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A Gammaherpesvirus G Protein-Coupled Receptor Homologue Is Required for Increased Viral Replication in Response to Chemokines and Efficient Reactivation from Latency¹,²

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The open reading frame (ORF) 74 of gamma-2-herpesviruses encodes a G protein-coupled receptor which is highly conserved in members of this subfamily and is homologous to the CXCR2 chemokine receptor. The viral G protein-coupled receptor has been implicated in viral pathogenesis. However, the advantage of such chemokine receptor homologues to the virus is currently unknown. To address this, we constructed ORF74 deletion mutants of a mouse gamma-2-herpesvirus (MHV-68) and examined the effect of the deletion on viral growth and reactivation from latency. Growth of the mutant viruses in NIH 3T3 cells was similar to that of wild-type virus. However, CXC chemokines with ELR motifs, KC, and macrophage-inflammatory protein 2, significantly increased viral replication of the wild-type, but not the mutant viruses, via a pertussis toxin-insensitive, mitogen-activated protein/extracellular signal-regulated kinase and phosphatidylinositol 3-kinase-dependent pathway. IFN-γ-inducible protein 10, a CXC chemokine lacking an ELR motif, was able to reverse the effect of KC on viral replication. The mutant viruses also showed significantly reduced reactivation from latently infected mouse splenocytes. Reinsertion of ORF74 into the mutant virus restored the wild-type phenotype. Utilizing a viral CXCR2 homologue to enhance replication and reactivation from latency represents a novel mechanism by which gammaherpesviruses can subvert the immune response. The Journal of Immunology, 2003, 170: 243–251.

Chemokines are a family of small structurally related cytokines whose functions include the induction of directional migration of cells. Members of the chemokine family are related by a four-cysteine motif and are divided into four groups, CC, CXC, C, and CX₃C, depending on the position of the first two cysteine residues. Chemokines bind to seven-transmembrane domain G protein-coupled receptors (GPCR). Several viruses, predominantly members of the Herpesviridae, express GPCR, some of which have been shown to be functional membrane-bound chemokine receptors (reviewed by Murphy (1)).

MURINE GAMMAHERPESVIRUS-68 (MHV-68) is a member of the gamma-2 subfamily of herpesviruses and is closely related to the human pathogen Kaposi’s sarcoma-associated herpesvirus (KSHV, human herpesvirus 8) (2–4). Sequence analysis has shown that the MHV-68 open reading frame 74 (ORF74) encodes a GPCR homologue of the cellular CXCR2 chemokine receptor (2). This gene is conserved in other gamma-2-herpesviruses such as KSHV, herpesvirus samiri, and equine herpes virus 2 (2, 5–8) and is, therefore, likely to play an important role in viral pathogenesis and/or immune evasion. The KSHV GPCR has been shown to be constitutively active, although further increases in activity can be achieved by the exogenous addition of CXC chemokines and can induce cell proliferation (7, 9). In both MHV-68 and KSHV, the viral GPCR (vGPCR) is expressed during lytic replication (10–12). It has been more difficult to detect expression of the vGPCR during latency for either MHV-68 or KSHV. However, using a highly sensitive nested RT-PCR, Wakeling et al. (13) were able to detect expression of MHV-68 GPCR in spleen and lung during viral persistence, whereas Virgin et al. (14) detected the MHV-68 GPCR in latently infected macrophages from B cell-deficient mice.

Since the MHV-68 ORF74 gene has significant sequence homology to the KSHV ORF74 (2), studies on MHV-68 deletion mutants are of considerable interest in delineating the role and significance of this vGPCR.

Recently, the cloning of several herpesviruses, including MHV-68, as infectious bacterial artificial chromosomes (BAC) has been described (15). This system has been useful for the introduction of various types of targeted mutation into the viral genome. In the present study, we used a BAC cloning system, that was described earlier (15), to generate MHV-68 viruses with mutations in the ORF74. Using these mutant viruses, we investigated the role of the vGPCR in MHV-68 replication and reactivation from latency.
Materials and Methods

