Brief Definitive Report

Highly Selective Escape from KSHV-mediated Host mRNA Shutoff and Its Implications for Viral Pathogenesis

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Abstract

During Kaposi's sarcoma (KS)–associated herpesvirus (KSHV) lytic infection, many virus-encoded signaling molecules (e.g., viral G protein–coupled receptor [vGPCR]) are produced that can induce host gene expression in transiently transfected cells, and roles for such induced host genes have been posited in KS pathogenesis. However, we have recently found that host gene expression is strongly inhibited by 10–12 h after lytic reactivation of KSHV, raising the question of whether and to what extent de novo host gene expression induced by viral signaling molecules can proceed during the lytic cycle. Here, we show by microarray analysis that expression of most vGPCR target genes is drastically curtailed by this host shutoff. However, rare cellular genes can escape the host shutoff and are potently up-regulated during lytic KSHV growth. Prominent among these is human interleukin-6, whose striking induction may contribute to the overexpression of this cytokine in several disease states linked to KSHV infection.

Key words: KSHV • DNA array • GPCR • Castleman's disease • herpesvirus • Kaposi's sarcoma

Introduction

Kaposi's sarcoma (KS)–associated herpesvirus (KSHV) is the etiologic agent of Kaposi's sarcoma (1) and is also associated with two lymphoproliferative disorders: multicentric Castleman's disease (MCD) and primary effusion lymphoma (PEL; reference 2). KS is an angioproliferative lesion whose predominant cells, spindle cells, are of endothelial lineage and are thought to be a major source of the angiogenic and inflammatory factors that drive KS histogenesis (3). Although most KS spindle cells are latently infected by KSHV, KS tumors also harbor a small subpopulation of lytically infected spindle cells (4) that may provide an important source of secreted, proangiogenic, and mitogenic factors contributing to disease progression (5, 6). For example, the KSHV lytic program encodes several transmembrane signaling molecules, including a viral G protein–coupled receptor (vGPCR; reference 7) whose expression in transfected cells induces transcription of multiple cellular genes including cytokines, signaling molecules, and transcription factors (8). KSHV vGPCR has attracted particular attention in KS pathogenesis because its expression can induce cell proliferation and endothelial tube formation in vitro, and angioproliferative lesions in transgenic mice in vivo (7, 9).

However, this simple picture is complicated by our recent finding that during the KSHV lytic cycle, there is a profound shutoff of host gene expression mediated by the viral shutoff exonuclease (SOX) protein (encoded by ORF37), which promotes degradation of cellular mRNAs (10). These observations raise important questions about the ability of lytic viral signaling molecules to stimulate production of host-encoded pathogenetic factors in infected spindle cells. Here, we demonstrate that the vast majority of cellular genes induced by exogenous vGPCR expression are not similarly up-regulated in lytically infected cells, despite abundant expression of the vGPCR. However, we have also identified a small subset of host transcripts that escape shutoff, and note that some of these, including IL-6, may play important roles in KSHV-related diseases.

Materials and Methods

Virus Preparation. The construction of Ad-RTA has been described previously (11). Ad-vGPCR was generated using the Adeno-X expression system according to the manufacturer's protocols (CLONTECH Laboratories, Inc.). KSHV stocks were generated as described previously (11).

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Cells, Transfections, and Virus Infections. Telomerase-immortalized microvascular endothelial (TIME) cells (12) and TIME cells stably expressing destabilized green fluorescent protein (TIME-dsGFP) were maintained in EBM-2 bullet kits (Clonetics). KSHV$^+$ BCBL-1 cells, human foreskin fibroblasts (HFFs), and 293 cells were maintained as described previously (11). 293T cells were transfected using Fugene 6 (Roche).

TIME cells were infected with KSHV in serum-free media and lytically reactivated as described previously (11). Adenovirus infection was performed as described previously (11) in serum-free EGM media. All cells were incubated in serum-free media for a minimum of 16 h before harvest.

