RANTES Binding and Down-Regulation by a Novel Human Herpesvirus-6 β Chemokine Receptor[1]

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The human herpesvirus 6 (HHV-6) U51 gene defines a new family of betaherpesvirus-specific genes encoding multiple transmembrane glycoproteins with similarity to G protein-coupled receptors, in particular, human chemokine receptors. These are distinct from the HHV-6 U12 and HCMV US28 family. In vitro transcription and translation as well as transient cellular expression of U51 showed properties of a multiple transmembrane protein with a 30-kDa monomer as well as high m.w. aggregates or oligomers. Transient cellularly expressed U51 also appeared to form dimeric intermediates. Despite having only limited sequence similarity to chemokine receptors, U51 stably expressed in cell lines showed specific binding of the CC chemokine RANTES and competitive binding with other β chemokines, such as eotaxin; monocyte chemoattractant protein 1, 3, and 4; as well as the HHV-8 chemokine vMIPII. In epithelial cells already secreting RANTES, U51 expression resulted in specific transcriptional down-regulation. This correlated with reduced secretion of RANTES protein into the culture supernatants. Regulation of RANTES levels may alter selective recruitment of circulating inflammatory cells that the virus can infect and thus could mediate the systemic spread of the virus from initial sites of infection in epithelia. Alternatively, chemokine regulation could modulate a protective inflammatory response to aid the spread of virus by immune evasion. Such mimicry, by viral proteins, of host receptors leading to down-regulation of chemokine expression is a novel immunomodulatory mechanism. ***The Journal of Immunology*****, 2000, 164: 2396–2404.**

Human herpesvirus 6 (HHV-6)[4] is a lymphotropic betaherpesvirus that replicates primarily in $CD4^+$ lymphocytes (reviewed in Ref. 1). In adults the virus is widespread and infects nearly 100% of some populations. Initial sites of infection and those necessary for host-to-host transmission have been demonstrated in secretory epithelium, with sites for latency within monocytic/macrophage cell types. Clinically it is significant during primary infection as the causal agent of a pediatric fever; some patients also develop the skin rash response that defines exanthem subitum (or roseola infantum), and most exanthem subitum is attributed to HHV-6. HHV-6 can account for 10–40% of hospitalizations of children under 2–3 yr for febrile illness. These primary infections can have serious and fatal complications. In secondary reactivated infections, HHV-6 infection may enhance HIV infection, and disseminated infection can occur in patients with AIDS. The virus has also been implicated as the cause of numerous complications following solid organ and bone marrow transplantation, including bone marrow suppression leading to engraftment inhibition, and has been proposed as the etiologic agent of multiple sclerosis. The virus can infect and persist in circulating cells that normally can mediate a protective inflammatory response to infection. In this paper we investigate proteins encoded by the virus that may mediate such infections.

The HHV-6 genome contains two genes, U12 and U51, which are predicted to encode proteins related to the family of G protein-coupled receptors (GCR) and most closely to the chemokine receptors (2). Chemokines are classified into four groups, CC, CXC, CX3C, and C, according to the distribution of conserved cysteine residues in the N-terminal region and their activities. They are proinflammatory cytokines that act to chemoattract specific subpopulations of circulating cells, for example monocytes or T lymphocytes, which are recruited during an inflammatory response (3). Receptors for chemokines comprise a distinct subgroup of the GCR superfamily. The receptors are multiply hydrophobic, with seven transmembrane domains, have numerous conserved sequence characteristics, and are classified according to the chemokine group specificity (4, 5).

Some chemokine receptors are also significant as coreceptors to CD4 for HIV infection (reviewed in Ref. 6). Expression of the CC chemokines RANTES, macrophage inflammatory protein-1α (MIP-1α) and MIP-1β contribute to the resistance of $CD4^+$ T cells from HIV-exposed-uninfected individuals to HIV infection, and mutations in both chemokine receptor and chemokine genes have been shown to confer resistance to HIV infection.

One family of betaherpesvirus-specific chemokine receptor homologues has been identified as the HCMV UL33-like family, whereas in our genomic studies of HHV-6, a distinct gene family encoding betaherpesvirus-specific chemokine receptor-like proteins was identified (2). HHV-6 U51 was the first member of the

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[4] Abbreviations used in this paper: HHV-6, human herpesvirus 6; GCR, G protein-coupled receptor; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; ORF, open reading frame; IVTT, in vitro transcription and translation; HA, hemagglutinin; HCMV, human CMV; MCMV, murine CMV.

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U51 family to be identified as a chemokine receptor-like protein by sequence analyses (2), but demonstration of function has yet to be shown. Here we show that together with its homologues in human CMV (HCMV), murine CMV (MCMV), and HHV-7, HHV-6 U51 comprises a betaherpesvirus-specific family of receptors. HCMV UL33 and UL78, MCMV M33 and M78, and HHV-7 U12 and U51 are homologues of the HHV-6 receptors U12 and U51, respectively (2, 7–9). HCMV contains two additional receptors, US27 and US28; the latter has been shown in vitro to bind the chemokines MCP-1, MIP-1a, MIP-1b, and RANTES (10, 11) and to act as a coreceptor for HIV-1 infection (12). Recently, HHV-6 U12 has also been shown to act as a β chemokine receptor (13). The importance of the herpesviral chemokine receptor-like genes for virus persistence has been demonstrated by gene deletions. Deletion of the MCMV receptor-like gene M33 and its rat CMV homologue R33 from the respective viral genomes generated viruses that were viable, but impaired for growth in vivo (14, 15).

Although other herpesviral chemokine receptors have been investigated, no functional studies have been reported for the U51 receptor family. The U51 gene is highly conserved between HHV-6 strains. Variant A and B strains, U1102 and Z29 (GenBank accession no. 116947), show 94% identity. Using FASTA analyses, comparisons with homologues in the other sequenced betaherpesviruses show that HHV-6 U51 is most similar to HHV-7 (38% identity over 290 aa). It is also a positional homologue with HCMV UL78 and MCMV M78, averaging 20% identity over 200 aa. This betaherpesvirus GCR family are all encoded in the middle of the long unique regions of the respective genomes and are distinct from the HCMV US28 GCR family encoded at the ends of the genomes. U51 is also related to the herpesvirus saimiri ORF 74-encoded receptor, which has been shown to bind CXC chemokines (16). There is 24% identity, across 258 aa, between U51 and ORF 74. However, this gene and its homologues in the other gammaherpesviruses, HHV-8 (17), murine herpesvirus 68 (18), and equine herpesvirus 2 (19), are in a divergent genomic location at the right-hand end of the genome analogous to the location of U12 at one end (left) of the HHV-6 genome.

