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KSHV *ORF K9* (vIRF) is an oncogene which inhibits the interferon signaling pathway

Shou-Jiang Gao[1,3], Chris Boshoff[2], Sukhanya Jayachandra[1], Robin A Weiss[2], Yuan Chang[1] and Patrick S Moore[1]

[1]*Division of Epidemiology, School of Public Health and Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, NY10032, USA;* [2]*Chester Beatty Laboratories, Institute of Cancer Research, London SW3 6JB, UK*

Kaposi's sarcoma-associated herpesvirus (KSHV) is a gammaherpesvirus linked to the development of Kaposi's sarcoma and a rare B cell lymphoma, primary effusion lymphoma. The KSHV gene *ORF K9* encodes vIRF which is a protein with low but significant homology to members of the interferon (IFN) regulatory factor (IRF) family responsible for regulating intracellular interferon signal transduction (Moore PS, Boshoff C, Weiss RA and Chang Y. (1996). *Science*, 274, 1739–1744). vIRF inhibits IFN-β signal transduction as measured using an IFN-responsive ISG54 reporter construct co-transfected with *ORF K9* into HeLa and 293 cells. vIRF also suppresses genes under IFN regulatory control as shown by inhibition of the IFN-β inducibility of $p21^{WAF1/CIP1}$, however, no direct DNA-binding or protein-protein interactions characteristic for IRF repressor proteins were identified. Stable transfectant NIH3T3 clones expressing vIRF grew in soft agar and at low serum concentrations, lost contact inhibition and formed tumors after injection into nude mice indicating that vIRF has the properties of a viral oncogene. Since vIRF is primarily expressed in KSHV-infected B cells, not KS spindle cells, this study suggests that vIRF is a transforming oncogene active in B cell neoplasias that may provide a unique immune escape mechanism for infected cells. This data is consistent with tumor suppressor pathways serving a dual function as host cell antiviral pathways.

Keywords: Kaposi's sarcoma-associated herpesvirus; interferon; oncogenesis; p21; lymphoma

Introduction

Several DNA tumor viruses have independently evolved specific mechanisms to inhibit tumor suppressor pathways suggesting that these pathways serve a dual purpose in acting as antiviral host cell defenses (Moore and Chang, 1997; Neil *et al*., 1997; Weinberg, 1997; Wold *et al*., 1994). This is a different view from the suggestion that tumor virus inhibition of tumor suppressor pathways is required to supplement the metabolic needs of the virus in otherwise quiescent cells (Jansen-Dürr, 1996; Moran, 1993). Host cell defenses to virus infection may include cell cycle shutdown, induction of apoptosis and enhanced immune recognition through upregulation of major histocompatibility complex (MHC) antigens (Moore and Chang, 1997).

The most recently discovered human tumor virus, Kaposi's sarcoma-associated herpesvirus (KSHV or HHV8), was identified in 1994 by representational difference analysis (Chang *et al*., 1994) and is etiologically implicated in the development of Kaposi's sarcoma (KS), body cavity-based/primary effusion lymphomas (PEL) and a subset of multicentric Castleman's disease lesions (Cesarman *et al*., 1995; Chang *et al*., 1994; Soulier *et al*., 1995). The evidence for KSHV infection being a necessary and causal factor for KS is extensive (for reviews, see Chang and Moore, 1996; Offermann, 1996; Olsen and Moore, 1997; Strathdee *et al*., 1996). DNA-based and serology-based studies demonstrate a consistent association of this virus with both AIDS-related and AIDS-unrelated forms of KS and the virus is detectable in 90–95% of KS lesions.

Within KS tumors, nearly all tumor cells show evidence of KSHV infection (Boshoff *et al*., 1995; Rainbow *et al*., 1997; Staskus *et al*., 1997). Cellular X-chromosome inactivation studies suggest that KS may have a monoclonal cellular origin (Rabkin *et al*., 1995; Rabkin *et al*., 1997) which is supported by preliminary virus terminal repeat analyses (Russo *et al*., 1996). However, reasonable disagreement exists as to whether or not KS tumors are composed of fully transformed tumors cells or represent a polyclonal expansion driven by endogenous or exogenous cytokines. In contrast, there is no disagreement that KSHV-associated PEL are of monoclonal cellular origin based on immunoglobulin rearrangement studies (Cesarman *et al*., 1995). These lymphomas are frequently coinfected with both KSHV and Epstein-Barr virus (EBV or HHV4) (Cesarman *et al*., 1995). Two cell lines (BC-1 and HBL-6) independently derived from the same tumor have the same polymorhpic terminal repeat pattern (Russo *et al*., 1996) providing strong evidence that KSHV is monoclonal in these tumors as well. Thus, identification of virus-encoded oncogenes may help in understanding the pathogenesis of this lymphoma and provide unique tools for studying cell growth and regulation.