Immunofluorescence Microscopy Assays. Immunofluorescence assays were performed as described previously (19). vGPCR antibodies were provided by G. Hayward (The Johns Hopkins University School of Medicine, Baltimore, MD).

mRNA Amplification, Microarray Hybridization, and Data Analysis. The human cDNA array has been described previously (8) and represents ~20,000 genes derived from PCR of an expressed sequence tag (EST) clone set using common primers as well as ~200 additional cDNAs amplified using gene-specific primers for KSHV sequences and select additional cellular genes. To generate mRNA for microarray analysis, 5 μg of total RNA from each sample was subjected to linear mRNA amplification by in vitro transcription of cDNA as described previously (13). To generate each probe, 2 μg of the amplified RNA was reverse transcribed in the presence of 300 μM amino-allyl dUTP as described previously (14) and coupled to either Cy3 or Cy5 (Amersham Biosciences). Probes were hybridized to the microarrays overnight at 65°C. Arrays were scanned using an Axon 4000B scanner, and Cy3 and Cy5 signals were normalized such that all good features ($r^2 \geq 0.75$) equaled 1. Arrays were analyzed using GenePix 3.0 and clustered using TreeView. Spots with obvious defects were excluded from the analysis. Each experiment was repeated three independent times, and genes up-regulated at least twofold greater than the reference sample in two or more of the experiments were considered significant. The fold up-regulation reported in Tables I and II represents the average fold up-regulation of that gene from the set of experiments. The complete array datasets can be viewed on the NCBI-GEO website (www.ncbi.nlm.nih.gov/geo, accession no. GSE1406).

Northern Blots and Immunoblots. Total RNA or poly(A) RNA isolated from TIME cells for array analysis was Northern blotted as described previously (8). Probes were generated using the Rediprime II random prime labeling system (Amersham Biosciences).

For immunoblots, cell lysates were prepared in radioimmunoprecipitation assay buffer and immunoblotted as described previously (15) using HIF-1 monoclonal antibodies (Novus Biologicals).

Results

Experimental Strategy. To determine if vGPCR-induced genes can escape KSHV-induced host shutoff, we used DNA microarray analysis to compare the profile of cellular genes up-regulated during lytic KSHV infection with those up-regulated upon expression of vGPCR alone. To this end, we infected TIME cells with an adenovirus construct expressing vGPCR (Ad-vGPCR), resulting in high-level expression of vGPCR mRNA (Fig. 1 A) and protein (Fig. 1 B). For examination of host gene expression during viral infection, we infected TIME cells with KSHV and induced lytic replication 2 h later by superinfection

