Characterization of a Chemokine Receptor-Related Gene in Human Herpesvirus 8 and Its Expression in Kaposi's Sarcoma

HONG-GUANG GUO,* PHILIP BROWNING,† JOHN NICHOLAS,‡ GARY S. HAYWARD,§ ERWIN TSCHACHLER,Ø YI-WEN JIANG,* MARIOLA SADOWSKA,* MARK RAFFELD,¶ SANDRA COLOMBINI,* ROBERT C. GALLO,* and MARVIN S. REITZ, JR.* 1

*Institute of Human Virology, Medical Biotechnology Center, University of Maryland Biotechnology Institute, Baltimore, Maryland 21201; †Vanderbilt Cancer Center, Vanderbilt University, Nashville, Tennessee 37232-6838; ‡Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; §Department of Dermatology, University of Vienna Medical School, Vienna, Austria; ØAdvanced BioSciences Laboratories, Kensington, Maryland 20895; and ¶Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland 20892

Received October 28, 1996; accepted December 5, 1996

Human herpesvirus 8 (HHV-8) is a recently discovered virus that is highly associated with Kaposi's sarcoma (KS) and AIDS-associated body cavity lymphomas, although it is also found in some normal individuals. HHV-8 is related by nucleotide sequence homology to herpesvirus saimiri (HVS), which causes T cell lymphomas in some New World monkeys, and to Epstein–Barr virus (EBV), a human herpesvirus linked etiologically with Burkitt's lymphoma and nasopharyngeal carcinoma. We report that, like HVS but unlike EBV, HHV-8 contains a gene (ORF74) with significant sequence homology to the high-affinity IL-8 receptor, a member of the \( \alpha \) (CXC) chemokine receptor family of transmembrane G protein-coupled receptors. We also show by reverse transcription PCR that the chemokine receptor-related HHV-8 gene is detectable in some RNA samples from KS tissue, and that its expression varies independently from that of ORF26, a minor capsid protein. The presence of a potential chemokine receptor in HHV-8 and its expression in KS tissue suggests that it may be important in the regulation of viral gene expression and may play a role in the etiology of KS and AIDS-related body cavity lymphomas.

Kaposi's sarcoma (KS) was first reported as a rather indolent tumor associated with older men of Mediterranean origin. It has subsequently been reported to be endemic to parts of Africa in both sexes, and is also seen in transplant patients (for reviews see Penn, 1979; Ziegler et al., 1984). It is a frequent feature in AIDS, particularly among homosexual men (for reviews see Friedman-Kien and Saltzman, 1990; Wahman et al., 1991), and takes a quite aggressive course in this setting, contributing substantially to the morbidity and mortality of the disease. Human immunodeficiency virus type 1 (HIV-1) does not appear to be present in AIDS-KS tumors, and its causative role must therefore be indirect.

Recently, using a subtractive DNA hybridization technique called representation difference analysis (Liston and Wigler, 1993), two DNA fragments homologous to gamma herpesviruses, specifically herpesvirus saimiri (HVS) and Epstein–Barr virus (EBV), were iso-

1 To whom correspondence and reprint requests should be addressed at the Institute of Human Virology, Medical Biotechnology Center, University of Maryland Biotechnology Institute, 725 West Lombard Street, Baltimore, MD 21201. Fax: (410) 706-4694. E-mail: reitz@umbi.umd.edu.

Copyright © 1997 by Academic Press
All rights of reproduction in any form reserved.
In spite of the apparent close association of viral sequences with KS, cultured KS-derived cells do not contain them and virus-infected cells appear to be selectively and rapidly lost in culture. Several immortalized cell lines have been reported from KS, and these also lack HHV-8 sequences (Lunardi-Iskandar et al., 1995). Recently, cell lines established from AIDS-associated body cavity lymphomas have been reported to contain the sequences and to express low but detectable amounts of extracellular transmissible virus (Cesarman et al., 1995; Renne et al., 1996).