The specificity of the U51 family to the betaherpesviruses makes them useful in investigations of the unique aspects of the biology of this virus subfamily. In this work we sought to express and characterize HHV-6 U51 and to investigate its effect on a host cell. We show that U51 has some of the characteristics expected of a seven transmembrane-spanning protein and that stable expression leads to morphological alteration as well as specific binding and down-regulation of the chemokine RANTES, which is a potent chemoattractant for T lymphocytes, NK cells, monocytes, and eosinophils (3, 5). We suggest that the specific down-regulation of RANTES expression by a chemokine receptor-like protein represents a novel herpesviral immunomodulatory mechanism.

Materials and Methods

Cells and virus, preparation of infected cell DNA

HHV-6A strain U1102 (2) was cultured in the $CD4^+$ T cell leukemic cell line JJhan in RPMI 1640 medium, supplemented with 10% (v/v) FCS and 2 mM glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. The human immortalized epithelial cell line, HaCaT (20), was maintained in the same medium. Vero cells were maintained in DMEM supplemented as described for RPMI. The human erythroleukemia (K562) and premonocytic (U937) cells were cultured in RPMI 1640 supplemented as described above. Transfected cell lines were maintained in 500 or 750 μg/ml of G418 for HaCaT or K562 lines, respectively.

HHV-6-infected JJhan DNA was prepared 5 days postinfection. Infected cells and uninfected controls were washed with PBS (Dulbecco's A), taken into lysis buffer (10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% SDS), digested with proteinase K (0.25 mg/ml), extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and then DNA precipitated from the aqueous phase with isopropanol.

Cloning of U51 gene into pCDNA3 and epitope tagging

The U51 gene was amplified using PCR with standard thermocycling procedures and Taq polymerase. The following primer set was used to amplify the coding region and to introduce *Bam*HI restriction enzyme recognition sequences: 5′-AAAGGATCCTTACTTCGTTTATTC-3′ and 5′-TATGGATCCAAAGGATCCACTCCT-3′. The amplified product was digested with *Bam*HI restriction enzyme, separated by agarose gel electrophoresis, and purified by phenol extraction followed by ethanol precipitation. The purified DNA fragment was ligated with *Bam*HI-digested, phosphatased plasmid pCDNA3 (Invitrogen) and transformed into *Escherichia coli* strain JM109. The selected clone was checked by sequence analyses and designated pcDNA3-U51. This plasmid was then epitope tagged with the sequence encoding the 9-aa epitope from influenza hemagglutinin recognized by mAb 12CA5 (Babco, Richmond, CA; Boehringer Mannheim, Indianapolis, IN) as described previously (21). The following primer was used to introduce this sequence into the N-terminal end of the U51 gene, such that the epitope-encoding sequences were inserted after the initiating methionine and glutamic acid codons: 5′-CTTCGTTTCTTTAGCATAATCCGGCACATCGTATGGATACTCCATCCTG-3′. Site-directed mutagenesis was performed following the Kunkel method (22), as described previously (21). This plasmid was designated pcDNA3-U51D.

In vitro transcription/translation and transient expression

In vitro transcription and translation (IVTT) was conducted using the transcription and translation-coupled rabbit reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, 0.5 μg of plasmid was used in an IVTT mix containing 12.5 μl of rabbit reticulocyte lysate, 20 U of RNasin, 15 μCi of [^{35}S]methionine (SJ1515, Amersham, Arlington Heights, IL), and 7.5 U of T7 RNA polymerase. Reactions were conducted at 30°C for 90 min, then analyzed directly by SDS-PAGE or immunoprecipitated.

For transient expression, the T7 RNA polymerase-vaccinia system (23) was used as described previously (21). Vero cells (3×10^5) were infected (input multiplicity 1 PFU/cell) with recombinant vaccinia virus vTF-3, expressing the T7 RNA polymerase (23). After 2.5-h incubation at 37°C, infected cells were transfected, using Lipofectin and OPTIMEM (Life Technologies, Gaithersburg, MD), with 7.5 μg of plasmid. After a further 4-h incubation, cells were washed with DMEM deficient in methionine and labeled at 37°C for 13 h with 2 μl (15 mCi/ml) of [^{35}S]methionine in methionine-deficient medium. Lysates were then prepared and immunoprecipitated.

Cell lysis, immunoprecipitation, and gel electrophoresis

Labeled cells from transient expression experiments were washed three times with ice-cold PBS, then lysed for 30 min into 0.5 ml of ice-cold RIPA buffer (21). Lysates were sonicated (1 min on setting 6; Heat Systems (Farmingdale, NY) XL2020 sonicator with cup horn), cleared by microcentrifugation (13,000 rpm, 30 min, 4°C), and stored at −20°C. After preclearing with 50 μl of protein A-Sepharose (50% swollen volume in RIPA buffer), equal amounts of lysate (quantitated by scintillation counting of TCA precipitates) were incubated at 4°C overnight with Abs (1 μl of anti-influenza hemagglutinin (HA) type 1 mAb 12CA5 (Boehringer Mannheim)) recognizing the epitope tag. Immune complexes were precipitated with protein A-Sepharose, washed three times with RIPA buffer, and eluted in sample buffer.

For direct analysis, IVTT reaction products were diluted with an equal volume of 2× SDS sample buffer (without DTT) and resolved by SDS-PAGE. For immunoprecipitation, IVTT reactions were diluted to 0.5 ml with RIPA buffer.

Except where noted in the text, to avoid aggregation of the hydrophobic U51 protein, all samples were sonicated, rather than heated, before separation by electrophoresis. SDS-PAGE was performed in 12% precast gels (Novex, San Diego, CA) as described previously (21).

