Activation of cyclin A gene expression by the cyclin encoded by human herpesvirus-8

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Human herpesvirus-8 (HHV-8), also known as Kaposi’s sarcoma-associated herpesvirus, encodes a protein, referred to as HHV8-Vcyc, with sequence similarity to human G1 cyclins, in particular of the D type. HHV8-Vcyc is expressed in Kaposi’s sarcoma and functional analysis suggests that it can activate cyclin-dependent kinases (cdk) and thereby trigger inactivation of the retinoblastoma protein (pRb), indicating that HHV8-Vcyc may contribute to the oncogenic potential of HHV-8. We show here that HHV8-Vcyc can activate transcription of the human cyclin A gene in quiescent cells, a property shared with known transforming oncogenes. Transcriptional activation by HHV8-Vcyc depends on an E2F-binding site in the cyclin A promoter, and cdk6 kinase activity is required. The ability of HHV8-Vcyc to activate cyclin A gene expression is shared by D-type cyclins and cyclin E. Unlike D-type cyclins, HHV8-Vcyc is unable to trigger phosphorylation of the pRb-related protein p107 and fails to induce dissociation of p107 from E2F. Unlike cyclin E, HHV8-Vcyc fails to interact physically with E2F complexes on the cyclin A promoter. These results provide additional evidence for the notion that the HHV-8-encoded cyclin differs in several properties from cellular G1 cyclins.

Introduction

Human herpesvirus-8 (HHV-8), also referred to as Kaposi’s sarcoma-associated herpesvirus, is consistently found in Kaposi’s sarcoma and in certain malignancies of lymphatic origin. Several open reading frames (ORFs) were identified that show similarity to cellular genes (for review, see Neipel et al., 1997). Among these is an ORF with sequence similarity to human D-type cyclin genes, referred to as HHV8-Vcyc (Cesarman et al., 1996; Chang et al., 1996; Li et al., 1997). Mammalian D-type cyclins can act as transforming oncogenes in vitro and in transgenic mice (Bodrug et al., 1994; Hinds et al., 1994; Wang et al., 1994) and the HHV8-Vcyc ORF is transcribed in primary effusion lymphomas and Kaposi’s sarcoma tissues, consistent with a potential role for HHV8-Vcyc in these diseases (Cesarman et al., 1996; Davis et al., 1997). It has been reported that HHV8-Vcyc binds to and activates cyclin-dependent kinase 6 (cdk6), and expression of HHV8-Vcyc in rodent cells promotes phosphorylation and inactivation of the retinoblastoma tumour-suppressor protein (pRb) (Godden-Kent et al., 1997; Li et al., 1997). While these observations indicate that HHV8-Vcyc may interfere with cellular growth control, it is unclear whether HHV8-Vcyc can indeed act as a transforming oncogene. In the case of D-type cyclins, deletion analysis revealed that the in vitro transforming potential of cyclin D1 co-segregates with its ability to activate cdk4/6 (Hinds et al., 1994).

One of the cellular targets for cyclin D-associated kinase is the cyclin A gene (Schulze et al., 1995), which encodes a key regulator of S-phase entry in mammalian cells (Pagano et al., 1992). Cyclin A transcription is negatively regulated through an E2F-binding site and repression of transcription in the G1 phase of the cell cycle is mediated by binding of E2F–p107
complexes to the promoter (Schulze et al., 1995). In untransformed cells, expression of the cyclin A gene is specifically prevented by several antiproliferative signals, such as loss of cell adhesion (Guadagno et al., 1993; Schulze et al., 1996) or DNA damage (Spitkovsky et al., 1997). Negative regulation of cyclin A gene expression is abolished in cells transformed by DNA tumour virus oncogenes (Zerfaß et al., 1995) and the ability of both human papillomavirus type 16 (HPV-16) E7 and adenovirus E1A to activate cyclin A gene expression is genetically linked to the transforming properties of these viral genes (Zerfaß et al., 1995, 1996).

It has been reported that binding of HHV8-Vcyc alters the substrate specificity of cdk4, as compared to activation of cdk4 by cellular D-type cyclins. Thus, cdk4 activated by HHV8-Vcyc is capable of phosphorylating histone H1 in addition to pRb (Godden-Kent et al., 1997; Li et al., 1997). However, the phosphorylation of other pocket proteins by HHV8-Vcyc–cdk4 was not studied. Given the key role of cyclin A gene expression in cell cycle checkpoint control and virus-induced transformation, we investigated whether HHV8-Vcyc could inactivate p107 and induce transcription of the cyclin A gene.