Virus stocks and plaque assay

The original stock of MHV-68 (clone G2.4) was obtained from Dr. A. A. Nash (University of Edinburgh, Edinburgh, U.K.). The virus was grown in baby hamster kidney cells (BHK-21, CCL10; American Type Culture Collection, Manassas, VA) maintained in Glasgow’s modified Eagle’s medium (Life Technologies, Grand Island, NY) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The BHK-21 cells were infected at a multiplicity of infection (MOI) of 0.01, and virus stocks were prepared when the cytotoxic effect was complete, by freezing and thawing the cultures. Virus titers were determined by plaque assay on NIH 3T3 cells (CRL1658; American Type Culture Collection) grown in DMEM (Life Technologies) supplemented with 10% FCS, 1% l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete DMEM). Serial 10-fold dilutions of virus were absorbed onto NIH 3T3 monolayers for 1 h at 37°C and then washed with fresh medium and overlaid with medium containing 1.6% carboxymethylcellulose (CMC). After 6 days, the monolayers were fixed with 10% paraformaldehyde and stained with 0.3% crystal violet solution to determine the number of plaques.

Generation of ORF74 mutants

Recombinant viruses with mutations in the ORF74 were made using MHV-68 cloned as BAC as recently described (15). Two different methods were used to delete regions of the ORF74. The first deletion mutant (ORF74FRT mutant) was generated by “ET-cloning” as described elsewhere (16). To this end, a linear PCR fragment containing a FRT-flanked tetracycline resistance cassette was generated from vector pCP16 (17) using two pairs of primers containing 24 nt for amplification of the tetracycline resistance gene and an additional 50 nt homologous to sequences flanking the region to be deleted, corresponding to nucleotide positions 105091–105140 and 105785–105834 (2), respectively. This PCR product was transferred into the MHV-68 BAC by homologous recombination in Escherichia coli strain DH10B already containing the MHV-68 BAC and the plasmid pSP72 (18). The plasmid pSP72 expresses the Red recombinase system under the control of a regulated (L-arabinose-inducible) promoter and mediates the recombination. Successful recombination led to the deletion of 105141–105784 of MHV-68. The tetracycline resistance gene was then removed by Flp-mediated recombination as described previously (19). Recombinant BAC plasmids were characterized by restriction enzyme analysis and sequencing.

A second, independent deletion mutant (ORF74DEL mutant) was generated by a two-step replacement procedure as described elsewhere (19, 20). For that purpose, a 3.495-kb SphI fragment of MHV-68 (nucleotide positions 103880–107375) was cloned into the shuttle plasmid pS776K-S (21) followed by this fragment, 440 bp of ORF74 were removed using two BsrGI sites (nucleotide positions 105376 and 105816), and the resulting fragment was transferred into the MHV-68 BAC by homologous recombination in E. coli strain DH10B. Recombinant BAC plasmids were characterized by restriction enzyme analysis and sequencing.

An additional mutant with a stop linker inserted in ORF74 (ORF74STOP mutant) was generated by the above-mentioned two-step replacement procedure. For that purpose, a 5.186-kb EcoRI fragment of MHV-68 (nucleotide positions 103164–108350) was cloned into the plasmid pK18 (21). A 16-bp stop linker containing a Hpal site and stop codons in all possible frames (22) was inserted in the MfeI site at nucleotide position 105568 of MHV-68. The resulting EcoRI fragment containing the stop linker was cloned into the shuttle plasmid pS776K-SR and transferred into the MHV-68 BAC by homologous recombination in E. coli strain DH10B. Recombinant BAC plasmids were characterized by restriction enzyme analysis and sequencing.

A revertant BAC plasmid to the ORF74 STOP mutant (ORF74STOP revertant) was generated by the two-step replacement procedure. For that purpose, a 3.495-kb SphI fragment of MHV-68 (nucleotide positions 103880–107375) was cloned into the shuttle plasmid pS776K-SR and electroporated into E. coli strain DH10B that already contained the ORF74 STOP mutant BAC. Recombinant BAC plasmids were characterized by restriction enzyme analysis and sequencing.