Generation of cell lines expressing U51

The plasmids pcDNA3-U51 (wild-type U51), pcDNA3-U51D (epitope tagged), and (pcDNA3) vector-only control were transfected into either HaCaT epithelial cells using Lipofectin (Life Technologies) according to the manufacturer's protocol or K562 cells using electroporation (electroporator with capacitance extender, Bio-Rad, Hercules, CA). HaCaT-expressing colonies were selected and grown using RPMI culture medium (10% FCS) supplemented with 500 μg/ml geneticin (G418, Life Technologies). Epithelial cell colonies were Perspex ring cloned and analyzed by DNA PCR using the SP6 and T7 primers to amplify across the multiple cloning site in pcDNA3. The U51 clones were screened for expression by

reaction with HHV-6 human sera in immunofluorescence assays as well as by RT-PCR as described below. Two each of independently derived vector (V)-only, U51 wild-type (U51), and U51tag (U51D) cell lines were used for further study and were labeled V-2, V-5, U51-3, U51-6, U51D-3, and U51D-5. The K562 cells were similarly selected using G418 at 750 μg/ml followed by cloning from the resistant population using limiting dilution. Four clonal lines of U51-expressing cells were selected. Cells were stored in liquid nitrogen before use, and only low passage clones (less then passage 5) were used in these studies.

RT-PCR

Confluent HaCaT or dense K562 cell lines cultured in 25-cm^2 flasks were washed twice with PBS, then lysed in 1 ml of cold RNA Isolator reagent (Genosys, The Woodlands, TX) and incubated at room temperature for 5 min. Chloroform (0.2 ml) was added, and the mixture was shaken until emulsified, then incubated for 15 min. After microcentrifugation (13,000 rpm for 30 min at 4°C), RNA was isopropanol precipitated from the aqueous phase, then pelleted by microcentrifugation. The RNA pellet was washed with 75% (v/v) ethanol (in water) and air-dried. Contaminating DNA was removed using DNase (Promega; 0.08 U/ml, 2 h at 37°C). DNase-treated RNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with ethanol, washed with 70% (v/v) ethanol (in water), and resuspended in water to 150 ng/ml.

Complementary DNA was synthesized from 2 μg of template RNA using 8 U/ml Moloney murine leukemia virus reverse transcriptase (Promega) in a 60-μl reaction containing 0.5 mM dNTP, 100 U of RNasin, 10 ng/ml random hexamers, 1× RT buffer (50 mM Tris-Cl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT). RNA was heated to 65°C for 10 min before cDNA synthesis, chilled on ice for 5 min, then added to the prepared reaction mix. Parallel reactions, excluding RT, were prepared for each RNA template to confirm the absence of contaminating DNA. Reactions were incubated at 37°C for 2 h, then diluted with an equal volume of water and stored at −20°C.

PCR was conducted in 30-μl reactions containing, as standard, 200 μM of each dNTP, 250 ng of each primer, and 1.5 U of Taq polymerase (Promega; storage buffer B) in 1× Taq reaction buffer. RANTES reactions contained 0.7 mM $MgCl_2$; all others contained 1.5 mM. Primer sequences were as follows (expected product sizes in base pairs are given in parentheses): β-actin: actin-F, GATGGAGTTGAAGGTAGTTT; actin-B, TGC TATCCAGGCTGTGCTAT (445 bp); HHV-6 U51: U51-F, TCGGTC GAGAATACGCTGTG; U51-B, AGATACGTAGTCACGGTCGA (493 bp); IL-8: IL-8-1, CTTCCTGATTTCTGCAGCTCTGTG; IL-8-2, CAA AAACTTCTCCACAACCCTCTG (245 bp); α_2 integrin: AL2-1, CCCT CTGGACAGCTTCTAGAG; AL2-2, GAAATCCCCGCTTACCTTGAC (189 bp); and RANTES: RAN-1, TCGCTGTCATCCTCATTGCTACTG; RAN-2, CATCTCCAAAGAGTTGATGTACTC (248 bp). Thermal cycling parameters for β-actin and U51 reactions were 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min, for 40 cycles; for IL-8 reactions the parameters were 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, for 40 cycles; for RANTES and α_2 integrin reactions the parameters were 95°C for 30 s, 49°C for 30 s, and 72°C for 2 min for 40 cycles. PCR product identities were confirmed by sequencing using the ABI Prism 377 automated sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Chemokine binding assay

Binding assays were conducted as described previously (24). Log phase cells (K562-U51 or K562-vector) were washed twice in unsupplemented RPMI, then resuspended at 2.5×10^7 cells/ml in binding medium (RPMI, 0.1% BSA, and 20 mM HEPES, pH 7.4) on ice. Where appropriate, U937 cells were used as positive controls. Assays, in duplicate or triplicate, contained 2.5×10^6 cells, 166 pM radiolabeled chemokine, and varying concentrations of unlabeled competitor chemokine. After 2-h incubation on ice with regular mixing, the cells were separated from the unbound chemokine by microcentrifugation through a pthlalate oil cushion (1.5 parts dibutyl phthalate to 1 part bis-(2-ethylhexyl)phthalate). Bound radioactivity was then counted using a gamma counter. Data analysis was conducted using the EBDA and LIGAND programs (version 2, 1985) (25). ^{125}I-labeled RANTES (sp. act., 2000 Ci/mmol), IL-8, and MIP-1α were obtained from Amersham, unlabeled chemokines were obtained from PeproTech (Rocky Hill, NJ), and RANTES was obtained from R&D Systems (Minneapolis, MN).

Quantification of RANTES secretion

A RANTES enzyme immunoassay was used according to the manufacturer's instructions (R&D Systems, DRN00). Each sample of culture supernatant was tested, in duplicate, undiluted as well as diluted 1/10 and 1/100. A series of standards was also tested in each assay, and the results were used to plot a standard curve from which the test results were determined. The range of sensitivity for the assay was 31.25–2000 pg/ml, with usually two of the three sample dilutions giving concentrations within this range. Unused culture medium without G418, processed in parallel to exclude any effect of the drug on results, gave readings equivalent to background. All within-assay range results for a sample were used to calculate a weighted mean concentration.

Results

Definition of a betaherpesvirus-specific GCR gene

U51 defines a new herpesvirus GCR family with only limited similarity to chemokine receptors. Although the degree of overall sequence identity between members of the U51 family is low, they have numerous common features and a conserved genomic location. The U51 family genes are located within block IV of the seven conserved herpesvirus gene blocks (2). Although the flanking genes within this block, notably those for glycoprotein H (HHV-6 U48) and the viral protease/assemblin (HHV-6 U53), are present in herpesviruses of all three subfamilies, the U51 homologues are found only in the betaherpesviruses. Using FASTA together with multiple alignment analyses, U51 was found to be a member of the class A family of GCRs that includes rhodopsin, adrenergic receptors, and chemokine receptors (26).