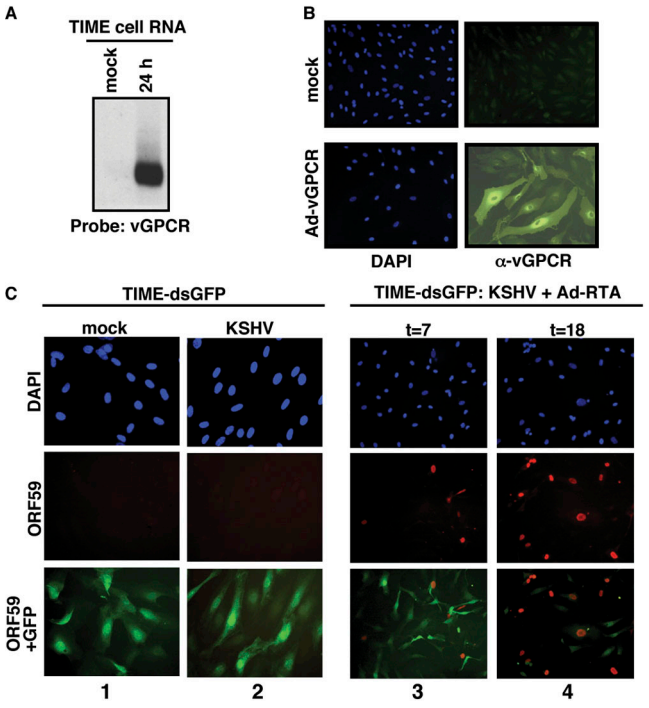

Figure 1. Expression of Ad-vGPCR and KSHV in TIME cells. (A) TIME cells were either mock infected or infected with Ad-vGPCR; 24 h after infection, total RNA was Northern blotted with a ^{32}P-labeled vGPCR DNA probe. (B) The same infected cells were examined by DAPI staining (left) or IFA with antibodies to vGPCR (right). (C) TIME cells stably expressing dsGFP were either mock infected, latently infected with KSHV, or infected with KSHV and lytically reactivated with Ad-RTA for 7 or 18 h. Each row represents the same field of cells showing either DAPI-stained nuclei (top), ORF59$^+$ cells (middle), or the merged image of ORF59 and dsGFP (bottom).

with an adenovirus encoding the KSHV lytic switch protein RTA (Ad-RTA; reference 11). IFA staining for the early lytic viral protein ORF59 confirms that this system allows lytic replication in the majority of the cells in the culture (Fig. 1 C, column 4). To affirm that this KSHV infection extinguished host gene expression under our experimental conditions, we used an assay for shutoff based on extinction of host GFP expression. TIME-dsGFP cells express a destabilized GFP (dsGFP); inhibition of host gene expression results in rapid loss of dsGFP staining (10). As shown in Fig. 1 C (column 2), latently infected TIME-dsGFP cells were uniformly GFP$^+$, whereas beginning at 7 h after lytic reactivation, ORF59$^+$ cells had become dsGFP$^-$ and by 18 h, dsGFP staining is virtually absent (Fig. 1 C, column 4).

Each microarray experiment consisted of six samples as follows: normal TIME cells (used as the reference), TIME cells infected with Ad-RTA or Ad-vGPCR alone for 20 h, or TIME cells infected with KSHV and immediately reactivated with Ad-RTA for 6, 12, or 20 h. Cy5-labeled probes were generated from each sample and mixed with an equivalent amount of a Cy3-labeled control reference probe derived from normal TIME cells and hybridized to the microarray. Only genes that were up-regulated greater