Electroporation of ~2–3 μg of the recombinant BAC plasmids into BHK-21 cells resulted in the reconstitution of recombinant viruses expressing the gfp gene. To remove the BAC vector sequences, rat embryonic fibroblasts expressing recombinase Cre (15) were infected, and viral clones with the BAC vector sequences deleted were purified by limiting dilution using loss of green fluorescent protein expression as a screening marker. DNA of all reconstituted, recombinant viruses was isolated from infected BHK-21 cells and analyzed by restriction enzyme digestion. Successful mutation was confirmed by Southern blot analysis. The DNA was transfected onto a Hybond N* membrane (Amer sham, Arlington Heights, IL). Blots were hybridized overnight with digoxigenin-labeled probes and developed using the ECL system (Boehringer Mannheim, Indianapolis, IN) according to the instructions of the manufacturer.

Replication of the ORF74 mutant viruses in vitro

Single-step and multistep virus growth experiments were conducted to compare the growth kinetics of mutant and revertant viruses to those of the parent virus. A series of replicate cultures of BHK-21 or NIH 3T3 cells was infected separately at a MOI of 1 for single-step and 0.01 for multistep assays. After 1 h of adsorption, the wells were washed with medium. Infected cultures were harvested at successive intervals postinfection, and the amount of infectious virus in the culture was determined by plaque assay on NIH 3T3 cells as described above.

Infectious center assay

The frequency of latently infected cells in mouse splenocyte suspensions was determined by infectious center assay. Splenocytes were removed from C57Bl/6 mice and spleen cell suspensions were prepared using cell strainers (BD Labware, Franklin Lakes, NJ). Splenocytes were infected with MHV-68 at a MOI of 10 for 24 h at 37°C and then plated at various densities (10^2–10^7/well) onto monolayers of NIH 3T3 cells in 12-well plates, incubated overnight, and then overlaid with medium containing 1.6% CMC. The cells were cocultured for 5–6 days, and plaques were counted as described above. Parallel cultures were set up using aliquots of 10^2–10^4 spleen cells and disrupted using a Tissue-Tearor homogenizer (Fisher, Pittsburgh, PA) to determine the amount of preformed infectious virus.

RT-PCR assays

Total RNA from NIH 3T3 cells infected with viruses was prepared using TRIzol (Life Technologies) as recommended by the manufacturer. The RNA was resuspended in 50 μl of diethyl pyrocarbonate-treated water, quantitated, and treated with DNase (Life Technologies) as recommended by the manufacturer. RNA (2 μg) was reverse transcribed by using the Superscript preamplification system (Life Technologies) for first-strand cDNA synthesis in a total volume of 25 μl, and a reverse transcriptase control was conducted in parallel. The primer sequences for the ORF73 genes were 5'-ATCTTTTTGTTGGAGGGGGACC-3' and 5'-ACCGAC TACACGCAACAAACCC-3'. The primer sequences for the ORF74 genes were 5'-GGATTCAGATGGGCGACG-3' and 5'-AGGTCTCTGTGAA CCCACCTTATGC-3'. The primer sequences for the ORF75 genes were 5'-CCGTCGTGTTGAAAGAGAGTG-3' and 5'-CTGTATACGGAT GGTTGAGAC-3'. The primer sequences for the M11 genes (Bcl-2 homologue) were 5'-GCAACCCGTATTACAGCTTCC-3' and 5'-CAG AATATCTCCTGGGCAACACC-3'. The products were analyzed on 1% agarose gel. The following conditions were used: for ORF73: 94°C for 2 min; 30 cycles of 94°C for 1 min, 58°C for 2 min, and 72°C for 3 min, followed by a 7-min final extension at 72°C. For ORF74 and ORF75c, 94°C for 4 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30s, followed by a 15-min final extension at 72°C.

Real-time quantitative PCR

Quantitation of viral genomes was performed by real-time PCR using the 7500 Sequence Detection System (PE Biosystems, Foster City, CA). This method is based on the continuous optical monitoring of a fluorogenic PCR.

PCR primers for the MHV-68 gpl590 gene were used. The upstream and downstream primer sequences were 5'-CAACAATCCCACTACAATT ATGGC-3' and 5'-TCGGGCTGCTTTTGTTG-3', respectively. The fluorogenic PCR probe located between the PCR primer was synthesized by PE Biosystems (5'-VIC)-ACTCTACATTCTGGGACCAC-3'. Two of the primers, as well as that of the probe, were selected from a region that does not share significant homology with other known nucleotide sequences in GenBank.

