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Short communication

The human cytomegalovirus US27 gene product enhances cell proliferation and alters cellular gene expression

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ABSTRACT

Human cytomegalovirus (HCMV) is a prevalent pathogen worldwide. Although generally harmless in healthy individuals, HCMV can pose a serious threat to immune compromised individuals and developing fetuses in utero. HCMV encodes four genes predicted to give rise to G protein-coupled receptors (GPCRs): US27, US28, UL33, and UL78. The US28 gene product is a functional chemokine receptor that enhances cell growth in some cell types but induces apoptosis in others. In contrast, the US27 gene product has not been demonstrated to signal either constitutively or in a ligand-induced manner. In this study, US27 was expressed in transfected cells, and both cell proliferation and DNA synthesis were significantly increased compared to control cells. PCR array analysis revealed that expression of US27 led to changes in a limited number of cellular genes, but genes that were up-regulated included the pro-survival factor Bcl-x, AP-1 transcription factor components jun and fos, and the IL-6 family cytokine oncostatin M. These results demonstrate that US27 can impact host cell physiology and may shed light on the function of this orphan viral GPCR.

Human cytomegalovirus (HCMV) is a member of the *Herpesviridae* family, and infection is widespread in the general population (Staras et al., 2006). While most healthy adults exhibit only mild or no symptoms of infection, serious disease can occur in immune compromised individuals, especially AIDS patients, transplant recipients, and newborn infants (Kesson and Kakakios, 2007). HCMV is the leading infectious cause of birth defects, and congenital HCMV infection continues to be problematic due to deficiencies in public awareness, diagnostic procedures, and treatment options (Revello et al., 2008).

Like all other herpesviruses, the HCMV virion consists of a large DNA genome enclosed in an icosahedral capsid surrounded by a dense tegument layer, all encircled in a lipid envelope containing numerous viral glycoproteins. HCMV has the largest genome of all the human herpesviruses at 230 kb and encoding at least 167 genes (Mocarski et al., 2006). More than half of these genes are not required for virus replication in vitro (Yu et al., 2003) but instead play roles in vivo in the manipulation of host immune responses and the establishment of latency (Jackson et al., 2011). The US27 gene, which encodes a putative G protein-coupled receptor (GPCR) found in the viral envelope, is one of these non-essential genes (Chee et al., 1990; Margulies and Gibson, 2007). Virus mutants lacking US27 are replication competent (Bodaghi et al., 1998), although a single log reduction in virus titers produced from both infected fibroblasts and endothelial cells was observed (O'Connor and Shenk, 2011). The US27 deletion mutant virus also exhibited a defect in extracellular spreading, but the virus was still able to infect neighboring cells, presumably via the cell-cell route (O'Connor and Shenk, 2011). The US27 gene is expressed late during infection, and the gene product is found mainly in the endosomes, the Golgi apparatus, and perinuclear compartments of infected cells (Fraile-Ramos et al., 2002).

The US27 gene product has many conserved features of the chemokine receptor subset of the GPCR superfamily, such as seven transmembrane domains, a DRY (aspartic acid–arginine–tyrosine) motif in the second intracellular loop, conserved cysteines (C104 and C176) in the second and third extracellular loops, and extensive glycosylation of the extracellular domains (Margulies and Gibson, 2007). Despite having these characteristics, US27 is considered an orphan since no human chemokine ligands have been shown to engage the receptor (Stapleton et al., 2012). Interestingly, HCMV encodes three other genes that give rise to proteins having similarity to human chemokine receptors (Beisser et al., 2002; Chee et al., 1990). One of these, US28, has been shown to elicit intracellular signaling both constitutively and in response to several human chemokines, including CCL3/MIP-1α, CCL5/Rantes, and CX_3CL1/Fractalkine (Gao and Murphy, 1994; Neote et al., 1993; Stropes et al., 2009). UL33 also has constitutive signaling ability (Casarosa et al., 2003), and rodent homologs of both UL33

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and UL78 have been shown to play a role in virus dissemination in vivo (Beisser et al., 1999, 1998). The M33 gene of murine cytomegalovirus, a homolog of HCMV UL33, was found to be required for salivary gland tropism and efficient reactivation from latency, and the HCMV US28 gene was able to complement and partially rescue those deficiencies (Cardin et al., 2009; Farrell et al., 2011).

Recent evidence suggests that US28 forms heteromeric complexes with US27, UL33 and UL78 (Tschische et al., 2011). While no functional changes were observed with the US28:US27 heteromer, the US28:UL33 heteromer and the US28:UL78 heteromer both ablated activation of NF-κB transcriptional activity by US28. This suggests a complex level of regulation in which these viral receptors may interact in particular combinations to either promote or block signaling through specific pathways in particular cell types or at specific times during the course of virus infection. US27, US28, UL33, and UL78 are all likely to play important roles in immune modulation and viral persistence, and the presence of multiple receptors in the viral genome could be due to the need to control cellular activity in the large variety of cell types infected by HCMV, which include monocytes, lymphocytes, epithelial cells, endothelial cells, and fibroblasts (Mocarski et al., 2006). Considering that GPCRs constitute a major target in pharmaceutical development, discerning the function of viral GPCRs during HCMV infection could be highly beneficial in the quest for novel anti-viral therapeutics.