Genomic sequencing of KSHV shows that the virus has a surprising degree of molecular mimicry (piracy) of cellular genes involved in cell replication and growth control (Russo *et al*., 1996). KSHV is poorly transmissible *in vitro* (Foreman *et al*., 1997; Moore *et al*., 1996b) and therefore transformation studies require examination of isolated genes. The KSHV genome possesses functional homologs to cellular protoonco-

Correspondence: Y Chang and PS Moore
[3]Current address: Department of Pediatrics, University of Texas Health Science Center, San Antonio, TX78284, USA
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genes (Russo *et al*., 1996) including a D-type cyclin (Chang *et al*., 1996; Godden-Kent *et al*., 1997; Li *et al*., 1997), antiapoptotic *bcl*-2 (Cheng *et al*., 1997; Sarid *et al*., 1997) and IL-6 homologs (Moore *et al*., 1996a; Nicholas *et al*., 1997), and a constitutively active IL-8-like receptor that induces cell proliferation in rat fibroblasts (Arvanitakis *et al*., 1997). KSHV ORF K13 also encodes a protein with motif similarity to virus-encoded FLICE-inhibitory proteins that prevent apoptosis through dominant negative inhibition of the APO-1/CD95 pathway (Bertin *et al*., 1997; Thome *et al*., 1997). These virus genes may directly inhibit cellular responses to infection such as shutdown of cell cycling and induction of apoptosis (Moore *et al*., 1996a). EBV, which is closely related to KSHV, induces many of the same cellular regulatory proteins after infection suggesting that both viruses achieve similar alterations in cell signaling and cell cycle regulation, but do so using different strategies (Russo *et al*., 1996). Many of these viral signaling transduction and regulatory protein homologs are expressed during quiescent (presumably latent) KSHV replication in B cells but are induced to high levels of expression during lytic replication (unpublished data, R Sarid, Y Chang, PS Moore).

Antiviral responses are also initiated or amplified by interferons (IFNs) induced by viral infection. Recent work has demonstrated an important effect of IFN signaling responses on cell growth control (for review, see Taniguchi *et al*., 1995) which may play a role in either the induction or inhibition of cellular transformation (Harada *et al*., 1993). The pleiotrophic activity of IFNs include tumor suppression through induction of the cyclin-dependent protein kinase inhibitor (CDKI) $p21^{WAF1/CIP1}$ cell cycle regulatory protein (Chin *et al*., 1996; Hobeika *et al*., 1997; Sangfelt *et al*., 1997) and through induction of apoptosis (Tamura *et al*., 1995; Tanaka *et al*., 1996). Class I IFN (i.e. IFN-α and IFN-β) also induce expression of major histocompatibility complex (MHC) class I antigens which facilitates immune surveillance for infected cells.

KSHV *ORF K9* encodes a unique viral protein called vIRF with sequence similarity to the IFN regulatory factor (IRF) protein family (Moore *et al*., 1996a; Russo *et al*., 1996). Members of the IRF family positively or negatively regulate IFN signal transduction through binding to IFN-stimulated response elements (ISRE) in the promoters of genes under IFN induction control (Taniguchi *et al*., 1995). Two members of this family, IRF-1 (Miyamoto *et al*., 1988) and IRF-2 (Harada *et al*., 1989), have antagonistic anti- and pro-oncogenic activities respectively when overexpressed in NIH3T3 cells (Harada *et al*., 1993). The 449-amino acid KSHV vIRF has 13% amino acid identity to several IRF members, but lacks the characteristic DNA binding sequence through which IRF proteins recognize specific ISRE in the IFN regulated promoters (Moore *et al*., 1996a). We show here that vIRF expression inhibits IFN signal transduction in reporter assays, downregulates expression of $p21^{WAF1/CIP1}$ and fully transforms NIH3T3 cells. The viral protein appears to provide a unique immune escape mechanism for the virus that may contribute to neoplasia particularly in virus-infected B cells.

Results

Effect of vIRF on IFN reporter assays

To determine whether KSHV vIRF inhibits IFN-β signal transduction as measured by gene expression reporter assays, we transiently cotransfected into HeLa cells a reporter plasmid pH1 containing IFN-β inducible chloramphenicol acetyltransferase (CAT) linked to ISRE elements of the wild-type hamster ISG54 promoter, together with the KSHV *ORF K9* cloned into a pMET7 expression vector (pMvIRF), or its reverse construct (pMFRIv). Whereas ISRE-linked CAT activity was inducible with IFN-β in HeLa cells transfected with the reverse construct, CAT induction was nearly completely abolished by increasing doses of the pMvIRF plasmid up to 5 μg (Figure 1a). Examination of transfection efficiency and protein expression levels (data not shown) indicated that the effect of vIRF was not due to a general inhibition of transcriptional activity. This effect was not cell line specific in that vIRF coexpression also inhibited IFN-β-induced CAT activity in 293 cells under similar conditions (Figure 1b).