Herpesviruses genomes have many genes and complex patterns of expression. The characterization of those viral genes expressed in KS and other diseases is required in order to understand how HHV-8 contributes to disease pathogenesis. We report here on the identification and sequence analysis of a part of the viral genome containing open reading frames (ORF) with homology to IL-8 chemokine receptors, which are G protein-coupled receptors (GCR) with seven transmembrane regions, and to part of the herpesvirus ORF75 virion protein. We also show that RNA for the chemokine receptor-related gene is expressed in some KS tissue, and that its expression does not coordinate with that of a viral minor capsid protein, a gene previously reported for HHV-8 (Moore et al., 1996).

Total DNA was prepared from a body cavity lymphoma (BCBL) sample from an AIDS patient as described elsewhere (Sambrook et al., 1989). DNA (100 mg) was partially digested with Sau3A (0.2 units, 37°C, 25 min) and then size fractionated by sedimentation in a sucrose gradient by standard techniques (Sambrook et al., 1989). A fraction containing DNA ranging from 13 to 20 kb was ligated into BamHI arms of λ phage EMBL3 (Stratagene, La Jolla, CA). Phage DNA was packaged and the library was plated out and screened by standard methods (Sambrook et al., 1989). The probe used was a 32P-labeled 473-bp insert from a plasmid clone of a PCR fragment generated from the uncultured lymphoma DNA using the published sequence (Chang et al., 1994) of the HHV-8 homolog of ORF75 of HVS. The primers used for amplification of this region were 2B1 (5'-GGGATCTGGTGGTACAGCA-3') and 2B2 (5'-ATTCGCCAAGGACGTACAGCA-3'). Five positive clones were identified out of 10^5 total plaques. These were plaque purified and grown for further study. One of these, λ B6-1, was subcloned as Sall fragments in pBluescript SK (Stratagene). The resultant subclones were screened with the same probe used for the λ library and a positive colony was picked. This plasmid (pB6-1) and λ B6-1 were sequenced directly by PCR cycle sequencing and primer walking.

RNA was extracted from KS biopsies or lymph node tissue from AIDS–KS patients by mincing the tissue finely with scissors or by grinding it in liquid N₂ in a mortar and pestle, homogenizing it in guanidinium thiocyanate and purifying the RNA by standard techniques (Sambrook et al., 1989). Total RNA was purified from pleural effusions, BCBL samples, cultured cells or KS-derived spindle cells, prepared as described elsewhere (Browning et al., 1994). Where noted, poly(A) RNA was prepared by oligo(dt)-cellulose chromatography. Total RNA (1 μg) was amplified by reverse transcriptase–PCR (RT–PCR) using Tth DNA polymerase (Tth reverse transcriptase RNA PCR Gene Amp Kit, Perkin-Elmer Applied Biosystems, Foster City, CA). Both the RT and amplification steps were performed under conditions recommended by the manufacturer. Thirty-five cycles of amplification were used, with 1 min at 95°C and 1 min at 60°C per cycle. Poly(A) RNA (200 ng) was amplified with random hexamers and MuLV Superscript RT (Gibco-BRL, Gaithersburg, MD) under conditions recommended by the manufacturer. Thirty-five cycles of amplification with recombinant Taq DNA polymerase (Perkin-Elmer) were then performed, with 1 min each at 94°C, 58°C, and 72°C per cycle. Two regions were amplified, one representing a region of plasmid pB6-1 related by sequence homology to HVS ORF74, a chemokine receptor-related gene, and the other representing the HHV-8 homolog of HVS ORF26, a minor capsid protein (Chang et al., 1994). The primer pair used for amplification of the HVS ORF74 homolog was 6L1R (5'-ACTGCGAGTGAAGCAGGTTCGTCTACATC-3') and 6L4 (5'-GATTGGTCACCTACACCTTGG-3'), which gives a fragment of 438 bp. Two primer pairs were used for the HVS minor capsid protein (ORF26) analog. One set of primers, used in the experiment with total RNA, was 2A1 (5'-CTCGGAGATTGCCACGGTTTAC-3') and 2A2 (5'-GAGCTCTGAAATGAGGAGAC-3'), which give a 732-bp fragment. The second pair, used for the amplification of poly(A) RNA, was based on primers used by Chang et al. (1994) and was 5'-AGCCGAAAGGATTTCCACCAT-3' and 5'-TCCGTGTGGTGTCTACGTCAG-3'. These yielded a fragment of 233 bp. RT–PCR fragments were electrophoresed and analyzed by staining with ethidium bromide or by Southern blotting and hybridization to 32P-labeled inserts from pB6-1 or a plasmid clone of the 732-bp fragment obtained by PCR amplification using primers 2A1 and 2A2.