Statistical analysis

Statistical analysis was performed using Student’s t test.

Results

Generation and characterization of ORF74 mutants

To analyze the effect of disrupting ORF74 of MHV-68, three independent mutants were constructed by different methods using MHV-68 cloned as a BAC (15) (Fig. 1A). A deletion mutant
(ORF74FRT mutant), lacking 644 bp of ORF74, was constructed by insertion of an FRT-flanked tetracycline resistance gene. After removing the tetracycline resistance gene by FLP-mediated recombination, a small residual insert consisting of an FRT site and some vector sequence is left in the disrupted ORF74 gene. To control for any effects of this small residual insert, a second deletion mutant (ORF74DEL mutant), lacking 440 bp of ORF74, was generated by a two-step replacement procedure which does not leave any foreign sequence behind. To exclude any effects the deletions introduced in ORF74 might cause on neighboring genes or regulatory sequences, a third independent mutant (ORF74STOP mutant) was made by insertion of a 16-bp stop linker into ORF74. This stop linker was first described for the construction of HSV-1 mutants (22) and has recently been used to construct a mutant of ORF72 (v-cyclin) of MHV-68 (23). To exclude the possibility that observed effects were due to rearrangements outside ORF74, a revertant of the ORF74STOP mutant (ORF74STOP revertant) was generated using an unmutated genomic fragment by a two-step

**FIGURE 1.** Construction of mutants and structural analysis of the genomes of reconstituted viruses. A, Schematic representation of the genomes of reconstituted viruses. P indicates the probe used for Southern blot analysis, corresponding to nucleotide positions 102215-103164. B, Southern blot analysis of DNA of reconstituted viruses digested with the restriction enzymes and hybridized with the probe indicated in A. FRT MUT, ORF74FRT mutant; WT, parental virus; STOP MUT, ORF74STOP mutant; REV, ORF74STOP revertant; DEL MUT, ORF74DEL mutant. Marker sizes (in kilobase pairs) are indicated on the left.
replacement procedure. The structure of all cloned genomes was verified by restriction enzyme digestion with different enzymes and by sequencing the site of mutation (data not shown). Recombinant viruses were reconstituted from all BAC-cloned genomes, the BAC vector sequences were removed, and the structure of all recombinant viruses was analyzed by restriction enzyme digestion of viral DNA and Southern blot (Fig. 1B). Bands of the expected size were observed, confirming the structure of the mutated viral genomes.

RT-PCR analysis of ORF74 mutant virus gene expression

The disruption of the ORF74 might inadvertently disrupt promoter/enhancer regions of neighboring genes. We used RT-PCR to confirm that the neighboring ORF73 and ORF75c were transcribed normally in the mutant viruses. Transcription of the M11 gene, which is expressed on a bicistronic transcript with ORF74 (13), was also analyzed. Our previous data showed that all four genes are expressed during lytic replication of MHV-68 in NIH 3T3 fibroblasts (S. R. Sarawar and B. J. Lee, unpublished data). NIH 3T3 fibroblasts were infected with the mutant, or wild-type virus, and the total cellular RNA was recovered. The RNA was briefly treated with DNase I to remove contaminating viral DNA and was then analyzed by RT-PCR. As shown in Fig. 2, wild-type virus yielded a PCR product corresponding to the predicted 759-bp fragment for ORF74, 362-bp fragment for ORF73, 405-bp fragment for ORF75c, and 400-bp fragment for M11. The stop mutant virus yielded a PCR product for ORF74 that was very slightly larger than that of the wild-type virus (due to the presence of the stop linker), whereas the two deletion mutants gave PCR products of around 600 bp, confirming the deletion. The products for the adjacent ORF73, ORF75c, and M11 genes were present as in wild-type virus, showing that the mutagenesis of ORF74 did not affect the transcription of neighboring genes. Control reactions without reverse transcriptase yielded no bands (data not shown), indicating that there was no contamination of the RNA preparation with viral DNA.

FIGURE 2. RT-PCR analysis of MHV-68 ORF73, 74, 75c, and M11. NIH 3T3 fibroblasts were infected with wild-type, stop mutant, FRT mutant, or DEL mutant virus at a MOI of 1. Three days later, cells were harvested and RNA was extracted for RT-PCR analysis. PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. Markers consisting of a 100-bp DNA ladder (Life Technologies) are shown to the left.