DNA-binding and protein interaction studies

No evidence for direct interaction of vIRF with ISRE or known IRF proteins was found under several different assay conditions (see Materials and methods). Conditions examined included use of purified bacterial recombinant vIRF protein and nuclear extracts prepared from vIRF-transfected COS7 or NIH3T3 cells in DNA-protein (South-western) assays or electrophoretic mobility shift binding assays (EMSA) with the ISG15 ISRE element. Under these conditions, clear evidence of ISG15 binding to IRF members, such as ISGF3, could be demonstrated. Since vIRF possesses only two of the five conserved amino terminus tryptophans critical for DNA binding motif, it appears unlikely that vIRF inhibits IFN signaling by direct competitive inhibition at the ISRE element. We cannot exclude the possibility that optimized conditions yet to be discovered are required to detect vIRF interactions with ISRE or interferon signaling proteins.

Effect of vIRF on $p21^{WAF1/CIP1}$ expression

The IFN-β inducible IRF1 and IRF2 transcriptional regulatory proteins compete for ISRE and have positive and negative IFN regulatory activity respectively. IFN induction of IRF1 may contribute to cell cycle arrest through increased expression of the CDKI $p21^{WAF1/CIP1}$ (Hobeika *et al*., 1997; Sangfelt *et al*., 1997; Tanaka *et al*., 1996). Overexpression of the negative regulator IRF2 can transform NIH3T3 cells, an effect reversed by IRF1 co-overexpression (Harada *et al*., 1993), which is consistent with the presumed roles for IRF1 and IRF2 in cell cycle regulation. To examine the effect of vIRF on IFN-inducible gene expression, stable NIH3T3 transfectants expressing vIRF cloned in-frame into a pcDNA3.1/His vector (pcvIRFin) or cloned out-of-frame as a negative control (pcvIRFout) were generated. The effect of vIRF expression on $p21^{WAF1/CIP1}$ expression was measured by Western

