 10×. The various panels correspond, respectively, to (A) SupT1 cells expressing an irrelevant siRNA (siNeg.Ctrl.) or (B) a gB-specific siRNA (si6gB) as well as two different clonal SupT1 cell sublines, each of which expresses a U51-specific siRNA, (C) si6U51-812 and (D) si6U51-130. It can be readily appreciated that virally induced syncytium formation was greatly reduced in the SupT1 cells that expressed either the gB-specific siRNA or the two U51-specific siRNAs. (E) Cell-free supernatants were collected from virus-infected SupT1 cultures at 6 days postinfection, and virus genomic DNA in the supernatant was measured by quantitative DNA PCR analysis using primers and TaqMan probes specific for the HHV-6 U38 gene. The data shown are from the same samples as in panels A to D; the results are representative of three separate experiments. The viral DNA copy number in SupT1 cells stably expressing either si6AgB or si6U51 were both significantly different from the viral DNA copy number in control SupT1 cells that were also infected with HHV-6 (P < 0.05 for each pairwise comparison between the three experimental cell lines and the control SupT1 cells). The detection sensitivity of the assay is about 10 copies.
The \[^{35}\text{S}]\text{GTP}_{\gamma}\text{S} \) assay was then used to determine if opioids or a selected subset of chemokines could stimulate \[^{35}\text{S}]\text{GTP}_{\gamma}\text{S} \) binding mediated by HHV-6B U51. Three different sets of HEK293 cell membranes were used in experiments, including those from wild-type 293 cells and cells stably transfected with a Tet-inducible expression plasmid carrying HHV-6B U51 (membranes were prepared from these cells either in the absence of U51 induction or following addition of 1 \(\mu\)g/ml tetracycline for 24 h, which resulted in a 50- to 100-fold induction of U51 expression at both the RNA and protein levels [Fig. 6]). Membranes from these different sets of HEK293 cells were tested with chemokines and opioids to determine if any chemokines or opioids stimulated the coupling of the U51 protein to G proteins. Table 1 shows that none of the chemokines or opioids tested had a significant effect on \[^{35}\text{S}]\text{GTP}_{\gamma}\text{S} \) binding.

Overall, we were unable to find any evidence for opioid ligand binding or opioid-induced G protein coupling by HHV-6B U51, and we therefore turned our attention to the possibility that U51 might influence cell membrane fusion events, as has been described previously for HCMV US28 (39).
FIG. 5. Virus infectivity was unaffected by an antibody specific for U51. Two-hundred microliters of an HHV-6A virus stock (strain U1102) was preincubated with either 5 μl of human plasma (“baby plasma”) or 6 μg of affinity-purified rabbit antisera specific for HHV-6B U51 (anti-6B U51) or HHV-7 U51 (anti-7 U51) for 1 h at 37°C. The virus-antiserum mixture was then added to SupT1 cells (approximate MOI of 0.1 TCID50/cell). Six days later, cell-free culture supernatants were collected and viral genomic DNA was measured by a quantitative PCR assay as previously described. The results are representative of three separate experiments. As expected, the human plasma efficiently neutralized HHV-6A infectivity (P < 0.05; two-tailed t test); in contrast, the U51-specific antisera had no such effect (P = 0.117; two-tailed t test).

Coexpression of U51 and vesicular stomatitis virus (VSV) G glycoprotein enhanced cell fusion. Membrane fusion events are important for viral entry into host cells and also for cell-to-cell spread of virus. To examine whether U51 facilitates virus replication and spread by contributing to membrane fusion, we used a luciferase-based gene reporter assay to quantify cell fusion events. This assay relies on the presence of the HIV-1 transactivating protein (Tat) in one cell and a Tat-inducible reporter gene cassette (firefly luciferase linked to the HIV-1 LTR) in the other cell. Upon fusion of the target and effector cells, Tat will activate luciferase transcription, and luciferase expression can then be detected and quantitated by a luminometer. Because the contents of the effector and target cells must mix in order for the HIV Tat to transcribe the luciferase gene, the level of luciferase activity represents the extent of fusion between the effector and target cells.