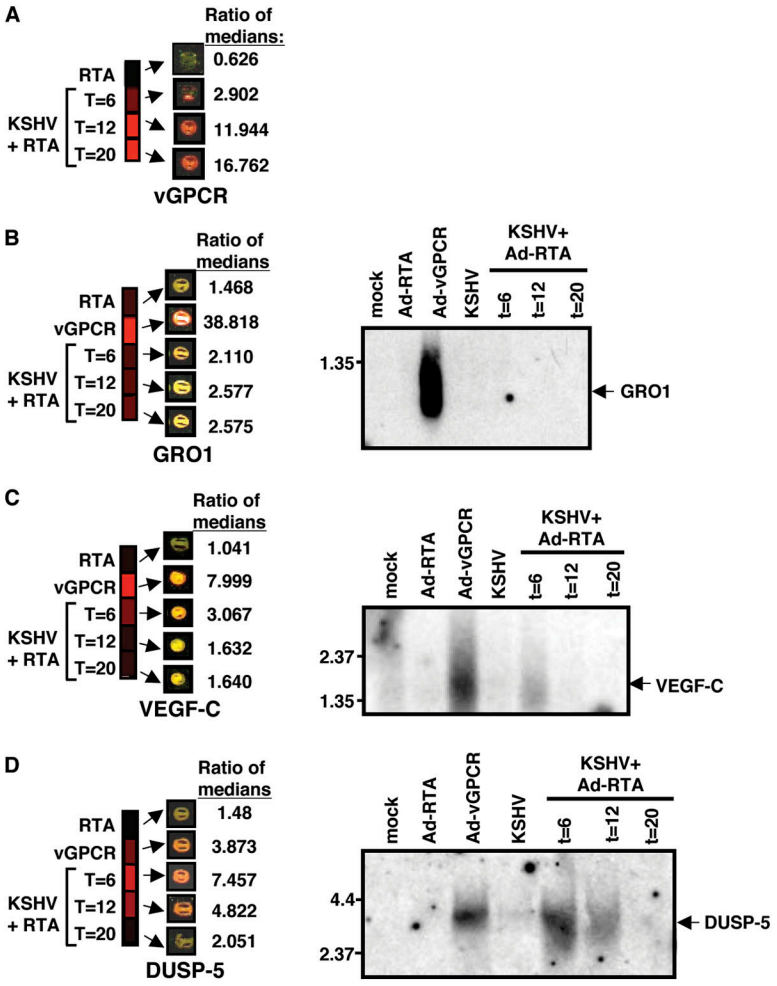

Figure 2. (A) vGPCR expression is detectable by 6 h after lytic reactivation. DNA arrays were performed on samples from TIME cells infected with Ad-RTA alone (RTA), or KSHV plus Ad-RTA for 6, 12, or 20 h. The colored bar represents an image taken from a TreeView cluster analysis, where increasing red intensity represents increasing vGPCR mRNA expression. Each array spot represented by the colored bar is shown on the right along with the median intensity of that spot. (B–D) Correlation of microarray and Northern blot data for three selected host mRNAs. DNA arrays were performed on samples from TIME cells infected with Ad-RTA alone (RTA), Ad-vGPCR alone (vGPCR), or KSHV and Ad-RTA for 6, 12, or 20 h. (left) As in A, increasing red intensity represents increasing mRNA expression of either GRO1 (B), VEGF-C (C), or DUSP-5 (D). (right) Northern blots of 100 ng of the corresponding poly(A) RNA from each indicated infection, as well as 100 ng of poly(A) RNA from TIME cells latently infected with KSHV, hybridized with ^{32}P-labeled GRO1 (B), VEGF-C (C), or DUSP-5 (D) DNA.

than twofold specifically in vGPCR-expressing and/or KSHV-infected cells, but not in mock-infected RTA-expressing cells, in at least two out of three experiments were tabulated.

The Majority of Cellular Gene Up-Regulation by vGPCR Is Blocked by KSHV-induced Host Shutoff. Infection of TIME cells with Ad-vGPCR induced up-regulation of several cellular genes (Table I), many of which overlap with those reported to be up-regulated in the endothelial cell line SLK upon transient transfection with a vGPCR plasmid (8). Significantly, we found that the majority of the vGPCR-induced genes were not up-regulated at any time during lytic KSHV infection. With the exception of IL-6, ADAMTS1, and nucleoside phosphorylase (Table I), all vGPCR-induced transcripts were absent by the 20-h reactivation time point, indicating that they failed to escape KSHV-induced gene shutoff. These findings are not attributable to the failure to express vGPCR during the lytic cycle, as vGPCR transcripts were weakly detectable by 6 h after infection, and accumulated strongly thereafter (Fig. 2 A). As expected, we detected strong induction of the majority of viral genes during the course of the lytic cycle (unpublished data), indicating that in these experiments productive KSHV replication occurred.

Northern blot analysis of the vGPCR targets GRO1, VEGF-C, and DUSP-5 (Fig. 2, B–D) verified that their mRNA expression profiles parallel the array intensity results. Given that vGPCR is transcribed as a delayed-early gene in midcycle, there exists only a short temporal window for vGPCR-induced transcripts to accumulate before being degraded by the shutoff machinery.

Selected Cellular Genes Escape KSHV-induced Host Gene Shutoff. Although most host genes were not up-regulated during viral infection, there existed a small subset of host genes, including IL-6, ADAMTS1, nucleoside phosphorylase, IL-1 receptor type I, solute carrier family 2, and general transcription factor IIB, whose transcripts accumulated during SOX-imposed shutoff (Tables I and II). Interestingly, although the array analysis indicated that HIF-1α mRNA expression is only increased through the 12-h time point in lytically infected cells, immunoblot analysis of HIF-1α demonstrates that the protein continues to accumulate throughout the viral lytic cycle (Fig. 3 A).