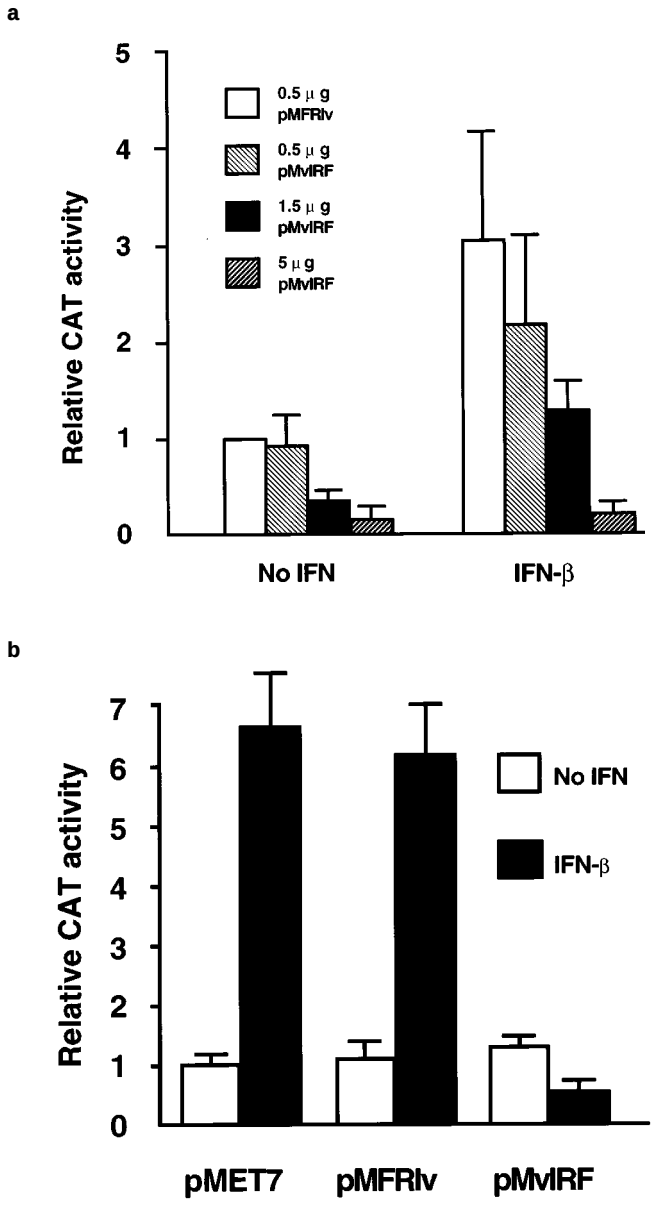

Figure 1 vIRF activity in HeLa and 293 cells after cotransfection with the ISG54 CAT reporter vector, pH1, and treatment with IFN-β. (**a**) In HeLa cells transfected with the reverse construct (pMFRIv), IFN-β treatment increased ISG-54-CAT reporter activity threefold compared to untreated cells. IFN-β induction was inhibited in a dose-dependent fashion by increasing amounts of the forward construct plasmid DNA, pMvIRF (0, 1.5 and 5 μg). At 5 μg of added pMvIRF plasmid DNA, CAT activity with or without IFN-β treatment was 15–24% of the untreated pMFRIv control cells and the effect of IFN-β induction was completely abolished. (**b**) In 293 cells transfected with the reverse construct (pMFRIv) or pMET7 vector alone, IFN-β induced ISG-54-CAT reporter activity increased 6.6-fold compared to untreated cells. IFN-β induced CAT activity was totally inhibited by cotransfection with 0.5 μg pMvIRF plasmid DNA. Results are the average of three experiments, with standard deviations, expressed relative to the CAT activity of untreated pMFRIv control cells

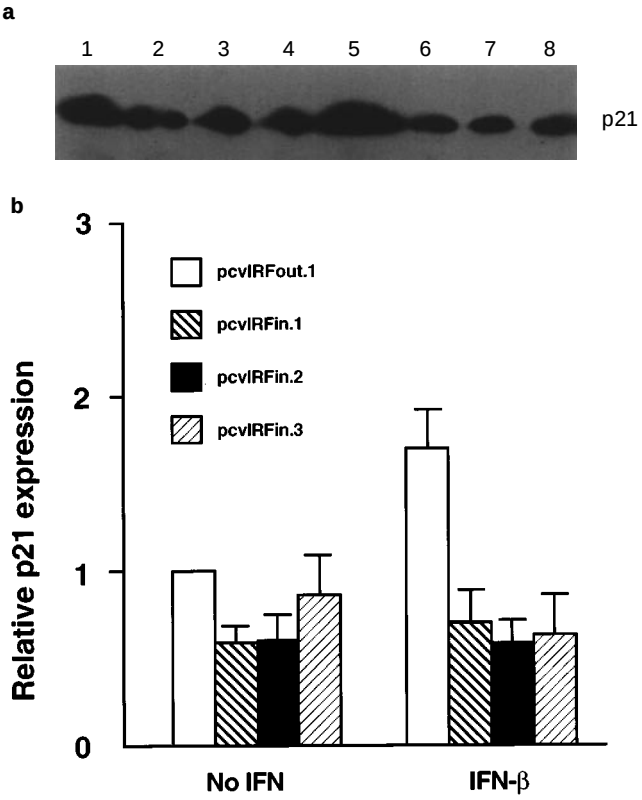

Figure 2 Expression of CDKI $p21^{WAF1/CIP1}$ in three vIRF transfected NIH3T3 clones (pcvIRFin.1, pcvIRFin.2 and pcvIRFin.3) and a control pcvIRFout.1 clone measured by immunoblotting. (**a**) A representative immunoblot shows that $p21^{WAF1/CIP1}$ protein levels increased in out-of-frame vIRF control cells treated with IFN-β but not in cells expressing in-frame vIRF. Lanes 1 and 5, pcvIRFout.1; lanes 2 and 6, pcvIRFin.1; lanes 3 and 7, pcvIRFin.2; lane 4 and 8, pcvIRFin.3. Lanes 1–4 are without IFN-β treatment; lanes 5–8 are treated with 500 units per ml of IFN-β. (**b**) Mean densitometric quantification of $p21^{WAF1/CIP1}$ expression measured by immunoblotting

blotting of whole cell extracts with each lane standardized by cell count and total protein.

Constitutive expression in the three stable in-frame vIRF cell clones (pcvIRFin.1, pcvIRFin.2 and pcvIRFin.3) but not an out-of-frame vIRF stable clone, pcvIRFout.1, downregulated $p21^{WAF1/CIP1}$ expression in NIH3T3 cells and prevented IFN-β induction of $p21^{WAF1/CIP1}$ (Figure 2a and b). The induction of p21 protein occurring after 500 μ IFN-β treatment of the pcvIRFout.1 control cells was completely abolished in all three clones (pcvIRFin.1, pcvIRFin.2, pcvIRFin.3) stably expressing KSHV vIRF in-frame.

NIH3T3 cell transformation studies

vIRF inhibits ISRE-containing reporter expression and downregulates $p21^{WAF1/CIP1}$ consistent with this protein's putative role in viral immune escape mechanisms. Although no direct vIRF-ISRE binding activity was found, the action of vIRF is functionally similar to that of IRF2. To determine if vIRF, like IRF2, has transformation capacity and can act as an oncogene, *in vitro* transformation assays were performed on in-frame and out-of-frame stable vIRF NIH3T3 transfectant clones.