Equal numbers of HEK293 cells were transiently transfected with a vector expressing either HIV Tat or luciferase under the transcriptional control of the HIV LTR. All cells also received a plasmid clone encoding pVSV-G, in the presence or absence of avian cells that carry HHV-6 U51, the rat kappa opioid receptor (as a negative control), or HCMV US28 (as a positive control) (39). Four hours after transfection, the two cell populations were trypsinized and mixed together at a 1:1 ratio. Forty-four hours thereafter, the cell fusion activity was quantitatively determined by measuring luciferase gene expression in the lysates of the cocultured cells (Fig. 7). As expected, cells coexpressing US28 and VSV-G exhibited an increased level of fusion activity (~3-fold) compared to cells transfected with VSV-G alone. Cells coexpressing VSV-G plus HHV-6A U51 also showed enhanced high fusion activity (~2-fold) compared to cells transfected with VSV-G alone, while the kappa opioid receptor expression plasmid had no effect on cell fusion.

DISCUSSION

Herpesvirus genomes contain homologs of many important cellular genes. Most notable among these are the numerous

![Image](http://jvi.asm.org/Downloaded from http://jvi.asm.org/ on November 11, 2017 by guest)
and U51, about which very little is known other than the fact viruses (HHV) 6 and 7. These viruses encode two GPCRs, U12 recently discovered human betaherpesviruses, human herpesviruses (7, 27, 55), indicating that RNAi represents a powerful tool with which to study protein function in the context of virus replication. In order to examine the function of U51 in virus replication, we used a retroviral RNAi system, applied this technology to the study of key genes in other herpesviruses (7, 27, 55), suggesting U51 may not play a role in the initial interaction between HHV-6B U51 and opioid ligands. Collectively, therefore, these findings suggest that despite the homology between U51 and opioid receptors (23), HHV-6B U51 does not appear to interact functionally with opioid ligands.

While we were unable to obtain any evidence for a functional interaction between HHV-6B U51 and opioid ligands, we did obtain strong evidence to suggest that U51 has the ability to modulate membrane fusion events triggered by other viral proteins. Our finding that the addition of a U51-specific antiserum to virus particles failed to neutralize virus infection suggests U51 may not play a role in the initial interaction between the virus particle and the host cell. This could be because U51 is not present in the virus particle, or it may reflect other possible explanations; further experiments will be needed to resolve this question. Nonetheless, the ability of U51 to enhance viral protein (VSV-G)-mediated cell-cell fusion suggests an alternative mechanism whereby U51 might facilitate the spread and replication of HHV-6. This finding is consistent with previous studies on HCMV US28, in which Pleskoff and coworkers reported that US28 enhanced cell-cell fusion mediated by different viral proteins (including the G protein of vesicular stomatitis virus, VSV-G) (39). These workers concluded that US28 might play a role in the fusion of the HCMV envelope with target cells (39). This view finds precedent in other studies, which show that US28 and cellular 7-tm receptors such as CCR-5 and CXCR-4 can promote fusion events between the HIV-1 envelope and its target cells (2, 19, 20, 40). Nonetheless, the precise mechanism by which HCMV

7-transmembrane (7-tm), G protein-coupled receptor (GPCR) homologs that are present within human herpesviruses (42, 46). In most cases, the biological function(s) of these proteins remains poorly understood, and this is especially true of the recently discovered human betaherpesviruses, human herpesviruses (HHV) 6 and 7. These viruses encode two GPCRs, U12 and U51, about which very little is known other than the fact that both receptors have the capacity to bind certain β-chemokines (notably, RANTES) (26, 33, 35).

In order to understand better the role that U51 plays in the life cycle of HHV-6, we decided to use RNA interference (RNAi) technology to selectively inhibit U51 gene expression in virally infected cells. Other researchers have successfully applied this technology to the study of key genes in other herpesviruses (7, 27, 55), indicating that RNAi represents a powerful tool with which to study protein function in the context of virus replication. In order to examine the function of U51 in virus replication, we used a retroviral RNAi system, since this allowed for the efficient and stable suppression of U51 expression in virus-infected cell lines. Silencing of U51 expression was found to reduce viral RNA replication by about 50-fold, and virally induced cytopathic effects were also blocked. Most importantly, virus replication was restored to normal when a human codon-optimized derivative of U51 was introduced into cells containing the U51 siRNA, indicating that the RNAi-mediated effect was specific to U51.

Previous studies on herpesvirus 7-transmembrane (7-tm) receptors have generally concluded that these genes are dispensable for in vitro replication of virus; examples include HCMV US28 (52), HCMV UL33 (30), mouse CMV (MCMV) M33 (15), rat CMV (RCMV) R33 (6), and HCMV UL78 (32). However, deletion of the MCMV M78 gene has been shown to reduce virus replication in cultured fibroblasts (37), and deletion of the RCMV R78 gene also results in attenuation of virus production in cell culture systems (5, 28). Furthermore, deletion studies have revealed that all of the herpesvirus 7-tm proteins exert profound effects on virus replication and pathogenesis in vivo (6, 15) and/or on virally induced effects on host cells (48, 49). Thus, strong precedents exist for the functional importance of HHV-6 U51 in virus replication.