Role of the MHV-68 GPCR in viral growth in vitro

One-step growth curve experiments were conducted to identify potential alterations in replication of the mutant viruses in vitro. We compared replication of wild-type, FRT mutant, DEL mutant, stop mutant, and revertant viruses in a single round of replication in both NIH 3T3 (Fig. 3A) and BHK-21 cells (data not shown). There was no difference in viral replication. Similar results were obtained in multistep growth assays (Fig. 3B). Furthermore, the plaque sizes were similar for the mutant, revertant, and wild-type viruses (Fig. 3C). This indicates that ORF74 is not essential for replication of MHV-68 in vitro.

Enhancement of MHV-68 replication by chemokines requires the vGPCR

Since both KSHV and HVS GPCR have been shown to signal in response to CXC chemokines (6, 9), we hypothesized that MHV-68 replication might be increased by the exogenous addition of chemokine ligands such as KC. The latter is a CXC chemokine with an ELR motif and is a ligand for the cellular CXCR2 chemokine receptor. Clear differences were observed in viral replication following treatment with KC (Fig. 4, A–C). Maximal responses were obtained at a concentration of 10 ng/ml KC (Fig. 4A). The effect of KC on viral replication peaked at 12–24 h after treatment (Fig. 4B). Wild-type virus titers were significantly higher in the presence, than in the absence, of KC at 12 or 24 h after infection (p < 0.0001, at each time point). RNase protection assays failed to detect cellular CXCR2 in either uninfected or MHV-68-infected fibroblasts (data not shown), suggesting that the effect of KC on viral replication might be mediated via the vGPCR. This contention was supported by the finding that KC did not increase the replication of STOP, FRT, or DEL ORF74 mutant viruses. In contrast, replication of the revertant virus was increased by KC to an extent similar to the wild-type virus, showing that the lack of response of the mutant virus to KC was due to the disruption of ORF74 and not to mutations inadvertently introduced elsewhere in the viral genome. These data show that MHV-68 replication is enhanced by the CXC chemokine KC and that the vGPCR is required for this effect.

Similar effects on viral growth were observed with another CXC ELR motif chemokine, macrophage-inflammatory protein 2 (MIP-2), which also significantly increased viral replication (p < 0.02), whereas CC chemokines RANTES and monocyte chemotactic protein 1 (MCP-1) had no effect (Fig. 4D). Crg-2 (mouse IFN-γ-inducible protein 10 (IP-10)) is a CXC chemokine lacking an ELR motif which binds to a different cellular receptor from KC and MIP-2. IP-10 has been shown to function as an inverse agonist for the KSHV GPCR (39, 40). Crg-2 had no effect on MHV-68 replication when added alone, but was able to significantly reduce the increase in viral replication induced by KC (p < 0.01, Fig. 4E). In contrast, RANTES and MCP-1 had no effect on KC-augmented viral replication (data not shown).

Signaling via the MHV-68 GPCR

To investigate the nature of the G proteins coupling with the MHV-68 CXCR2 homologue, pertussis toxin (100 μg/ml) was added to virus-infected NIH 3T3 cells in the presence and absence of KC. Pertussis toxin did not affect KC-enhanced viral replication (Fig. 5A), suggesting that G proteins of the pertussis toxin-insensitive Gq family rather than Gi/o proteins, which are generally pertussis-toxin sensitive, were used in the effects on viral replication mediated via the MHV-68 GPCR.
To further investigate the signaling pathway utilized by the MHV-68 GPCR, the effect of inhibitors of mitogen-activated protein/extracellular signal-regulated kinase (MEK; mitogen-activated protein kinase kinase) and phosphatidylinositol 3-kinase (PI3-kinase) was determined. PI3 kinase inhibitor LY294002 and MEK inhibitors PD98059 and U0126, but not a structurally related control compound, U0124 (each added at a concentration of 10 μM), were able to block KC-enhanced replication of the wild-type and revertant viruses \( (p < 0.001 \text{ in each case, Fig. 5B}) \). In contrast, replication of the wild-type virus in the absence of added chemokines or of the ORF74 STOP or DEL mutant viruses in the presence of KC was unaffected by LY294002 or PD98059 (Fig. 5B). U0126 reduced replication of the wild-type, revertant, and mutant viruses in the presence of KC to below the level of replication in the absence of added chemokine (Fig. 5B). The percentage reduction was 16–28% and was statistically significant for the wild-type, STOP mutant, and revertant \( (p < 0.01 \text{ in each case}) \), but not for the FRT mutant. A similar small reduction was observed in the replication of the wild-type virus in the absence of KC (data not shown). Because both mutant and wild-type viruses were affected and because the other MEK inhibitor PD98059 did not have the same effect, it is possible that this reflects a minor nonspecific effect of U0126.