Three in-frame vIRF NIH3T3 clones lost contact inhibition, had shorter doubling times, grew at higher cell densities and had higher growth rates under low serum conditions than untransfected parental NIH3T3 cells or two NIH3T3 clones transfected with out-of-frame vIRF (Figure 3 and Table 1). Colony forming

efficiency in soft agar was 17–41-fold higher for the three pcvIRFin cell clones than the pcvIRFout cell clones or untransfected control cells (Figure 4 and Table 1).

Stable vIRF expression in NIH3T3 cells readily resulted in tumor formation after infection into nude mice, whereas no tumor formation occurred after injection of either parental or out-of-frame vIRF expressing NIH3T3 (Table 1). Each cell line was injected into both flanks of four nude mice, which were observed until tumor formation or up to 70 days. The three pcvIRFin clones generated tumors at 23 of 24 sites while none of 24 sites injected with the two pcvIRFout cell clones or parental NIH3T3 cells developed tumors after 70 days of observation. The latency of tumor formation was typically short (5–28 days, average 17 days). Tumors had typical histologic features of fibrosarcomas (Figure 5) and were positive for vIRF expression on Northern blotting (not shown).

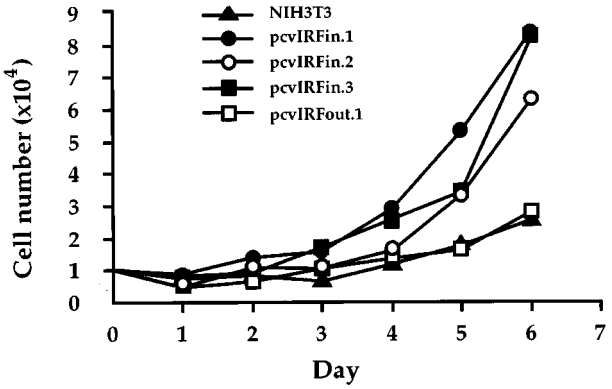

Figure 3 Growth of NIH3T3 stable clones transfected with pcvIFRin or controls transfected with pcvIRFout in 1% FBS medium. vIRF transfectant cells (pcvIRFin.1, pcvIRFin.2, pcvIRFin.3) grew approximately four times faster than the controls (pcvIRFout.1 and non-transfected NIH3T3 cells) in low serum media

Discussion

vIRF is the first KSHV-encoded protein found to transform NIH3T3 cells and induce tumor formation.

Table 1 Tumor formation and phenotypes of control NIH3T3 cells and NIH3T3 cells constitutively expressing KSHV in-frame and out-of-frame vIRF

Cells	*Saturation density, $\times 10^6$ cells* (s.d.)*	*Colony efficiency in soft agar, % (s.d.)*	*Tumorigenicity in nude mice*	
			Tumors per injection	*Latency, days*
pcvIRFin.1	5.39 (0.70)	24.4 (1.2)	8/8	5–14
pcvIRFin.2	6.00 (0.75)	24.6 (2.9)	7/8	5–28
pcvIRFin.3	5.23 (0.50)	13.6 (2.0)	8/8	5–14
NIH3T3	2.28 (0.29)	0.8 (0.3)	0/8	NA
pcvIRFout.1	2.17 (0.26)	0.6 (0.2)	0/8	NA
pcvIRFout.2	2.36 (0.35)	0.8 (0.2)	0/8	NA

