Splicing of Rhesus Rhadinovirus R15 and ORF74 Bicistronic Transcripts during Lytic Infection and Analysis of Effects on Production of vCD200 and vGPCR

Carly L. Pratt, 1,2,† Ryan D. Estep, 1,2,† and Scott W. Wong 1,2,3,*

Vaccine and Gene Therapy Institute, Oregon Health and Science University, West Campus, 1 and Division of Pathobiology and Immunology, Oregon National Primate Research Center, 2 Beaverton, and Department of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, Oregon.

Received 30 January 2004/Accepted 1 November 2004

Rhesus macaque rhadinovirus (RRV) is the rhesus macaque homologue of human herpesvirus 8 (HHV-8). Here we examine expression of RRV R15 and ORF74, homologues of K14 and ORF74 of HHV-8, respectively. As in HHV-8, transcripts encoding RRV R15 and ORF74 are bicistronic. However, unlike what has been suggested for HHV-8, RRV R15- and ORF74-encoding transcripts are expressed late during lytic infection and undergo unique splicing events that result in the production of transcripts capable of encoding vGPCR, as well as membrane-associated and secreted forms of vCD200. The alternative splicing for vCD200 has implications for viral pathogenesis.

Rhesus macaque rhadinovirus (RRV) is the rhesus macaque homologue of human herpesvirus 8 (HHV-8)/Kaposi’s sarcoma-associated herpesvirus (2, 10). DNA sequence analysis of RRV indicates that the virus encodes several of the open reading frames (ORFs) present in HHV-8 thought to be involved in virus-mediated pathogenesis. For example, like HHV-8, RRV encodes a constitutively signaling GPCR (G-protein-coupled receptor) with transforming potential (3) and a viral interleukin-6-like homologue (7). Another RRV ORF of interest is R15, which is homologous to human CD200 and to ORF K14 of HHV-8, which encodes a viral CD200 homologue (7), capable of binding the CD200 receptor on cells of myeloid lineage and initiating an anti-inflammatory (TH2) response (4–6, 13).

Transcriptional studies of HHV-8 ORF74 have revealed that this ORF is transcribed as the second ORF of spliced bicistronic messages with ORF K14. These studies also suggest that vCD200 and vGPRC are made in full length from these transcripts; however, this has not been demonstrated. In addition, detection of the K14 and ORF74 transcripts has been reported to occur early during lytic HHV-8 gene expression (1, 8, 9, 11). We sought to determine the expression patterns of R15 and ORF74 during lytic RRV infection in vitro, to compare transcription of these ORFs to HHV-8.

Northern blot analysis of R15 and ORF74 transcripts. Figure 1A depicts the region of the RRV genome encoding R15 and ORF74. To examine expression of RRV R15 and ORF74, primary rhesus fibroblasts were infected with RRV strain 17577 (multiplicity of infection of 5) (12) in the presence of cycloheximide (CHX; Sigma, St. Louis, Mo.) to inhibit protein synthesis or phosphonoacetic acid (PAA; Sigma) to inhibit DNA replication or were left untreated to detect immediately early, early, or late viral transcripts, respectively. The time points for collection of RNA were 24 (CHX), 48 (PAA), and 72 (untreated) h. Northern blot analysis with double-strand ORF74 and R15 probes demonstrated that transcripts are detected only in 72-h-untreated cultures, indicating that these ORFs are transcribed as late-lytic genes, and that similar-size transcripts are detected, suggesting that R15 and ORF74 are bicistronic (Fig. 1B and C). An antisense oligonucleotide probe specific for R15 further confirms the specificity of both transcripts for R15 (Fig. 1D).

RT-PCR analysis of R15-ORF74 bicistronic message. To confirm that R15 and ORF74 are bicistronic, we utilized PCR primers specific for the 5’ end of R15 and the 3’ end of ORF74 in reverse transcription-PCR (RT-PCR) analysis with 72-h-untreated RNA and amplified two predominant products, a 2.1-kb product and a more abundant 1.7-kb product (Fig. 2A). RT-PCR products were sequenced, confirming the identity of the unspliced 2.1-kb cDNA and demonstrating the presence of a splice donor at nucleotide (nt) 123555 (within R15 sequence) and a splice acceptor at nt 123916 (8 nt upstream of the ORF74 ATG) (Fig. 2C), which results in the 1.7-kb species. The donor sequence motif (G’TGTTGGGT) is identical to the donor sequence identified in the splice of the K14 and ORF74 transcript in HHV-8, while the acceptor motif (TGTCA’G) is more divergent from that found in HHV-8 (TTGTA’G) (1, 8, 9, 11).