A number of other herpesvirus 7-tm proteins exhibit either constitutive or ligand-mediated signaling characteristics (3, 8, 11, 12, 21, 22, 53). This prompted us to wonder whether U51 might exhibit cell signaling properties that could perhaps influence the efficiency of viral replication. Since U51 has previously been noted to share significant sequence similarity with opioid receptors (23), we elected to focus particular attention on the possibility that U51 might interact with opioid ligands. The panel of opioid ligands that was evaluated for U51 binding included molecules with both broad opioid receptor binding characteristics and compounds with well-defined selectivity for each of the three human opioid receptor subtypes (δ, μ, κ). The opioid ligands induced efficient G protein coupling in membrane preparations from CHO cell sublines that overexpressed the relevant cognate receptors (Table 1), but none of the opioid ligands tested was found to bind to U51 (data not shown) or to induce G protein coupling by U51 (Table 1). Similarly, none of the chemokines tested was able to induce G protein coupling by U51, including RANTES. These findings are consistent with previously reported studies, since RANTES has been shown to bind to HHV-6A U51 but not to transduce any intracellular signaling events following such binding (33). Collectively, therefore, these findings suggest that despite the homology between U51 and opioid receptors (23), HHV-6B U51 does not appear to interact functionally with opioid ligands.

FIG. 7. U51 enhances cell-cell fusion in the presence of VSV-G in vitro. Equal numbers of HEK293 cells were transfected either with a HIV-1 Tat expressing plasmid (pcTat) (50) or with a plasmid containing a luciferase reporter gene under the transcriptional control of the HIV-1 LTR (17). All of the cells were also transfected with plasmid expression vectors encoding the following proteins: none (pcDNA3 lane), VSV-G alone (VSV-G lane) or VSV-G plus HCMV US28, HHV-6A U51 (6AU51CO), or the rat kappa opioid receptor (KOR), which was included as a negative control. The pcTat and LTRluc cell pools were then trypsinized 4 h posttransfection, mixed, and allowed to re-adhere to tissue culture plastic; 44 h later, luciferase activity was measured. The experiment shown is representative of three independent experiments. Shown are the mean relative light units (RLU) and standard deviations for three replicate samples obtained. As previously reported, HCMV US28 enhanced cell fusion initiated by US28 (39) (P < 0.05). HHV-6A US1 had a similar, though slightly less pronounced, effect (P < 0.05), while KOR had no such effect (P = 0.431; two-tailed t test).

11922 ZHEN ET AL. J. VIROL.
US28 or HHV-6A U51 may enhance cell-cell fusion is uncertain. For example, while we know that U51 is expressed on the cell surface of transfected 293 cells (9), we cannot be certain whether intracellular U51 may also contribute to the fusogenic effects associated with coexpression of U51. We can, however, exclude an effect on the steady-state expression of VSV-G, since flow cytometric analysis revealed no difference in the levels of VSV-G expression on the cell surface, regardless of the coexpression of U51 (data not shown).

Our current working hypotheses are that (i) U51 and/or US28 may interact with cell membrane-tethered chemokine ligands such as fractalkine (29) and (ii) these viral proteins may form a complex with RANTES and cellular glycoaminoglycans, thereby altering viral infectivity and/or promoting cell-cell fusion (51). Since the ligand repertoire and signaling properties of HHV-6A U51 remain largely unknown, it is also possible that U51 may interact with other chemokines or nonchemokine ligands and/or that it may have the capacity to induce signaling events in either a ligand-specific or a constitutive fashion (akin to HCMV US28 and other herpesvirus 7-tm proteins) (3, 8, 11, 12, 22, 53).

Overall, the studies reported here establish that U51 plays an important role in the replication and spread of HHV-6A. Our work also suggests a possible mechanism for this effect, which may reflect the ability of U51 to enhance cell-cell fusion and thus spread of this highly cell-associated human herpesvirus (872). Further studies will be required to further examine this hypothesis and to unravel the full ligand binding and signaling properties of HHV-6A U51 as well as to determine whether HHV-6B U51 also has similar activity (34).

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