Unlike other herpesvirus GCRs characterized, U51 shares only limited similarity to the chemokine receptors. The closest homologues show only borderline overall similarity, with 20–24% identity to the functional CXC receptor from herpesvirus saimiri, ORF 74, as well as the cellular receptor, CCR7 (EBI1), inducible by both HHV-6 and EBV (16, 27, 28). However, although only distantly related, U51 does have a similar length and overall structure as the chemokine receptors. Other similar features to chemokine receptors are shown by amino acid sequence alignment using CXCR2 (IL-8β receptor) and CCR1 (MIP-1α and RANTES receptors) as prototypes for CXC and CC receptors, respectively (Fig. 1). U51 shares some features with the human chemokine receptors and GCRs (4, 29). It has the characteristic seven-transmembrane predicted structure (Fig. 1) together with basic residues in the first intracellular loop as well as conserved tyrosines in the first and fifth transmembrane domains; prolines in the second, fifth, and sixth transmembrane domains; tryptophan in the first extracellular loop; and cysteine in the second extracellular domain. It also has conserved cysteine, proline, and histidines in the seventh transmembrane domain, although these cannot be exactly aligned. It has potential sites for chemokine ligand binding at the acidic residues in the extracellular N-terminal domain and charged residues in the third extracellular domain, whereas regions in the transmembrane and intracellular domains have potential for mediating intracellular signaling. Here, like other GCRs, it has conserved prolines in the sixth and seventh transmembrane domains, and in common with the CXC chemokine receptors, U51 has a predicted *N*-glycosylation site in the second extracellular domain. However, like herpesvirus saimiri ORF 74, it does not have the conserved aspartate in the second transmembrane domain and has only a few clusters of serines in the C-terminal intracellular loop.

U51 also significantly diverges from other chemokine receptors in an unusually short N-terminal extracellular domain with acidic residues; although it includes characteristic acidic residues, it lacks the conserved cysteine and predicted *N*-glycosylation site common in these receptors (4, 29). This cysteine residue in other chemokine receptors is predicted to be disulfide-bonded with a conserved cysteine in the third extracellular domain, which is also absent from HHV-6 U51, although the U51 family features more conserved cysteines at distinct positions that may play a compensatory role (Fig. 1).

FIGURE 1. Multiple alignment of U51 family compared with U12 and prototypes of the chemokine receptor family. CCR1 represents the CC chemokine receptor family (binds RANTES); CXCR2 represents the CXC family (IL-8β, binds IL-8). CLUSTALV (Higgens algorithm) was used for the multiple alignment followed by manual adjustment to maximize conserved positions. The predicted seven-transmembrane regions (TM) found in the GCR family are marked I–VII (26). Potential extracellular loops are before TMI and between TM II and III, IV and V, and VI and VII. *N*-linked glycosylation sites in these exposed loops are indicated as well as two conserved cysteines, marked in bold, in loops between TM II and III, and IV and V. Consensus residues (identity of more than three, using HHV-6 strains as one count) are marked in small letters; capital letters mark sites where the majority (more than six) or all are conserved. HHV6A is strain U1102, HHV6B is strain Z29, and HHV7J is strain JI. Asterisks mark conserved residues specific for the U51 family.

Overall, the U51 family appears to define a new betaherpesvirus-specific family of G protein-coupled receptor genes, with only distant similarity to the chemokine receptors. Amino acid residues specific to this family are marked in Fig. 1.

Biochemical characterization of U51

For a preliminary investigation of the properties of U51, the HHV-6A strain U1102 U51 gene and a modified version (termed U51D) containing the HA epitope tag at the N-terminus were each inserted into the eukaryotic expression vector pcDNA3, putting them under the control of the T7 promoter and the human CMV major immediate-early promoter.

IVTT of the U51 constructs generated products with properties typical of a seven-transmembrane-spanning protein. Wild-type U51 showed a discrete band of 28 kDa, consistent with the U51 monomer, although smaller than the predicted M_r of 34.7 kDa, and a broad range of higher M_r species centered around 180 kDa (Fig. 2*A*). IVTT of U51D gave rise to a similar band profile, with the monomer slightly increased in size to 30 kDa, most likely due to a conformational effect of the 9-aa epitope tag (Fig. 2*A*). When samples were heated to 95°C before electrophoresis, the 28-kDa (U51) or 30-kDa (U51D) band disappeared (Fig. 2*A*), consistent with aggregation of a multiply hydrophobic protein. The ladder of bands seen with IVTT U51 and U51D (Fig. 2*A*, *lanes 1* and *2*) increase in size by ~8-kDa increments, consistent with the size of ubiquitin, which may be added to misfolded hydrophobic proteins synthesized by IVTT (30). Inclusion of canine pancreatic microsomal membranes in the IVTT mix caused an increase in the size of the discrete band, consistent with use of the single predicted

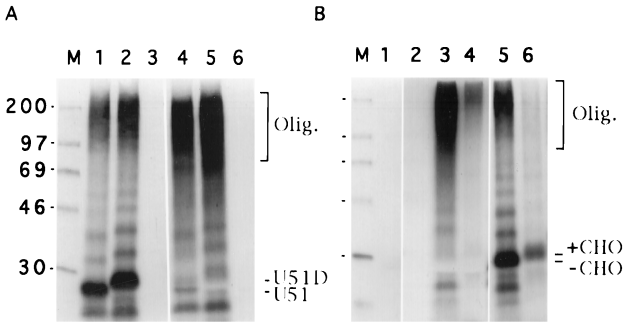

FIGURE 2. In vitro transcription and translation of U51 and U51D. *A*, IVTT of U51 (*lanes 1* and *4*), U51D (*lanes 2* and *5*), and pcDNA3 vector (*lanes 3* and *6*). Samples were sonicated (*lanes 1–3*) or were heated to 95°C (*lanes 4–6*) before gel electrophoresis. (M, M_r markers shown in K_d). *B*, IVTT with canine pancreatic microsomal membranes (CMM). The pcDNA3 vector with CMM (*lane 1*) and without CMM (*lane 2)* (the white area is from gel tearage); U51D with CMM (*lanes 4* and *6*) or without CMM (*lanes 3* and *5*) is shown. Samples were sonicated (*lanes 1, 2, 5*, and *6*) or heated to 95°C (*lanes 3* and *4*) before gel electrophoresis. M, M_r markers shown in kilodaltons. Discrete U51 bands, glycosylated and unglycosylated U51D, and the oligomeric forms are indicated.