Figure 1 shows the nucleic acid sequence and translation of the left-hand part of pB6-1 and the phage clone flanking region in λ B6-1. This sequence contains a long ORF coding for a potential 341-residue protein and starting with an ATG which is part of a Kozak consensus sequence (Kozak, 1983). There are several possible polyadenylation signals downstream of the termination codon, one of which is around position 1190, just beyond the position of the termination codon for the homolog of the HVS ORF75, the reading frame of which is in the opposite sense. This is the position in HVS of ORF74, a viral chemokine receptor homolog (Nicholas et al., 1992). Analysis by the program SOAP ( included in the PC/Gene sequence analysis package, Intelligenetics, Mountain View, CA), which calculates hydrophobicity by the
FIG. 1. Sequence of HHV-8 chemokine receptor analog. Partial sequence of pB6-1, including homolog of HVS ORF74. The nucleotide sequence of a portion of pB6-1 and λ B6-1 including the ORF homologous to HVS ORF74 as well as its inferred amino acid sequence is shown. A probable polyadenylation signal is underlined.
method of Klein et al. (1984), predicts that the translated HHV-8 protein is an integral membrane protein that contains seven transmembrane regions, characteristic of GCRs, a family of proteins which includes chemokine receptors.

The inferred reading frame was used to screen the SwissProt database by a FASTA (Pearson and Lipman, 1988) search using the GCG sequence analysis package on the NIH mainframe computer and by a BEAUTY search (Worley et al., 1995) using the Baylor University Molecular Biology server. The strongest match was indeed found with the HVS ORF74 GCR homolog (Nicholas et al., 1992) (optimized FASTA score = 548, 36% amino acid identity in a 308-residue overlap). Significant optimized FASTA scores (>200) were also obtained with a variety of other GCR-related proteins. The closest matches were with various chemokine receptors, particularly those belonging to the high-affinity CXC chemokine receptor B chain (IL8RB) (Table 1). Other close matches include US28, a GCR homolog of human cytomegalovirus (HCMV), as well as fusin, a GCR-related protein recently identified as a cofactor for fusion of T cell line-tropic strains of the HIV (Feng et al., 1996).

Figure 2 shows an alignment, performed using the CLUSTAL program of Higgins and Sharp (1988) (contained in the PC/Gene package), between the inferred amino acid sequence of the putative HHV-8 chemokine receptor and several other viral and cellular chemokine receptors. The putative transmembrane regions are indicated with brackets. Positions of amino acid identity between the HHV-8 chemokine receptor homolog and those of HVS or HCMV or between HHV-8 and IL8RB or fusin are shaded. Several other features found in most GCRs are conserved in the inferred HHV-8 protein, including several glycosylation signals in the N-terminal putative extracellular domain, proline residues in putative transmembrane regions V, VI, and VII, and cysteine residues in the putative second, third, and fourth extracellular domains.

RNA from KS tissue was analyzed by RT-PCR for expression of RNA from several HHV-8 genes, including the chemokine receptor-related gene. In one set of experiments, as shown in Fig. 3A, gene transcripts for the HVS minor capsid protein (ORF26) homolog were detected in one of four samples of total RNA at relatively high levels, in a second at low levels, and were not detectable in two other samples. RNA for the chemokine receptor homolog was detectable in three of four samples (Fig. 3B), including the one weakly positive for minor capsid protein RNA, but was not detectable in the sample strongly positive for minor capsid protein RNA.