*In 35 mm culture dishes

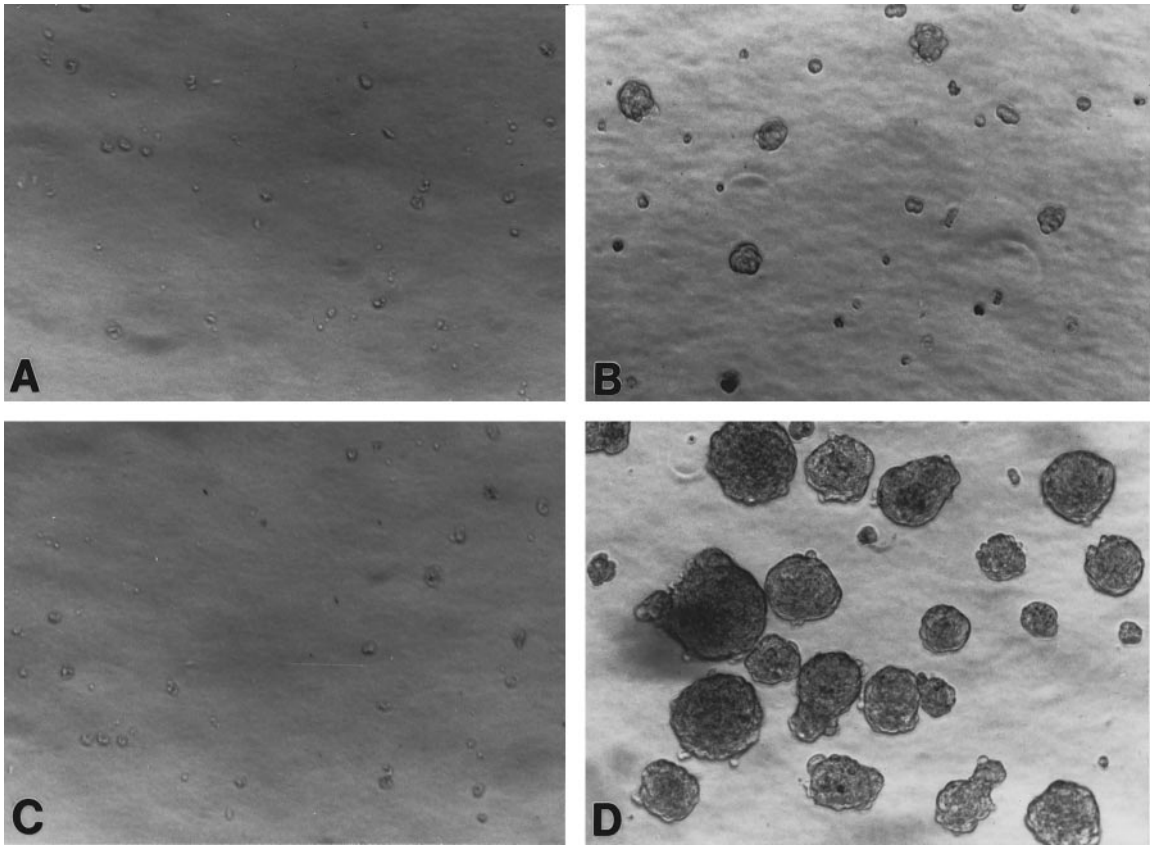

Figure 4 Colony formation of NIH3T3 stable transfectants pcvIRFin.1 (**b** and **d**) and pcvIRFout.1 control clone (**a** and **c**) in soft agar at 6 days (**a** and **b**) and 12 days (**c** and **d**). pcvIRFin.1 cells formed large numbers of healthy colonies while controls had no or very few small, dead cell colonies after 12 days

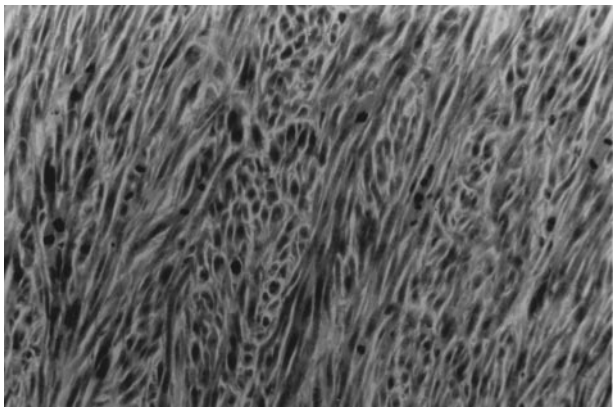

Figure 5 Tumors in nude mice subcutaneously injected with NIH3T3 cells stably expressing KSHV vIRF demonstrate features of a malignant fibrosarcoma. The tumor is composed of pleomorphic spindle-shaped cells with hyperchromatism and a high mitotic rate. (H&E, 40× magnification)

Preliminary studies demonstrate that it is not significantly expressed in KS tumors (as detected by Northern blotting) and therefore may not play an important role in this disease. vIRF is expressed, however, in persistently (latenly) infected PEL cells, and expression markedly increases after treatment with chemical agents which induce lytic virus replication (Moore *et al*., 1996a).

The mechanism by which vIRF antagonizes IFN-β signaling as measured by our reporter assay is unknown; we found no direct evidence for either ISRE-binding or protein-protein interactions with known IRF family members. It remains possible that optimized conditions may reveal direct vIRF-ISRE or vIRF-IRF interactions although this was examined in our study using a variety of methods. It appears unlikely that vIRF inhibition of IFN-β signaling results from competitive inhibition at the ISRE since the DNA binding regions of vIRF is poorly conserved (Moore *et al*., 1996a). Nonetheless, the functional activity of vIRF appears to be similar to IRF2 which acts as a negative regulator of IFN signal transduction by competitive inhibition with IRF1 at ISRE sequences (Harada *et al*., 1989).