Of these genes, one whose induction is particularly striking is IL-6. To confirm the up-regulation of IL-6 mRNA during lytic growth, RNA from mock or lytically infected TIME cells was examined by Northern blotting for IL-6 and hsp70, a gene we demonstrated previously to

Table I. *Genes Up-Regulated in TIME Cells by vGPCR Alone or by vGPCR during Lytic KSHV Infection*

	Average fold up-regulation[a]					
			KSHV + AdRTA			
Name	RTA	vGPCR	t = 6	t = 12	t = 20	GenBank ID[b]
GRO-1	1.4	26.0	1.8	1.6	2.1	W4690
MCF.2-derived transforming sequence	0.7	20.7	0.7	0.9	0.7	H05800
TNF-α–induced protein 3	0.7	19.7	0.8	1.2	0.7	AA476272
Microvascular endothelial differentiation gene	1.7	9.1	1.4	1.3	1.6	AA045793
Speroxide dismutase 2, mitochondrial	1.0	9.2	1.3	1.9	1.5	AA488084
VCAM-1	1.0	7.5	0.9	0.9	1.0	H07071
CD83 antigen	0.8	5.3	1.3	0.8	0.9	AA111969
TRAF1	1.0	4.6	1.0	1.0	1.3	R71725
Suppressin	0.9	3.9	1.0	1.1	0.7	AA425806
TNF-α–induced protein 2	1.2	3.2	1.3	0.9	1.0	AA457114
Apoptosis inhibitor 2	0.9	2.8	1.0	1.0	0.8	H48706
Apoptosis inhibitor 1	1.3	2.8	1.1	0.5	1.1	AA702174
VEGF-C	1.4	11.6	3.5	1.5	2.0	H07991
IKB-α	1.0	5.0	2.1	1.3	0.8	W55872
Mannose-binding lectin (protein C) 2, soluble	0.9	4.6	2.0	1.3	1.6	T69359
Cyclin D1	0.9	3.9	3.3	2.3	1.4	AA487700
Cytochrome *c*-1	1.2	2.6	2.7	2.7	1.7	AA865265
DUSP5	1.5	2.9	4.5	3.3	2.0	W65461
Plasminogen activator, tissue	1.0	2.8	3.5	3.3	1.3	AA453728
Metallothionein 1G	0.8	1.7	3.9	3.3	1.2	H53340
IL-6	1.2	3.3	4.4	4.5	4.7	N98591
ADAMTS1	1.0	3.9	5.4	5.8	14.3	R76553
Nucleoside phosphorylase	1.0	3.4	5.1	4.4	4.1	AA430382

[a]Average ratio as described in Materials and Methods of genes up-regulated in TIME cells either infected with AdRTA alone (RTA), AdvGPCR alone (vGPCR), or lytically infected with KSHV (KSHV + AdRTA) for either 6, 12, or 20 h.
[b]GenBank/EMBL/DDBJ accession no. of microarray EST clone.

be susceptible to KSHV-induced host shutoff (10). The lytically infected cells showed complete shutoff of the hsp70 message, but strong up-regulation of IL-6 (Fig. 3 B, left). IL-6 protein levels are similarly induced upon lytic KSHV infection of TIME, HFF, and 293 cells (Fig. 3 C). Thus, induction of IL-6 appears to be a conserved response to lytic KSHV infection across multiple lineages. IL-6 mRNA accumulates to levels far greater than those present preinfection, suggesting that its accumulation is not simply due to evasion of SOX-induced degradation, but also may be actively induced. vGPCR has been shown to induce IL-6 transcription (8) and, indeed, infection of TIME cells with Ad-vGPCR leads to augmented IL-6 expression, though this increase is modest relative to its induction during the KSHV lytic cycle (Fig. 3, B and C). RTA has also been shown to induce IL-6 in 293T and R1T cells (16), though we observed virtually no RTA-induced IL-6 in TIME cells (Fig. 3, B and C, and Table I), perhaps suggesting that up-regulation of IL-6 by RTA is cell type specific. In agreement with this notion, we did observe somewhat elevated levels of IL-6 in RTA-expressing HFFs (Fig. 3 C). We suspect that additional viral regulators may also be involved in IL-6 up-regulation in KSHV-infected cells.