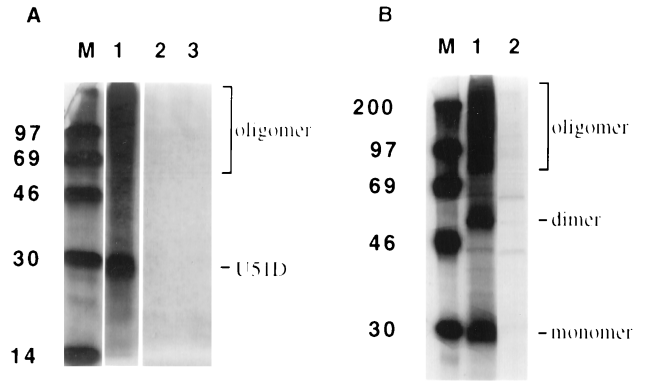

FIGURE 3. Immunoprecipitation of U51D. *A*, IVTT (without CMM) of U51D (*lane 1*), U51 (*lane 2*), or pcDNA3 vector (*lane 3*), immunoprecipitated with the anti-HA mAb 12CA5. M, M_r markers shown in kilodaltons. *B*, Transient cellular expression of U51D (*lane 1*) or pcDNA3 vector (*lane 2*) transfected into Vero cells amplified by vaccinia T7 recombinant superinfection and then immunoprecipitated with anti-HA mAb 12CA5. Discrete U51 bands, dimers, and oligomers are indicated. Identical results were shown with transfection and superinfection of HaCaT cells.

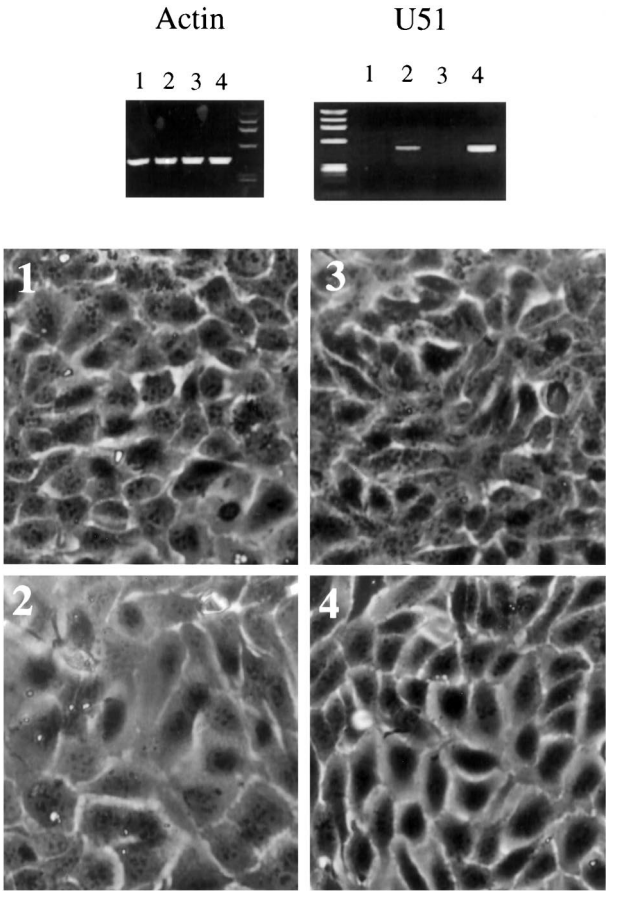

FIGURE 4. Expression of U51D in HaCaT cells. RT-PCR for U51 and β-actin in HaCaT cell lines V7 (*lane 1*), U51D-5 (*lane 2*), V2 (*lane 3*), and U51D-3 (*lane 4*). Light micrographs demonstrate the morphology of HaCaT cell lines V7 (*panel 1*), U51D-5 (*panel 2*), V2 (*panel 3*), and U51D-3 (*panel 4*). Original magnification, $\times$200.

N-glycosylation site in the second extracellular domain of U51 (Fig. 2*B*). As with unglycosylated U51, heating the glycosylated form caused increased aggregates of high M_r oligomers at the top of the gel (Fig. 2, *A* and *B*).

Both the discrete band and the high M_r aggregate were immunoprecipitated by the anti-HA mAb 12CA5 from IVTT reactions with U51D (Fig. 3*A*). Transient expression of U51D in Vero cells using the T7 RNA polymerase vaccinia system followed by immunoprecipitation gave rise to a similar pattern of bands (Fig. 3*B*). The discrete band comigrated with the glycosylated IVTT U51D (data not shown), suggesting that the glycosylation site was used in vivo, as had been shown in the in vitro expression system. An additional band, which had not been seen in IVTT experiments, was prominent in the immunoprecipitations of the cellularly expressed U51D (Fig. 3*B*), and its size of 58 kDa was consistent with that expected for a U51 dimer. Similar results for Vero cells were found for transient expression in epithelial, HaCaT, cells.

These results showed that the U51 protein has characteristics of a protein containing multiple hydrophobic domains, that the conserved *N*-glycosylation site in U51 can be used, and that a proportion of the protein might exist as a dimer.

Stable expression of U51 in epithelial cells

HHV-6 in vivo can infect and be secreted from epithelial cells, which are important for host-to-host transmission in saliva and also as a barrier in systemic infections (31–33). However, the virus then permissively infects circulating cell types. Virus chemokines and their receptors may modulate interactions between these cell types. To investigate the effect of U51 expression on an epithelial cell, HaCaT cells were used as a model system, because they can undergo normal differentiation (20), and epithelial in vitro culture systems that are fully permissive for HHV-6 replication have not been defined. Furthermore these cell lines provide an unlimited standardized system for characterization. Transient and stably transfected cell lines were established with the same plasmid clones used to demonstrate protein expression in the Vero-vaccinia T7 transient expression assays (pcDNA3-U51, pcDNA3-U51D, or vector alone). U51D could easily be detected in the transiently transfected, vaccinia T7-infected HaCaT cells by immunoprecipitation with the tagging mAb, 12CA5, with results similar to those shown above for Vero cells. However, in the absence of amplification of gene expression by the T7 polymerase, the U51 gene product could not be detected by immunoprecipitation in transient or stably transfected cell lines, although the U51 transcript was detected by RT-PCR (Fig. 4, *upper panels*). Thus, this level is lower than that detectable using the tagging mAb, and possibly higher levels are not consistent with cell survival, as shown for other multiple membrane proteins. The U51 cell lines were further screened by immunofluorescence for reactivity with HHV-6-seropositive sera, and three of seven sera tested showed positive fluorescence with no reaction to the vector-only cell lines.