The effect of vIRF on IFN signal transduction may have a functional effect on cell cycle regulation as well. Cyclin-dependent kinase inhibitior $p21^{WAF1/CIP1}$ levels did not increase in NIH3T3 cells expressing vIRF in response to IFN-β treatment although the increase seen in our control cells lines was relatively modest. Hobeika *et al*. (1997) found similar levels (approximately twofold) of $p21^{WAF1/CIP1}$ induction in DU145 prostatic carcinoma cells after treatment with 2500 units IFN-α. This level of $p21^{WAF1/CIP1}$ induction correlated with cell cycle arrest in DU145, suggesting that the level of $p21^{WAF1/CIP1}$ inhibition found in our study may also contribute the dysregulated growth control of the pcvIRFin NIH3T3 clones. Similar (threefold) levels of $p21^{WAF1/CIP1}$ induction by IFN-α were found for Daudi cells by Sangfelt and colleagues while higher levels of induction were found in other cell lines (Sangfelt *et al*., 1997). While the effect of vIRF expression on other cell growth control regulation pathways requires further investigation, vIRF inhibition of the IFN-inducibility of $p21^{WAF1/CIP1}$ provides *in situ* evidence that this viral protein can inhibit intracellular IFN signaling.

By inhibiting $p21^{WAF1/CIP1}$ expression, vIRF may allow KSHV-infected cells to escape cell cycle shutdown initiated as an antiviral response by IFN. It is possible that vIRF expression may also inhibit immune responses against infected cells. NIH3T3 clones stably expressing vIRF had lower baseline levels of major histocompatibility (MHC) class I surface antigen expression than out-of-frame controls (data not shown); however, these cells show poor MHC class I induction after IFN-β treatment and thus the effect of vIRF on IFN-inducibility of MHC class I antigen could not be determined.

A by-product of this survival mechanism may be dysregulated cell proliferation contributing to the transformed phenotype of infected B cells. While KSHV is evolutionarily distant from most other human tumor viruses (with the exception of EBV), it appears to share with other transforming viruses such as adenovirus and papillomavirus the functional capacity to alter retinoblastoma protein-mediated control checkpoints and apoptosis mediated through both p53-dependent and independent pathways (Moore and Chang, 1997). Further, the adenoviral E1a oncoprotein may induce resistance to IFN signaling (Reich *et al*., 1988) by inhibitory binding to the p300/CBP transcriptional adaptor protein (Bhattacharya *et al*., 1996; Zhang *et al*., 1996), and downregulation of MHC processing (Rotem-Yehudar *et al*., 1996), an analogous effect to that seen in our study of KSHV vIRF.

The convergent evolution of tumor suppressor inhibition by distantly-related tumor viruses suggests that tumor suppressor pathways also serve to control viral infection. The activation of tumor suppressor pathways by interferons, which are antiviral cytokines induced by viral infection, provides additional support to the notion that these pathways serve important antiviral functions. Thus, the specific inhibition of tumor suppressor pathways by KSHV-encoded genes leading to cell transformation may be an unintended consequence of this virus' strategy to overcome host cell defense pathways (Moore *et al*., 1996a; Moore and Chang, 1997). While vIRF is the first KSHV-encoded protein found to have transforming activity *in vitro*, other viral gene products, such as v-*cyc* and v-IL6 are also likely to contribute to KSHV-mediated dysregulation of cell growth control *in vivo*. Our current study confirms that interferon signaling pathway activation plays an important role in tumor suppression and that tumor suppressor pathways are likely to have dual roles as antiviral pathways.

Materials and methods

Cell lines

Human embryonic 293T ('293') cells, human cervix epitheloid carcinoma HeLa cells and monkey COS7 cells obtained from American Type Culture Collection (Rockville, MD) were maintained in Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), 1% penicillin + streptomycin and 1% of 200 mM L-glutamine.

Expression vectors

A 1368 bp vIRF PCR product generated with the primer set 5′-AAA GAA TTC ATG GAC CCA GGC CAA A-GA CC-3′ and 5′-GGG GTC GAG TTA TTG CAT GGC ATC CCA TA-3′ was cloned in-frame (pcvIRFin) and out-of-frame (pcvIRFout) into the *Bam*HI and *Eco*RI sites of pcDNA3.1/His, a CMV promoter driven mammalian expression vector (Invitrogen, Carlsbad, CA). pMvIRF and pMFRIv are vIRF and its reverse sequence cloned into the Klenow blunted *Sac*I site of pMET7 mammalian expression vector (a gift of Dr J Culpepper) (Takebe *et al*., 1988) after excising the vIRF insert from pcvIRF with *Bam*HI and *Eco*RI then blunting with Klenow fragment. pTvIRFin and pTvIRFout are in-frame and out-of-frame vIRF cloned into the *Bam*HI and *Xho*I sites of pTrcHis prokaryote expression vector (Invitrogen) after isolation of the vIRF insert from pcvIRFin using the same restriction enzymes. The sequence fidelity of all constructs was verified by bidirectional sequencing on an ABI 377 sequenator (Applied Bio-systems Inc., Foster City, CA). pH1 is a CAT reporter plasmid containing the hamster ISG54 promoter (Bluyssen *et al*., 1993) with an IFN-β responsive ISRE sequence (a gift from Dr D Levy). PSV is a CMV promoter-driven β-galactosidase expression vector used for determining transfection efficiency (Stratagene, La Jolla, CA).