However, transcriptional induction alone is unlikely to account for the strong accumulation of IL-6 mRNA during infection because many other vGPCR-induced transcripts fail to accumulate under these conditions (Table I).

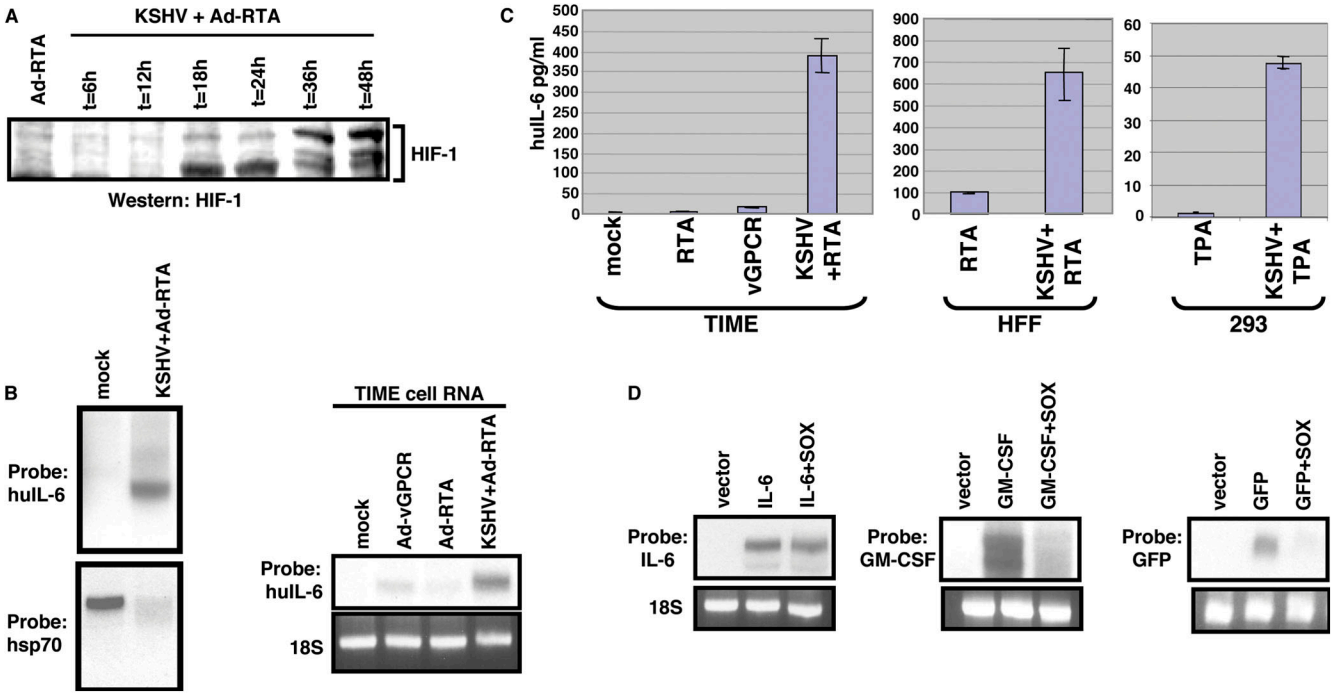

Figure 3. HIF-1 and IL-6 are potently up-regulated during lytic KSHV infection of cells. (A) TIME cells were infected with either Ad-RTA for 48 h or KSHV plus Ad-RTA for the indicated times and harvested, and equivalent amounts of each lysate were immunoblotted with HIF-1 antibodies. (B) TIME cells were either mock infected or infected with the indicated viruses for 20 h, and total RNA was probed with either ^{32}P-labeled human IL-6 or hsp70 DNA. (left) Cells were heat shocked at 42.5°C for 2 h before harvest to demonstrate efficient shutoff of the *hsp70* message. (C) TIME, HFF, and 293 cells were either mock infected, infected with Ad-RTA or Ad-vGPCR alone, or infected with KSHV and lytically reactivated with Ad-RTA or TPA for 24 h. The cells were incubated in serum-free media for the last 12 h of the infection, and IL-6 levels in the supernatants were measured using the BD Opti EIA human IL-6 ELISA kit. (D) 293T cells were transfected with either empty vector or a plasmid expressing GFP, GM-CSF, or IL-6 or cotransfected with plasmids expressing SOX and either GFP, GM-CSF, or IL-6 at a 4:1 ratio. Total RNA was Northern blotted using the indicated ^{32}P-labeled DNA probes.

To explore the possibility that IL-6 mRNA may also escape SOX-induced degradation, we cotransfected SOX and IL-6 expression vectors into 293T cells and examined the resulting levels of IL-6 mRNA. Although accumulation of control RNAs (e.g., GFP, GM-CSF) was strongly inhibited by SOX, no loss of IL-6 mRNA occurred in the presence of SOX (Fig. 3 D). These data indicate that cis-acting sequences or structures in rare cellular transcripts such as IL-6 may rescue the mRNA from the SOX-dependent degradative pathway.

Discussion

We demonstrated previously that lytic KSHV replication promotes a widespread shutoff of cellular gene expression that likely occurs via enhanced mRNA turnover (10). In this report, we have further characterized the consequences of this mRNA shutoff using microarray-based expression profiling. Our findings reveal that very few human transcripts accumulate during infection, including most of those induced by vGPCR.