Six independent clonal HaCaT lines were studied, U51-3 and U51-6 expressing wild-type U51; U51D-3 and U51D-5, expressing the epitope-tagged U51D; and V2 and V7, containing the vector alone. RT-PCR analysis of the cell lines with primers specific for a sequence within the U51 gene and standardized with RT-PCR for β-actin showed expression of U51 in the U51 cell lines but not in the vector controls (Fig. 4). Representative results are shown for the tagged lines (Fig. 4), and similar results were found for the wild-type lines. The cell lines showed altered cellular morphology compared with the two vector cell lines. In Fig. 4 differences in cell spreading or flattening are shown in the U51-expressing cell lines. The morphological differences were not accounted for by growth differences or viability, as cell yields were similar (see below and Fig. 6).

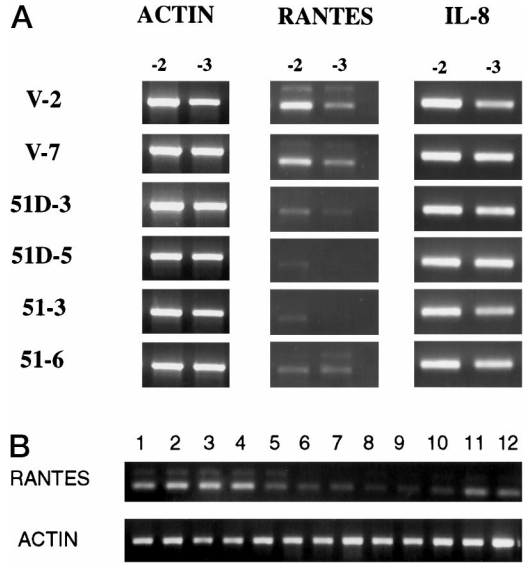

FIGURE 5. Down-regulation of RANTES RNA in U51-expressing cell lines. RT-PCR analysis of IL-8, RANTES, and β-actin expression in U51- and U51D-expressing cell lines. Randomly primed cDNA samples generated from equal amounts of total RNA. Products corresponding to the linear range of amplification are shown. *A*, 10^{-2} and 10^{-3} dilutions are shown. *B*, Products from two separate flasks of each cell line were further tested to show reproducibility (1/50 dilution of cDNA for the RANTES PCR and 1/1000 dilution of cDNA for the β-actin PCR). Lanes are: HaCaT cell line V-2 (*lanes 1* and *2*), V-7 (*lanes 3* and *4*), 51D-3 (*lanes 5* and *6*), 51D-5 (*lanes 7* and *8*), 51-3 (*lanes 9* and *10*), and 51-6 (*lanes 11* and *12*).

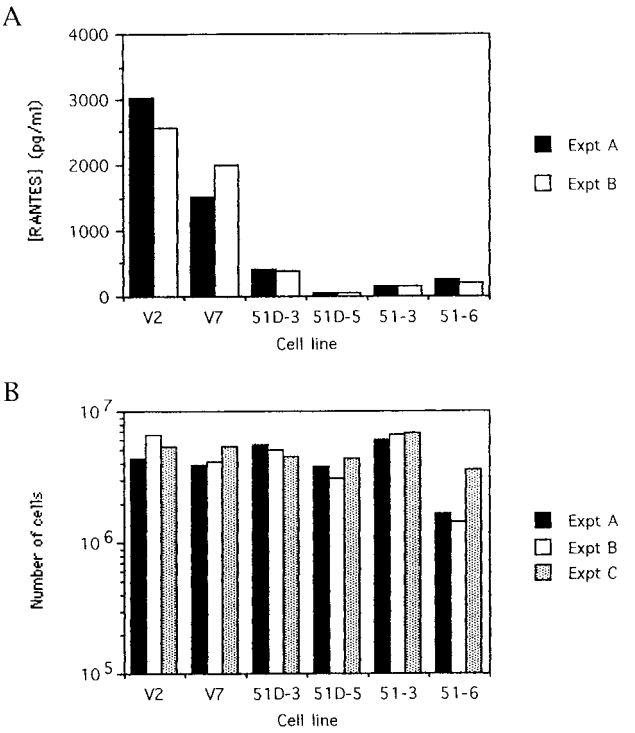

FIGURE 6. Down-regulation of chemokine RANTES secretion in epithelial cell lines stably expressing U51 and U51D. Cleared supernatants from confluent monolayers of HaCaT cell lines were assayed for RANTES levels using a commercial ELISA. Cells were seeded at 8×10^5 cells/flask. Supernatants were taken, and cells were counted after 4 days. *A*, RANTES titers per milliliter of supernatant for two vector lines, two U51 lines, and two U51D lines. Expt. A and B represent data from two sets of flasks processed in parallel. *B*, Cell counts from Expt. A and B and from a third identical experiment, Expt. C. Data are the means of two counts for each cell line. The results show that RANTES levels are significantly reduced in the U51- and U51D-expressing cell lines, with no significant variation in cell number.

Down-regulation of RANTES expression

Chemokine expression has been shown to alter cellular morphology (34), and infection with HHV-6 or HCMV can alter both chemokine expression (35, 36) as well as cellular morphology (1). In HCMV infections, RANTES can be sequestered via HCMV US28 chemokine receptor (36). It was therefore of interest to investigate the effect of U51 on chemokine expression and possible autologous feedback mechanisms. To this end, RT-PCR was used to test for expression of RANTES and IL-8, as representative CC and CXC chemokines, with β-actin used as a control. α_2 integrin was used as an additional control, with indistinguishable results from actin (not shown).

Amplification from equal amounts of cDNA template generated from the HaCaT cell lines U51-3, U51-6, U51D-3, U51D-5, V2, and V7 showed that, compared with the control cell lines V2 and V7, the level of RANTES RT-PCR product was reduced in all U51 and U51D lines by at least 10-fold (Fig. 5).Titrations of cDNA template in serial dilutions followed by separate PCR amplifications confirmed that the reduction was ~10-fold, whereas β-actin, α_2 integrin, and IL-8 RT-PCR product yields were similar in all cell lines (Fig. 5). On the basis of these results, it seemed that U51 was specifically causing regulation of RANTES expression.