Transfection

CAT cotransfection experiments were performed in 35 mm well tissue culture plates using standard calcium-phosphate method (Fujita *et al*., 1985) with 0.5 μg of pH1 reporter plasmid DNA and 0.5 μg to 5 μg of plasmid DNA of pMvIRF or pMFRIv. Transfection efficiency was standardized by cotransfection of 0.3 μg of PSV DNA. Total amounts of DNA was equalized by addition of salmon sperm carrier DNA. One day after transfection, cells were treated with 500 units per ml of human recombinant IFN-β (Sigma Biosciences, St Louis, MO) for 16 h and harvested for CAT determinations (Harada *et al*., 1989). Cell extracts for the CAT assay were standardized according to the transfection efficiency as measured by β-galactosidase production.

Generation of vIRF stable cell lines

NIH3T3 cells were transfected with 3 μg of pcvIRFin or pcvIRFout linearized with *Sca*I (Fujita *et al*., 1985). The transfected cells were then selected in medium containing G418 (700 μg per ml), and resistant colonies were isolated after 2–3 weeks. The stable transfectant colonies were further cloned by end-point limiting dilution.

Electrophoretic mobility shift assays (EMSA)

vIRF bacterial recombinant proteins (generated with pTvIRFin or control pTvIRFout using the Xpress protein expression system (Invitrogen)) or whole extracts (Schindler *et al*., 1992) of pMvIRF or control pMFRIv transiently-transfected COS7 cells were used in EMSA (Hassanain *et al*., 1993). DNA probes used contained either the CAG TTT CGG TTT CCC sequence of the ISRE site (Levy *et al*., 1989) or the CTT TCA GTT TCA TAT TAC TCT AAA TCC AT sequence of the GAS site (Silvennoinen *et al*., 1993). vIRF interaction with other cellular factors was assayed using 1 μg of bacterial recombinant vIRF protein or COS7 cell preparations with IFN-β treated WI38 (for ISRE probe EMSA) or IL-6 treated HEPG2 (for GAS probe EMSA) cell nuclear extracts. The authors thank C Schindler and C Lee for assistance with these experiments.

Protein analysis

Immuno-blotting was performed as previously described (Gao *et al*., 1996) using antibody to $p21^{WAF1/CIP1}$ (Calbiochem, Cambridge, MA) and developed with enhanced chemiluminescence (ECL) kit (Amersham Life Science, Arlington Heights, IL). For each determination, 100 μg of total cell protein was loaded per lane and confirmed by Coomassie Brilliant Blue R-250 staining.

Cell-proliferation assays

Cells were seeded in DMEM supplemented with 10% FBS at 2×10^4 cells per 35 mm dishes and media was changed every 3 days. Doubling time was determined by counting cells every 2 days and calculating the growth rate for exponentially growing cells. Saturation density was determined by counting the numbers of cells in culture 3–4 days after reaching confluency. For growth in low serum media, cells were grown in DMEM supplemented with 1% FBS and cell numbers were determined daily. All the above experiments were repeated at least three times with six replicates in each determination. Results are the average of the experiments with standard deviations.

Soft-agar assay

Cells (1×10^4) were suspended in a 0.35% agar solution in DMEM supplemented with 10% FBS, and overlaid onto a 0.5% agar solution in DMEM containing 10% FBS in 35 mm plates prepared 1 day before and incubated in a 37% and 5% CO_2 incubator. One day after incubation, 2 ml of DMEM supplemented with 10% FBS was added. Cells grown in soft agar were counted 12 days after plating. Cloning efficiency is the number of colonies $\times 100$ divided by the numbers of cells plated. Each determination is the average of three experiments with standard deviations.

Nude mice assay

Cells (1×10^6) suspended in 100 ml PBS were injected subcutaneously into both flanks of 4–6 week old nude mice. Mice were checked daily for tumor formation for at least 70 days. Latency was the time required to produce visible tumors. Nude mice experiments were approved by the Ethics Committee of the Institute of Cancer Research and the Home Office of the UK.

Transcript analysis

Northern blotting was performed under standard conditions with random-labeled probes derived from gel purified, *Bam*HI and *Eco*RI digested, pcvIRFin vIRF insert.

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