In a separate set of experiments, using polyadenylated RNA (Fig. 3C), chemokine receptor transcripts were detected in a pleural effusion from AIDS – KS, but were negative in a second pleural effusion, a peripheral blood mononuclear cell (PBMC) sample from AIDS – KS, uncultured and early passage (p6) BCBL cells, two short-term cultures from KS tumors, and six samples of spindle cells purified from AIDS – KS patients. The positive AIDS – KS pleural effusion was also positive for RNA of the HVS minor capsid protein (ORF26), as were the uncultured BCBL sample and one short-term culture from a classical KS. The BCBL cell culture was also negative by PCR for HHV-8 DNA, suggesting that it did not contain HHV-8. Although the classical KS cell culture in lane 8 was negative for actin, the RNA otherwise appeared to be of sufficient quality for RT-PCR because it was positive for several HHV-8 transcripts other than the ORF75 or chemokine receptor homologs (see below). The cell line BCBL-1 (Renne et al., 1996) was negative for chemokine receptor homolog RNA, but became positive after induction with sodium butyrate (not shown). None of the RNA samples shown in Fig. 3C were positive when the reverse transcription step was omitted, indicating that the amplified fragments indeed represented RNA transcripts.

These data indicate that although HHV-8 DNA sequences are almost always present in KS tissue, detectable expression of the different viral genes is variable. Consistent with this conclusion, we have shown elsewhere that, in contrast to the detection of RNA for the chemokine receptor and ORF74 homologs in a minority of the samples in Fig. 3C, RNA transcripts of a recently reported HHV-8 cyclin D homolog (Chang et al., 1996a) were detectable in all of them except for the AIDS – KS PBMC, one AIDS – KS culture, and the BCBL short-term culture (Browning et al., manuscript in preparation). PBSC-33 and -39, the two classical KS cultures, both pleural effusions, and the uncultured BCBL sample are also positive for RNA transcripts of one of the two chemokine homologs (MIP-1B) recently described by Nicholas et al. (submitted for publication).

We have identified and characterized a gene from HHV-8 that is closely related to the ORF74 chemokine sequence of the putative HHV-8 chemokine receptor B chain (IL8RB) (Table 1). Other close matches include US28, a GCR homolog of human cytomegalovirus (HCMV), as well as fusin, a GCR-related protein recently identified as a cofactor for fusion of T cell line-tropic strains of the HIV (Feng et al., 1996).

Table 1: Relationship of HHV-8 GCR-like Sequence to Other gcr Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>FASTA score</th>
<th>(% identity/n residues)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HVS GCR homolog</td>
<td>559</td>
<td>(36/308)</td>
</tr>
<tr>
<td>IL8RB α CXC chemokine receptor</td>
<td>439</td>
<td>(28/323)</td>
</tr>
<tr>
<td>IL8RB β CXC chemokine receptor</td>
<td>397</td>
<td>(28/314)</td>
</tr>
<tr>
<td>CMV US28 GCR homolog</td>
<td>326</td>
<td>(24/312)</td>
</tr>
<tr>
<td>Type 1A angiotensin receptor</td>
<td>297</td>
<td>(21/305)</td>
</tr>
<tr>
<td>EBV-induced GCR homolog</td>
<td>297</td>
<td>(23/308)</td>
</tr>
<tr>
<td>Burkitt’s lymphoma GCR homolog</td>
<td>270</td>
<td>(22/285)</td>
</tr>
<tr>
<td>CKR1 C-C chemokine receptor</td>
<td>269</td>
<td>(20/312)</td>
</tr>
<tr>
<td>Bradykinin receptor</td>
<td>263</td>
<td>(24/294)</td>
</tr>
<tr>
<td>Anaphylatoxin chemotactic receptor</td>
<td>209</td>
<td>(24/324)</td>
</tr>
<tr>
<td>Fusin (HIV-1 fusion accessory)</td>
<td>205</td>
<td>(21/270)</td>
</tr>
</tbody>
</table>