To determine whether the transcriptional down-regulation of RANTES was reflected in reduced levels of RANTES secretion, culture supernatants from the six cell lines were tested using a RANTES-enzyme immunoassay. Cells were seeded at equivalent density, and assays were conducted on supernatants 4 days postinfection of now confluent monolayers. RANTES levels in control supernatants were consistently high, averaging 2000–3000 pg/ml for the vector-only controls, V2 and V7, whereas the U51 and U51D cell line supernatants contained much lower levels of RANTES, averaging 30–300 pg/ml (Fig. 6*A*). This down-regulation was not related to decreased cell numbers, as there was no significant difference between the final cell densities of the cell lines (Fig. 6*B*).

Ligand binding characteristics of HHV-6 U51

Having identified U51 as a chemokine receptor-like protein and shown that its expression is associated with down-regulation of RANTES, but not that of IL-8, it was important to determine ligand binding. To investigate this, U51 was stably expressed in K562 cells, which have been used previously for similar assays, for example HCMV US28 and HHV-6 U12, and do not express the MIP-1α/RANTES receptor or show specific competable binding of chemokines (11, 13). Binding assays using ^{125}I-labeled chemokines showed clearly that U51 binds RANTES, but not IL-8 or MIP-1α (Fig. 7*A*). The positive control cell lines used were U937 cells that express functional receptors (Fig. 7*A*).

Titration of unlabeled RANTES into the binding assay showed that ^{125}I-labeled RANTES binding by U51 was saturable and competable, indicating that the binding was specific. The titration was repeated in three separate experiments with similar results, and a representative titration is shown in Fig. 7*B*. Scatchard analysis of the competition curve was performed using EBDA and LIGAND programs (25) (Fig. 7*B*). The results identified a high affinity binding site, with a K_d of 0.83 $\pm$ 0.13 nM (Fig. 7*B*). The number of receptors per cell assayed, as calculated from the *r* value, was 9375 $\pm$ 750. Thus, as well as regulating the expression of RANTES, U51 binds RANTES in a saturable and competable manner.

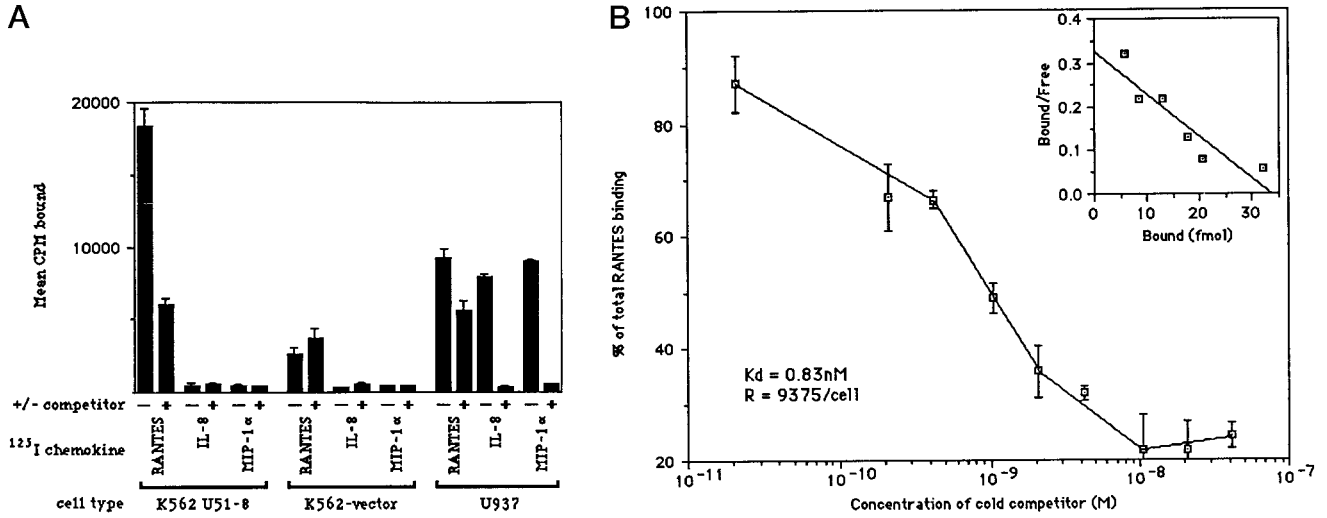

FIGURE 7. Chemokine binding profile of U51. *A*, ^{125}I-labeled RANTES, IL-8, and MIP-1α binding was tested in the presence and the absence of cold competitor (1,000-fold molar excess for IL-8 and MIP-1α, 250-fold molar excess for RANTES). Assays using U51-8 (U51 clone 8) and K562 vector cells were performed in triplicate; control assays with U937 cells were performed in duplicate. Mean bound counts are shown, with error bars representing $\pm$1 SD for U51-8 and K562 vector cells and $\pm$1 range for U937 cells. Background binding was not subtracted from these data. *B*, Binding of ^{125}I-labeled RANTES to K562 cells stably transfected with pcDNA3-U51. Displacement of ^{125}I-labeled RANTES binding by unlabeled RANTES. Each point represents the mean $\pm$1 SD for determinations performed in triplicate. The mean total binding shown is 20,516 $\pm$ 729 cpm. Nonspecific binding was 3,710 $\pm$ 64 cpm (vector control). The parameters for binding are shown in the *lower corner* derived from the Scatchard analyses shown in the *upper corner*, which gave a high affinity binding site with a K_d of 0.83 $\pm$ 0.13 nM.

U51 is therefore the most divergent member of the chemokine receptor family to have been shown to bind ligand to date.

To compare the ligand binding profile of U51 with those of other receptors, we tested a panel of unlabeled chemokines for competition of RANTES binding to U51. Of those tested, MCP-1, MCP-3, MCP-4, eotaxin, and vMIPII did compete binding, while, consistent with the direct binding assays described above (Fig. 8) MIP-1α and IL-8 did not. Thus, U51 shows a unique ligand binding specificity compared with data on known chemokine receptors.

Discussion

In this report we demonstrate the specific binding of RANTES and selected CC chemokines. We further show down-regulation of RANTES expression in epithelial cells by an HHV-6 protein, U51, which is related to chemokine receptors. This potential immunomodulatory mechanism may also participate in the spread of virus infection.