Unlike what has been reported for HHV-8, in which neither the K14 nor the ORF74 coding sequence is altered, the splice event in this region removes 72 bp from the 3’ end of R15, making the stop codon for this shortened form of R15 a TGA located 8 bp downstream of the ATG for ORF74 (Fig. 2C). This splice would affect the predicted vCD200 expression from the transcript, creating a form of vCD200 with 24 amino acids deleted from the C terminus (amino acids 230 to 254) and replaced with 6 new amino acids. Importantly, the 24-amino-
acid region deleted from vCD200 contains the predicted transmembrane domain of this protein (amino acids 229 to 250) (Fig. 2D).

Examination of expression patterns of R15 and ORF74 from spliced and unspliced bicistronic messages. Since antibodies specific to vCD200 and vGPCR are not available, we examined protein expression from the 2.1- and 1.7-kb cDNAs by introducing an internal FLAG epitope upstream of the identified splice site of R15 and a hemagglutinin (HA) epitope tag to the C terminus of vGPCR within the context of 1.7- or 2.1-kb cDNAs. The internal FLAG-tagged R15 constructs are 1.7-kb R15-FLAG and 2.1-kb R15-FLAG, while the C-terminal HA-tagged ORF74 constructs are 1.7-kb ORF74-HA and 2.1-kb ORF74-HA. CHO cells were then transfected with empty vector or tagged versions of the cDNA constructs; stained with antibodies specific for the FLAG or HA epitopes (Sigma); and examined by immunofluorescent confocal microscopy, fluorescence-activated cell sorting (FACS), and Western blot analyses.

CHO cells expressing the 2.1-kb R15-FLAG showed specific staining, with vCD200-FLAG and vGPCR not available, we examined protein expression from the 2.1- and 1.7-kb cDNAs by introducing an internal FLAG epitope upstream of the identified splice site of R15 and a hemagglutinin (HA) epitope tag to the C terminus of vGPCR within the context of 1.7- or 2.1-kb cDNAs. The internal FLAG-tagged R15 constructs are 1.7-kb R15-FLAG and 2.1-kb R15-FLAG, while the C-terminal HA-tagged ORF74 constructs are 1.7-kb ORF74-HA and 2.1-kb ORF74-HA. CHO cells were then transfected with empty vector or tagged versions of the cDNA constructs; stained with antibodies specific for the FLAG or HA epitopes (Sigma); and examined by immunofluorescent confocal microscopy, fluorescence-activated cell sorting (FACS), and Western blot analyses.

CHO cells expressing the 2.1-kb R15-FLAG showed specific staining, with vCD200-FLAG appearing predominately cytosolic, with areas of membrane staining (Fig. 3A). A similar staining pattern was observed in cells expressing 2.1-kb ORF74-HA (Fig. 3B), demonstrating that vCD200 and vGPCR are synthesized from this cDNA.

Cells expressing the spliced 1.7-kb R15-FLAG cDNA displayed vCD200 staining that was exclusively cytosolic, indicating that the truncated version of vCD200 is absent from the plasma membrane (Fig. 3C) and suggesting that the transmembrane region is required for cell surface expression. vGPCR staining from the 1.7-kb R15-ORF74 cDNA was similar to that from the 2.1-kb cDNA product (Fig. 3D), indicating that splicing of the 2.1-kb R15-ORF74 transcript does not inhibit vGPCR expression.

To further demonstrate that the transmembrane region is necessary for cell surface expression of vCD200, live transiently transfected CHO cells were stained for surface-expressed vCD200 with anti-FLAG antibody and subjected to FACS analysis. Compared to vector-transfected cells, cells expressing vCD200 from the 1.7-kb R15-FLAG construct demonstrated very low levels of surface staining (2.24%), while cells transfected with the 2.1-kb R15-FLAG construct demonstrated significant levels of surface staining (33.4%), confirming that full-length-membrane-associated vCD200 is strictly expressed from the unspliced 2.1-kb R15-ORF74 transcript (Fig. 3E). FACS analysis of fixed-permeabilized cells expressing either the 1.7- or the 2.1-kb R15-FLAG construct indicated equal levels of total vCD200 expression from the two constructs (data not shown).

Western blot analysis of lysates from transfected CHO cells demonstrated that a band of approximately 40 kDa for vCD200-FLAG was detected in cells expressing either 1.7- or 2.1-kb R15-FLAG (Fig. 4A). The predicted size of full-length vCD200-FLAG is 28 kDa; however, the protein has several
predicted glycosylation sites, which likely accounts for the discrepancy in the size of the protein detected by Western blotting. Also, since the splicing event that removes the transmembrane region of vCD200 deletes 18 amino acid residues, both the full-length and spliced species of vCD200 are likely represented by the same approximately 40-kDa band in cells expressing the 2.1-kb R15-FLAG construct.