* FASTA scores were derived as described in text.
receptor homology of HVS. Among cellular genes, the predicted HHV-8 gene product is most closely related to the chemokine receptor family of GCR proteins, particularly receptors for the CXC chemokine IL-8. HSV ORF74 has in fact been shown to code for a functional IL-8 receptor (Ahuja and Murphy, 1993). The presence of a chemokine receptor in HHV-8 is of possible significance in vivo. In fact, HHV-8 is not present in several neoplastic KS-derived cell lines, suggesting that it is not necessary for the maintenance of KS lesions. Induction of its expression by butyrate in BCBL-1 cells suggests that it is expressed primarily during viral lytic replication. In view of its potential activity as a mediator of chemotaxis and inflammation, however, it may well be involved in pathogenesis in vivo.

It is plausible that HHV-8 could play a role in KS development, especially given the apparent high prevalence of viral antibodies and DNA in KS. Against this possibility, HHV-8 is not present in several neoplastic KS-derived cell lines.

FIG. 2. HHV-8 G protein-coupled receptor homolog. Comparison of HHV-8 chemokine receptor homolog with cellular and viral chemokine receptors. Amino acid alignment was performed using the program CLUSTAL. Amino acid residues in the HHV-8 inferred amino acid sequence that are identical to those of the other proteins are shaded. Dashes indicate gaps in the amino acid sequences.
FIG. 3. Detection of HHV-8 RNA by RT-PCR. (A and B) Total RNA was subjected to reverse transcription and PCR amplification as described in the text and analyzed by Southern blot. Lanes 1–3 contain RNA samples from KS biopsies, lane 4 contains RNA from a lymph node biopsy from an AIDS-KS patient, lane 5 contains RNA from the Jurkat T cell line, and lane 6 contains pAW109 RNA from the RT-PCR kit, used here as a negative control. A used primers and a probe for the HHV-8 minor capsid protein (ORF26) homolog, and B used primers and a probe for the HHV-8 homolog to HVS ORF74. (C) Polyadenylated RNA was analyzed as described for A and B, except that the fragments were detected by staining with ethidium bromide. The top panel was analyzed for the ORF74 chemokine receptor homolog, the middle panel was analyzed for the minor capsid protein (ORF26) analog, and the bottom panel represents a control RT-PCR analysis for actin. Lane 1 was a PBMC AIDS-KS sample; lane 2 was an AIDS-KS cell line; lane 3 was a pleural effusion from an AIDS-KS patient; lane 4 was an uncultured BCBL sample from an AIDS-KS patient; lane 5 was a second pleural effusion from an AIDS-KS patient; lane 6 was a short-term culture (passage 6) from a BCBL sample from an AIDS-KS patient; lanes 7 and 8 were short-term cultures (passage 2) from classical KS patients; lanes 9–14 were samples of spindle cells purified from peripheral blood of AIDS-KS patients; lane 15 was a negative control which did not contain any RNA. The numbers on the left give the position and size in kb of specific fragments from a molecular weight marker containing HindIII-digested λ phage and HaeIII-digested φX174.

lines which bear many similarities to the spindle cells thought to be the tumor cell in KS (Lunardi-Iskandar et al., 1995), and HHV-8 are not detectable in KS cells grown in vitro (Dictor et al., 1996; Colombini, unpublished data). Although we have shown that HHV-8 chemokine receptor RNA is detectable in KS tissue, the only consistent expres-
sion of viral genes reported so far is of two small RNAs with little protein coding potential (Zhong et al., 1996), which may represent latency genes. Furthermore, there are reports of a varying, but significant, incidence of HHV-8 detection in apparently healthy people (Rady et al., 1995; Luppi et al., 1996; Monini et al., 1996). Obviously, with a virus as genetically complex as HHV-8, much work needs to be done on the expression of individual genes in order to properly assess its possible role in KS.

ACKNOWLEDGMENTS

We thank A. Mazzucca for expert editorial assistance. E.T. is supported by a grant (PO 1437-MED) from the Austrian Science Foundation. The DNA sequence has been deposited in GenBank under the accession number U82242.

REFERENCES