In summary, the data in Fig. 5B show that the signaling pathway by which KC stimulates viral replication via the MHV-68 GPCR involves both MEK and PI3-kinase.

**Role of vGPCR in viral reactivation from latency**

In addition to its function in chemokine-stimulated viral replication, the role of the MHV-68 GPCR in the reactivation of latent virus was examined. Mouse splenocytes were infected in vitro with mutant, wild-type, or revertant virus at a MOI of 1 for 24 h and then washed thoroughly with medium. Infected splenocytes were then used in an infectious center assay, which measures viral reactivation in culture. Reactivation of latent virus requires intact viable cells. Therefore, mechanically disrupted splenocytes were used as a control to detect preformed infectious virus. Cultures using disrupted splenocytes yielded only low numbers of plaques showing that the majority of plaques in the infectious center assay were the result of viral reactivation from latency (data not shown). The frequency of infectious centers was significantly lower in splenocytes infected with the STOP mutant virus than in those infected with either the wild-type or revertant virus \( (Fig. 6A, p < 0.0001) \). Similar results were obtained with the FRT and DEL mutants \( (p < 0.0001, \text{ in each case}) \). In contrast, the level of viral genomes, determined by real-time quantitative PCR, was similar in splenocytes infected with the STOP mutant virus than in those infected with either the wild-type or revertant virus \( (Fig. 6A, p < 0.0001) \). Similar results were obtained with the FRT and DEL mutants \( (p < 0.0001, \text{ in each case}) \). In contrast, the level of viral genomes, determined by real-time quantitative PCR, was similar in splenocytes infected with wild-type and mutant viruses \( (Fig. 6B) \). This suggests that the mutant virus is able to establish latency in splenocytes to the same extent as the wild-type virus, but reactivation from latency is impaired.

**Discussion**

Several viruses have been shown to encode membrane-bound vGPCR, at least some of which have been shown to be functional chemokine receptor homologues (reviewed by Murphy (1)). Beta-herpesviruses such as CMV, human herpesvirus (HHV) 6, and HHV7, encode CC chemokine receptor homologues (24–29) which have been implicated in viral dissemination (26, 30–32) and sequestration of chemokines in vitro (33), a potential mechanism of immune evasion. Interestingly, a murine CMV GPCR, M78, has been shown to facilitate the accumulation of immediate early viral mRNA (34).
Members of the gamma-2-herpesvirus subfamily, on the other hand, encode CXC chemokine receptor homologues (2, 5–8). These vGPCR are encoded by ORF74 and are conserved in all members of the gamma-2-herpesvirus subfamily that have been sequenced to date, suggesting that they play an important role in viral growth and/or immune evasion. Since the gamma-2-herpesvirus family includes the KSHV (HHV8), which has been implicated in the induction of disease in humans (7, 35, 36), it is especially important to understand the function of these viral genes. However, suitable ORF74 mutant viruses and culture systems for exploring the effect of the vGPCR on KSHV replication are not currently available. Therefore, we investigated the role of the vGPCR in vitro using a mouse y-2 herpesvirus. The approach that we chose was to construct MHV-68 mutants lacking functional vGPCR. The vGPCR of both MHV-68 and KSHV (Refs. 11–13, 37, 38; Fig. 2) are expressed during lytic replication. Expression of the MHV-68 GPCR during persistence has also been reported (13, 14).