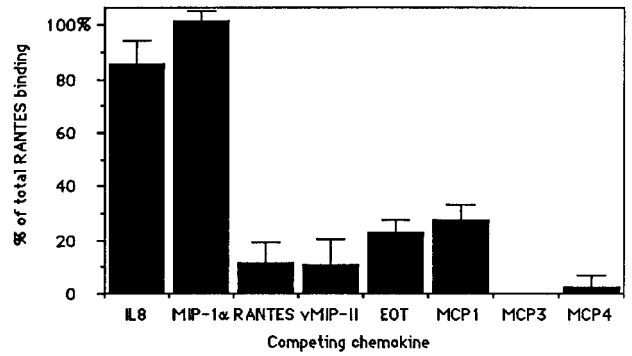

FIGURE 8. Competition for RANTES binding to U51 by heterologous chemokines. Unlabeled chemokines were added at a 625-fold molar excess (125-fold molar excess for RANTES) over ^{125}I-labeled RANTES. The amount of ^{125}I-labeled RANTES bound in the presence of each competitor is shown as a percentage of the amount bound in the absence of competitor. Background binding (to vector K562 cells) was subtracted in each case. The mean background count was 4119 cpm, and mean uncompeted binding was 9420 cpm. Each chemokine was tested in triplicate. The mean is shown with error bars representing $\pm$1 SD.

Analysis of epitope-tagged and wild-type U51 expressed in vitro and transiently in cells showed that, as predicted from the hydrophobicity analysis of the predicted amino acid sequence, U51 has the characteristics of a highly hydrophobic multiple membrane protein, aggregating when heated. Interestingly, a prominent 58-kDa form of U51, consistent with a dimer, was observed during cellular expression but not by in vitro transcription-translations. This form may be favored in vivo and require modifications or correct membrane insertion to form. Dimerization is functionally relevant for some of the human chemokine receptors; CCR5 is present in a dimeric form on the cell surface (37), and CCR2 forms dimers in a ligand-dependent manner (38).

U51 binding specificity resembled those of HHV-6 U12 and CCR3. These results have shown that U51 has a high affinity for the CC chemokine, RANTES (K_d of 0.8 nM), as well as specificity for MCP-1, but not for the CXC chemokine, IL-8. These properties are similar to those of U12, which has an affinity for RANTES of 1.3 nM (13). Both HHV-6 U12 and U51 differ from HCMV US28 in that they do not bind MIP-1α efficiently. HCMV US28, which most closely resembles U12 and some other CC chemokine receptors, also binds RANTES, MCP-1, as well as the viral chemokine, HHV-8 vMIPII (10, 11, 40, 39). We also show U51 can bind HHV-8 vMIPII. This binding is of interest, as HHV-6 has been identified in HHV-8-associated Kaposi sarcoma lesions, and chemotaxis of HHV-6-infected cells chemoattracted via U51 specificity for vMIPII could affect migration of HHV-6-infected cells to an HHV-8-associated lesion, possibly affecting pathology (41). Additionally, we have shown that U51 has some specificity for eotaxin, like CCR3, but also to MCP-3 and MCP-4, thus displaying a binding profile to a subset of CC chemokines that is distinct from those of receptors described to date. Interestingly, HHV-6 U12 and U51 appear to have different regulatory mechanisms, in that U12 is expressed late and U51 is expressed at early times

postinfection (13, 42). Thus, binding of RANTES or other chemokines at different times in the replicative pathway may result in different downstream signaling.

Stably expressed U51 had two effects on HaCaT cells: down-regulation of RANTES expression (with no effect on IL-8) and morphological alteration, both possible downstream effects of signaling through this receptor. The ability of U51 to bind RANTES, but not IL-8, was consistent with the effects of U51 expression on these two chemokines. Initial investigations of the effect of U51 on chemokine expression using RT-PCR showed an ~10-fold reduction in the level of RANTES-specific RT-PCR signal in the U51- and U51D-expressing HaCaT cell lines. The reduced RT-PCR signal for RANTES was reflected in RANTES levels in culture supernatants of the cell lines; both U51 and U51D cell lines had lower levels than vector control lines. Similar results were found for the K562 U51 lines, although at a lower overall level of RANTES that did not affect binding affinity measurements (not shown). It is possible that the receptor is acting constitutively or is using an undefined mechanism, but the cell lines are secreting RANTES, which could bind the receptor. Both the U51 wild-type and epitope-tagged lines gave similar results, which suggest that insertion of the epitope at the N-terminus does not affect the activity on RANTES expression. In cellular studies, levels of RANTES mRNA and protein correlate, indicative of transcriptional regulation (43). The RANTES down-regulation shown in the U51 cell lines was also similar between RNA and secreted protein levels. This is consistent with a transcriptional mechanism of RANTES inhibition caused by U51. The U51-expressing epithelial cells also showed moderately increased spreading and flattening independent of any effect on growth or viability. This is of interest, as such changes in cell morphology, motility, or differentiation may enhance the spread of HHV-6, which is primarily cell associated and disseminates predominantly via direct fusogenic cell-cell spread, with cytopathic effects on morphology causing enlarged cells (1). All the cell lines maintained their characteristic epithelial spreading responses to collagen-, laminin-, and fibronectin-coated dishes (not shown). Binding of RANTES to its receptors can cause morphological alterations and adhesion molecule redistribution (34). The relationship of U51 expression to such effects and cellular differentiation will be further investigated.

Interestingly, although it retains chemokine binding activity despite low identity with other chemokine receptors, U51 does not appear to function as an HIV coreceptor, at least with the expression levels obtained with the pcDNA3-U51 construct used in a transient expression system and the strains analyzed (44). However, possible interactions could have an effect on HIV infection with RANTES-sensitive strains via the chemokine regulation as demonstrated here.

HCMV has a biphasic effect on RANTES expression, down-regulating initially but then later, after infection, up-regulating (45). HCMV deleted of the US28 gene are unable to induce this effect; however, the down-regulation was shown to be mediated by chemokine sequestration from the medium and not via a transcriptional mechanism (36). HCMV does inhibit transcription of another CC chemokine, MCP-1, but the US28 receptor is not required for this effect (46). Our data provide evidence for an effect on chemokine expression by a viral chemokine receptor, HHV-6 U51. Altering the levels of chemokines such as RANTES may affect selective recruitment of circulating inflammatory cells such as T lymphocytes and monocytes, which may enhance lytic or latent infections with HHV-6 of these cell types.

In summary, we have shown that stable expression of HHV-6 U51, a member of the GCR superfamily distantly related to chemokine receptors, can specifically bind RANTES and is associated with morphological alteration in epithelial cells and down-regulation of RANTES expression, both transcripts and secreted protein. The U51 homologues comprise a betaherpesvirus-specific gene family that may have a conserved immunomodulatory role. This represents a novel mechanism by which a betaherpesvirus can modulate the host immune response and may subvert it to enhance infection.

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