This result has significant implications for the role of the vGPCR in KS development. Isolated expression of vGPCR in mammalian cells activates a variety of signaling pathways, including the PI3K, p38, and JNK kinase cascades (7, 9, 17). These signaling events stimulate mitogenic and antiapoptotic pathways, but the oncogenic role of these cell-autonomous signals in KS has been disputed because lytically infected cells are destined to die (6). However, vGPCR signaling also leads to up-regulation of VEGF (9), a powerful endothelial mitogen that can promote angiogenesis in a paracrine fashion. This activity could be of great importance in KS pathogenesis, as it does not require long-term survival of individual lytically infected cells, only a continuous supply of them.

Given the powerful constitutive signaling activity of vGPCR and its intrinsic proangiogenic character, one paradox has been why KS develops so infrequently after KSHV infection. In Western societies, where 2–7% of the population is KSHV infected, KS is largely limited to those developing HIV infection or receiving iatrogenic immunosuppression. The attenuation of VEGF and other vGPCR-dependent gene induction events by KSHV-induced shutoff leads us to propose that only infections with extensive lytic replication, such as those that may occur during cellular immune dysfunction, can generate sufficient VEGF to contribute meaningfully to KS progression. This inference accords well with clinical observations that link KS progression to elevated levels of circulating KSHV DNA (18).

Table II. *Cellular Genes That Escape KSHV-induced Host Shutoff*

Name	Average fold up-regulation[a]					GenBank ID[b]
			KSHV + AdRTA			
	RTA	vGPCR	t = 6	t = 12	t = 20	
vGPCR inducible genes						
Cyclin D1	0.9	3.9	3.3	2.3	1.4	AA487700
Cytochrome *c*-1	1.2	2.6	2.7	2.7	1.7	AA865265
DUSP5	1.5	2.9	4.5	3.3	2.0	W65461
Plasminogen activator, tissue	1.0	2.8	3.5	3.3	1.3	AA453728
Metallothionein 1G	0.8	1.7	3.9	3.3	1.2	H53340
vGPCR noninducible genes						
Metallothionein 1H	0.8	0.7	3.3	2.7	1.4	H77766
Snail1, zinc finger protein	1.3	0.7	3.8	3.2	1.1	AA464983
Hypoxia-inducible factor 1 α	1.5	1.6	3.8	3.7	2.5	AA598526
vGPCR inducible genes						
IL-6	1.2	3.3	4.4	4.5	4.7	N98591
ADAMTS1	1.0	3.9	5.4	5.8	14.3	R76553
Nucleoside phosphorylase	1.0	3.4	5.1	4.4	4.1	AA430382
vGPCR noninducible genes						
KIAA0808	1.0	1.5	5.3	3.4	2.4	N66992
IL-1 receptor type 1	1.2	1.0	3.9	3.2	2.7	AA464525
Solute carrier family 2, member 3	1.1	1.1	3.6	3.3	2.5	T97889
General transcription factor IIB	1.0	1.1	3.5	3.1	2.9	H23978