To study the function of US27, the gene from HCMV strain AD169 was cloned into the p3XFLAG expression vector and transiently transfected into HEK293 cells, as described (Stapleton et al., 2012). The cells were seeded into 96-well plates at a density of 1×10^4 cells per well, and cell proliferation was monitored using the CellTiter-Glo Assay (Promega, Madison, WI). Briefly, a luciferin substrate that is converted to oxyluciferin in the presence of O_2 and ATP was added to each well. The resulting luminescence is proportional to the amount of ATP present, reflecting the number of viable cells in the well. Cells expressing US27 had greater luminescence, suggesting that they had an enhanced growth rate compared to controls, as shown in Fig. 1A. The control cells included mock transfected cells treated with transfection reagent only, cells transfected with the empty p3XFLAG vector, and cells transfected with the p3XFLAG vector expressing HCMV US28 or human chemokine receptor CXCR3, which was cloned from human peripheral blood mononuclear cells as previously described (Stapleton et al., 2012). US27-expressing cultures consistently exhibited 14–26% greater luminescence over the course of the experiment than most of the other cell lines, which is indicative of higher cell numbers and a faster growth rate. Each of the other control cell lines had comparable growth rates except for the US28-expressing cells, which exhibited reduced luminescence, indicating fewer viable cells. The transfection efficiency was comparable among the cell lines at 60–70%, as indicated by staining with an anti-FLAG antibody (Sigma–Aldrich, St. Louis, MO), followed by FITC-conjugated secondary antibody and flow cytometry (Fig. 1B).

To rule out any possibility that the FLAG tag might be affecting US27 functional activity, the US27, US28 and CXCR3 genes were also cloned into a pEGFP vector (Clontech, Mountain View, CA) and expressed as fusion proteins linked to the C-terminal domain of EGFP. As shown in Fig. 1C, HEK293 cells transiently transfected with pEGFP-US27 also exhibited greater cell proliferation than corresponding control cells. The increase in growth rate for these cells was more modest, with an increase of 9–16% over each of the control cell lines over the course of the experiment. The growth of the pEGFP-US27 transfected cells was 26–30% higher than pEGFP-US28 transfected cells. Transfection efficiency with the pEGFP plasmids was comparable among the cell lines, but lower than the p3XFLAG plasmids, at 46–52% (Fig. 1D). Overall, the US27-expressing HEK293 cultures grew faster while US28 cells grew at a reduced rate compared to the control cell lines. These results suggested that the US27 gene product might enhance cell growth.

Previous studies have shown that the US28 gene product may have different effects on cell growth and survival depending on cell type. Transient transfection of GFP-US28 induced apoptosis in 293T, HeLa, and Cos cells (Pleskoff et al., 2005), whereas US28 was found to enhance cell growth and cell cycle progression in stably transfected NIH-3T3 cells (Maussang et al., 2006). Since we found increased growth rates associated with US27 and decreased growth rates with US28 in HEK293 cells, we next transfected and evaluated two additional cell types, HeLa and Cos cells (Fig. 1E and F). The results indicate that these cell types also exhibited greater proliferation when expressing HCMV US27 than control cells. Cells expressing US28 were found to have the slowest growth rate, possibly due to the loss of some cells undergoing apoptosis, a result that would be consistent with previous studies (Pleskoff et al., 2005). Although we also attempted to examine the effects of US27 in NIH-3T3 cells, the rate of transfection was extremely low (10%) and prevented their inclusion in this study.

In order to confirm that the differences in growth rates that we observed were specifically due to the presence of US27 and not the result of well-to-well differences in plasmid purity, transfection efficiency, or transgene expression, stable HEK293 cell lines were created. Following transfection with p3XFLAG-US27, cells were cultivated in the presence of 1 mg/ml geneticin to eliminate untransfected cells, and clonal cell lines were created using limiting dilution. As shown in Fig. 2A, comparable levels of FLAG-tagged protein were expressed in each of the cell lines. CXCR3 was detected as a 42 kD band, US28 as a 44 kDa band, and US27, which is extensively glycosylated, was detected as a 45–55 kD smear. Immunofluorescence microscopy also indicated that protein expression levels were comparable among the cell lines (Fig. 2B). When the growth rate of the stable cell lines was examined via cell counting, the US27 cultures were found to have significantly higher cell numbers after 72 h (Fig. 2C). Although one representative clonal cell line is shown here, three individual 293-US27 clonal cell lines were examined and all had comparable expression levels and growth rates (data not shown), suggesting that the integration of the expression cassette did not account for the enhanced proliferation of cells expressing US27. The mean doubling time was 12.1 h for the 293-US27 cultures, compared to 13.9 h for empty vector transfected cells, 14.4 h for 293-CXCR3 cells and 17.5 h for 293-US28 cells (Fig. 2D). When cell proliferation was examined using the Cell Titre-Glo assay, 293-US27 cells still exhibited significantly greater proliferation (14–25% higher) than control cells (Fig. 2E). In addition, DNA synthesis was examined using BrdU incorporation, and the rate of DNA synthesis was found to be significantly higher (26–49%) in cells expressing US27 compared to the control cell lines (Fig. 2F). These results clearly demonstrate that the HCMV US27 gene product stimulates cell proliferation and increases the rate of DNA synthesis.

In order to identify cellular genes that might be affected by the US27 gene product and contribute to the enhanced proliferative effect observed here, PCR array analysis was performed on the stable cell lines. RNA was extracted from each cell type (HEK293, 293-US27, 293-US28, and 293-CXCR3) and expression levels of 84 genes involved in the JAK-STAT signaling pathway were compared using RT2 Profiler Arrays (SABiosciences, Valencia, CA). For each of the stable cell lines, three biological replicates were assayed, averaged, and compared to control HEK293 cells, as shown in Fig. 3. Some genes were found to be up-regulated in a non-specific manner, for example, JUNB expression was increased by more than 2-fold in all three cell lines compared to control HEK293 cells. Other genes that were up-regulated by all three receptors included MYC, CEBP, and JAK1, suggesting that overexpression of any GPCR might yield the induction of a certain subset of inflammatory genes. In