We found that CXC chemokines, such as KC, enhanced MHV-68 replication in permissive fibroblasts by a mechanism that was dependent on the vGPCR. Thus, stimulation of viral replication by KC was significantly impaired in mutant viruses lacking the viral chemokine receptor. Another striking feature of the ORF74 mutant virus was its significantly reduced ability to reactivate from latently infected splenocytes. Several mutants, constructed using different strategies, all showed the same phenotype, both in terms of reactivation from latency and the replicative response to KC. Thus, it is unlikely that the observed phenotype was an artifact caused by the method of construction of the mutants. Reinsertion of the ORF74 into the mutant virus (construction of a revertant virus) restored the wild-type phenotype, again showing that the observed properties of the mutant were indeed due to the lack of the ORF74 gene product and not due to a secondary mutation that had occurred elsewhere in the genome. The fact that neighboring genes were expressed normally showed that mutation of the ORF74 gene had not inadvertently affected their transcription.

The CC chemokines RANTES and MCP-1 did not affect MHV-68 replication. Neither did the CXC chemokine crg-2 (mouse IP-10), which lacks an ELR motif. Therefore, only the

FIGURE 4. Effect of chemokines on MHV-68 replication. A, Dose-response curve for the effect of the CXC chemokine KC on viral replication. Viral titers were determined in the presence or absence of chemokine. Monolayers of NIH 3T3 cells were infected with virus at a MOI of 1 and treated with KC at various concentrations. Production of virus was determined by plaque assay. Data are expressed as mean PFU/ml ± SD from three independent experiments. B, Time course of KC stimulation of MHV-68 replication in NIH 3T3 cells. Monolayers of NIH 3T3 cells were infected with virus at a MOI of 1 and treated with KC (10 ng/ml). Production of virus was determined by plaque assay. Data are expressed as the mean ratio of viral titers in the presence and absence of chemokine ± SD from three independent experiments at 12 and 24 h and two at 48 h. C, Stimulation of viral replication by KC is dependent on the vGPCR. Monolayers of NIH 3T3 cells were infected with wild-type, revertant, Stop, FRT, or Del ORF74 mutant viruses at a MOI of 1 and treated with KC (10 ng/ml). Production of virus 24 h after infection was determined by plaque assay. Data are expressed as the mean ratio of viral titers in the presence and absence of KC ± SD. D, The effect of CC and CXC chemokines on MHV-68 replication. The effect of CC chemokines MCP-1 and RANTES or CXC chemokines MIP-2 and crg-2 (mouse IP-10) on replication of MHV-68 was determined as in C. All chemokines were used at a concentration of 10 ng/ml. Data are expressed as the mean ratio of viral titers in the presence and absence of chemokine ± SD of triplicate determinations from one of two independent experiments that gave similar results. E, Reduction of KC-induced viral replication by crg-2. Monolayers of NIH 3T3 cells were infected in triplicate with wild-type or Stop mutant viruses at a MOI of 1 and treated with KC (10 ng/ml) and/or crg-2 (10 ng/ml). Production of virus 24 h after infection was determined by plaque assay. Data are expressed as the mean PFU/ml ± SD.
CXC chemokines with ELR motifs appear to meet the structural requirements for binding and/or signaling through the MHV-68 GPCR that are necessary for the increased viral replication. Although crg-2 (mouse IP-10) did not affect viral replication alone, it was able to inhibit the increase in viral replication induced by KC. This is reminiscent of the ability of IP-10 to function as an inverse agonist for signaling of the KSHV GPCR (39, 40). Thus, our data confirm and extend previous findings on the inhibitory effect of IP-10.

The stimulation of viral replication via the vGPCR was insensitive to pertussis toxin, indicating that the G protein used was more likely to be Gq than the pertussis toxin-sensitive Gi/o proteins. Previous studies have documented signaling of the KSHV GPCR via both pertussis toxin-sensitive and -insensitive pathways, depending on the cell type (7, 40, 41). The increase in viral replication induced by KC was sensitive to specific inhibitors of both PI3-kinase and MEK, showing that both of these kinases were involved in signaling via the MHV-68 GPCR. Again, this parallels a reported signaling pathway for the KSHV GPCR (42). Interestingly, it has recently been reported that, in mice expressing the KSHV GPCR as a transgene, activation of the receptor by chemokines is necessary for its tumorigenic effect in vivo (43).