The lack of a transmembrane region in the 1.7-kb cDNA suggests that a soluble version of vCD200 can be secreted from cells expressing spliced 1.7-kb R15-ORF74 cDNA. To test this, supernatants from transfected CHO cells were analyzed by Western blotting. As hypothesized, supernatants from cells expressing 1.7-kb cDNA contained significant amounts of vCD200, indicating that the 1.7-kb cDNA is capable of producing soluble secreted vCD200 (Fig. 4C). Interestingly, the 2.1-kb R15-FLAG construct also expressed a secreted form from transfected CHO cells, suggesting that the 2.1-kb cDNA also encodes a soluble form or that the 2.1-kb transcript is spliced in these cells to yield the 1.7-kb transcript. RT-PCR analysis of RNA isolated from the 2.1-kb R15-FLAG-transfected cells indicated that the 1.7-kb transcript is produced in these cells, and DNA sequence analysis of the products confirmed the identity of both cDNAs (data not shown). Taken together, these data show that there are two species of vCD200 produced from the 2.1-kb cDNA construct, a cell-associated form (from unspliced 2.1-kb message) and a secreted form (from spliced 1.7-kb message).

Further confirmation that the 1.7-kb cDNA product is capable of expressing vGPCR was evident by Western blot analysis of lysates from transfected CHO cells. With the use of anti-HA antibody, a band of the predicted size of the RRV vGPCR was detected in cells transfected with either the 1.7- or the 2.1-kb ORF74-HA construct (Fig. 4C), although the signal appears more prevalent in the lysates from 2.1-kb-transfected cells (Fig. 4C). This result indicates that full-length protein is produced from either spliced or unspliced cDNAs but that splicing may decrease translation levels of vGPCR.

The presence of soluble RRV vCD200 during RRV infection could have major implications in altering the function of the immune system of an infected host. RRV vCD200 is similar in sequence to human CD200 and to HHV-8 vCD200, which have been demonstrated to play important roles in controlling the inflammatory response induced by cells of myeloid lineage, inducing these cells to produce an anti-inflammatory (TH2)
The function of RRV vCD200 is being investigated.

This work was supported by NIH grants AI07472 (R.D.E.), RR00163 (S.W.W.), and CA75922 (S.W.W.). We thank Anda Cornea for assistance with confocal microscopy.

REFERENCES


FIG. 3. Immunofluorescent confocal microscopy and FACS analysis of CHO cells transiently expressing 1.7- or 2.1-kb bicistronic R15-ORF74 cDNAs. vCD200 contains an internal FLAG epitope tag, while vGPCR contains a C-terminal HA epitope tag. Immunofluorescence analysis (A to D) demonstrates that vCD200 expressed from the 2.1-kb cDNA (A) is detected throughout the cytoplasm and at the cell surface (arrowheads), while vCD200 expressed from the 1.7-kb cDNA (C) displays only a cytoplasmic staining pattern. The staining pattern for vGPCR is similar for the 2.1-kb (B) and 1.7-kb (D) versions of cDNA, with protein detected throughout the cytoplasm as well as at the cell surface. The field shown in panels A, C, and D is 512 by 512 μm; that shown in panel B is 1,024 by 1,024 μm. (E) Live cells were stained for FLAG (vCD200) expression and analyzed by FACS. Compared to vector control cells, cells transfected with the 1.7-kb R15-ORF74 cDNA display low levels of surface staining, while cells transfected with the 2.1-kb R15-ORF74 cDNA show significant levels of surface staining, confirming that only the 2.1-kb cDNA is capable of expressing a membrane-associated form of vCD200.

FIG. 4. Western blot analysis of vCD200 and vGPCR expression. (A) Anti-HA Western blot analysis of immunoprecipitated lysates from CHO cells transfected with empty vector, 1.7-kb ORF74-HA, 2.1-kb ORF74-HA, or HA-ORF74-expressing control vector. (B and C) Total cell lysates (B) or concentrated supernatants (C) from CHO cells transfected with empty vector, 1.7-kb R15-FLAG, or 2.1-kb R15-FLAG were analyzed for recombinant vCD200-FLAG expression.

This work was supported by NIH grants AI07472 (R.D.E.), RR00163 (S.W.W.), and CA75922 (S.W.W.). We thank Anda Cornea for assistance with confocal microscopy.

FIG. 3.

FIG. 4.