[a]Average ratio as described in Materials and Methods of genes up-regulated in TIME cells infected with AdRTA alone (RTA), AdvGPCR alone (vGPCR), or lytically infected with KSHV (KSHV + AdRTA) for either 6, 12, or 20 h.
[b]GenBank accession no. of microarray EST clone.

Another way that vGPCR could influence angiogenesis in spite of the global SOX-induced host shutoff would be if it were to be expressed outside the context of lytic infection. Attempts to detect vGPCR mRNA during latency have failed, though given the potency of vGPCR's signaling activity, it is possible that transcript levels too low to detect might still be functionally significant. Alternatively, vGPCR might be induced outside of the lytic program in a manner analogous to the KSHV K2 gene, which can be selectively expressed upon exposure to IFN-γ in the absence of authentic lytic induction (19).

Notably, a small subset of host transcripts are spared from shutoff during lytic infection, and these include transcripts with potential roles in the pathogenesis of KSHV-related diseases. IL-6 is one such transcript. Enhanced IL-6 expression is a feature common to several KSHV-associated neoplasms, including MCD and PEL. Many PEL-derived cell lines, which display both latent and lytic infection, secrete substantial quantities of IL-6, and some of these appear to depend at least in part on IL-6 for survival or growth (20). Furthermore, although much of the IL-6 action in KSHV-related MCD is likely encoded by the vIL6 (K2) gene (21), at least one paper reports high levels of circulating human IL-6 (22), and in such cases the mechanism we describe could be one source of this IL-6.

The up-regulation of IL-6 in infected cells also raises important mechanistic questions. As noted earlier, the large accumulation of IL-6 transcripts suggests a dual mechanism involving both enhanced production and diminished degradation. Indeed, we observed that even in the absence of KSHV infection, IL-6 mRNAs were selectively refractory to SOX-mediated degradation, suggesting the presence of one or more cis-acting sequence elements or structures that facilitate their escape from shutoff. Interestingly, it has been shown recently that HSV-1 vhs-mediated targeting of some cellular mRNAs for degradation is based on sequence specificity (23), perhaps indicating that there may also exist sequence elements that protect select

mRNAs from host shutoff in that virus as well. The definition of these elements and the factors that recognize them may shed considerable light on the mechanism of SOX-induced RNA turnover as well as how viral mRNAs escape this shutoff.

It is interesting that three of the induced genes in Table II are involved in cellular transcription. In particular, HIF-1α is a heterodimeric basic helix-loop-helix transcription factor that becomes stabilized under hypoxic conditions. Stabilized HIF-1α dimerizes with HIF-1β and transcriptionally activates several genes responsive to low oxygen whose products play critical roles in tumor progression including angiogenesis, cell growth, and energy metabolism (24). Although we did not observe the escape of many HIF-1 target genes in infected cells, one HIF-1 target is up-regulated; that is, the transcript for solute carrier family 2, member 3 (GLUT3). Interestingly, it has been reported that hypoxia also induces KSHV lytic reactivation (25), an event likely mediated by the presence of functional hypoxia response elements within the RTA and ORF34 promoters. Thus, an additional function for HIF-1 in lytic cells may be to enhance KSHV replication via the stimulation of select viral promoters, the products of which are not subject to shutoff.

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