The KSHV GPCR can signal constitutively, although its activity can be further increased by CXC chemokines (7, 9). In the case of MHV-68, we do not know whether the GPCR signals constitutively, although it does appear to respond to CXC chemokine ligands. Our studies confirm the published work on chemokine stimulation of

**FIGURE 6.** MHV-68 vGPCR is required for efficient reactivation from latency. A., Infectious center assay. Mouse splenocytes were infected with wild-type or revertant MHV-68 viruses at a MOI of 1.0 in vitro. After 24 h, splenocytes were plated on fibroblast monolayers and overlaid with CMC. Plaques were counted after 6 days. Data are presented as mean ± SD for triplicate assays in one of two independent experiments performed. In each experiment, there was a highly significant difference in the numbers of infectious centers obtained with wild-type and mutant viruses (***, p < 0.0001, in each case). B, Real-time quantitative PCR. Quantitation of viral genomes was performed by real-time PCR with the 5700 Sequence Detection System (PE Biosystems) using primers specific for the MHV-68 gp150 gene. Data are shown for one of two separate experiments performed. Similar results were obtained in each experiment.
signaling via a gammaherpesvirus GPCR and extend these findings to show an effect on viral replication. The general lack of effect of PI3-kinase and MEK kinase inhibitors on the basal replication suggests that the signals via these pathways that affect viral replication are not constitutive. However, this does not rule out the possibility that the MHV-68 GPCR signals constitutively via other pathways. Furthermore, the constitutive activity of the KSHV GPCR depends on the cell type in which it is expressed (41).

The stimulation of viral replication by CXC chemokines in vitro was tested in permissive fibroblasts and a moderate enhancement was seen. Nevertheless, this enhancement was highly significant, was specific for CXC chemokines with ELR motifs, and could be blocked by highly specific inhibitors of both PI3-kinase and MEK. In vivo, macrophages, B cells, dendritic cells, and lung endothelial cells have been shown to harbor MHV-68. Therefore, it is possible that in vivo the degree of stimulation of viral replication via the MHV-68 GPCR may differ from that observed in vitro dependent on the infected cell type.

One of the most interesting features of the MHV-68 GPCR revealed by these studies was its role in viral reactivation from latency. Thus, reactivation of ORF74 mutants from latently infected splenocytes was significantly impaired. This appeared to reflect a problem with reactivation rather than with viral entry into the cells, since real-time quantitative PCR showed that the levels of viral genome in splenocytes were similar for wild-type and mutants.

Signaling via the KSHV GPCR has been shown to differ from that via its cellular counterpart (7, 41). We do not know whether the same applies to the MHV-68 GPCR and its cellular counterpart, mCXCR2, or whether chemokines can stimulate viral replication via the cellular receptor. We were unable to detect expression of the cellular receptor in cultured fibroblasts. However, preliminary results suggest that there is no difference in MHV-68 replication in CXCR2+/- and CXCR2-/- mice (B. J. Lee and S. R. Sarawar, unpublished data). MHV-68 has been shown to establish latency in splenic macrophages, dendritic cells, and B cells (44–47). Thus, both CXCR2+ (macrophages and dendritic cells) and CXCR2- (B cells) harbor MHV-68 in splenocyte cultures. As shown in Fig. 6, reactivation of the viral CXCR2 mutants from latently infected splenocytes was dramatically reduced compared with that of the wild-type virus. We have also seen the same effect in splenocyte cultures from B cell-deficient mice (B. J. Lee and S. R. Sarawar, unpublished data). Hence, even when the cellular CXCR2 is present on the infected cell types, the viral chemokine receptor still has a significant effect.

Our observations suggest that herpesvirus vGPCR may play a role in viral replication and reactivation from latency in vivo. This role may be more prominent in immunocompromised individuals. However, this does not exclude the possibility that the vGPCR plays additional roles in viral dissemination and immune evasion, as do the CC chemokine receptor homologues in betaherpesviruses. We are currently examining the pathogenesis of the mutant viruses in both normal and immunocompromised mice.

We conclude that the gammaherpesvirus vGPCR modulates both viral replication and reactivation from latency and that this represents a novel mechanism by which gamma-2-herpesviruses can subvert components of the immune system.

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