Methods

■ Plasmids and reporter gene assays. Human cytomegalovirus (CMV) promoter-driven expression vectors for Myc-tagged HHV8-Vcyc, cdk6 (Godden-Kent et al., 1997), E2F-4 (Ginsberg et al., 1994), haemagglutinin (HA)-tagged p107, cyclin E, cdk2, DP-1 (Zerfaß-Thome et al., 1997), cdk4 and p16INK4a (Schulze et al., 1995) and the expression vector pMo-E7 (Zerfaß et al., 1995) have been described previously. The reporter gene construct pCAwt was constructed by removing sequences between +11 and +245 from the parental construct pALUC (Schulze et al., 1995). pCAE2F was constructed by site-directed mutagenesis, as described previously for pCA(−89/+11)∆E2F (Schulze et al., 1995). NIH 3T3 cells were transfected with reporter gene constructs (3 µg) and expression vectors (3 µg) for HHV8-Vcyc, cdk6, cdk6ΔKN and p16INK4a. After 16 h of incubation, cells were washed and placed in DMEM containing 0.5% newborn calf serum (NBCS). Luciferase assays were performed on cell extracts prepared 36 h after transfection. In all transfection experiments, the amount of vector sequences was kept constant. Variations in transfection efficiency were accounted for by normalizing luciferase values to the activity a co-transfected CMV promoter-driven β-galactosidase control plasmid.

■ Analysis of p107 and E2F-4 phosphorylation. In order to analyse phosphorylation of p107, C33A cells were co-transfected with expression vectors for E2F-4 and DP-1 in parallel with vectors encoding various cyclin and cdk subunits. Cell extracts were immunoprecipitated with monoclonal antibodies to E2F-4 (WUF-11; a gift from E. Harlow, Massachusetts General Hospital, Cambridge, MA, USA) and DP-1 (TDF10; a gift from K. Helin, International Oncology Institute, Milan, Italy) and immunoblotted with WUF-11. In control experiments, cell extracts were either mock-treated, treated with lambda phosphatase or treated with lambda phosphatase in the presence of phosphatase inhibitors, as described previously (Ginsberg et al., 1994).

■ Analysis of E2F–p107 complexes. For immunoprecipitation of E2F–p107 complexes, transfected cells (C33A or U2O-S) were extracted in a buffer containing 50 mM HEPES (pH 7.0), 150 mM NaCl, 0–1% NP-40, 0–2 mM PMSF, 1 mM sodium fluoride and 10 mg/ml aprotinin, as described previously (Zerfaß-Thome et al., 1997). Pre-cleared lysates were subjected to immunoprecipitation with antibodies to p107 (C-18; Santa Cruz) and DP-1 (TDF10). After electrophoresis and transfer onto PVDF membrane, immunoblotting was performed with antibodies to E2F-4 (WUF-11), DP-1 (TDF10) and HA (12CA5; Boehringer Mannheim). In order to study E2F–p107 complexes by electrophoretic mobility shift assay (EMSA), extracts from transfected cells were incubated with a double-stranded oligonucleotide corresponding to the E2F-binding site of the human cyclin A promoter (5’ TTCAATTGTCGCCGAGATCTT 3’) and analysed by antibody supershift experiments, as described previously (Schulze et al., 1995).

Results and Discussion

Activation of the cyclin A promoter by HHV8-Vcyc

To address the question of whether HHV8-Vcyc can trigger expression of the cyclin A gene, co-transfection experiments were performed. When an epitope-tagged version of HHV8-Vcyc was co-expressed with a cyclin A promoter–reporter gene construct, we observed a threefold activation of transcription (Fig. 1, a), similar to results obtained with HPV-16 E7 (Zerfaß et al., 1995) in a control experiment. As shown by Western blotting of extracts from transfected cells (Fig. 1b), transfection with the HHV8-Vcyc expression vector resulted in a high level of expression of HHV8-Vcyc protein. Transactivation could be further enhanced by co-expression of cdk6, indicating that cdk6 cooperated with HHV8-Vcyc in transactivation and that under the experimental conditions shown in Fig. 1, cdk6 may have been limiting. The moderate transactivation obtained with cdk6 alone suggests that endogenous D-type cyclins may have contributed to some extent to transactivation of the cyclin A promoter in the experiments reported here. However, this appears to be a minor effect, in keeping with the finding that expression of D-type cyclins is strongly down-regulated in growth factor-deprived cells (Won et al., 1992). To test whether the ability of HHV8-Vcyc to stimulate cdk6 kinase activity is required for transcriptional activation, we analysed activation of the cyclin A promoter by HHV8-Vcyc in the presence of a kinase-negative mutated cdk6 (cdk6KN; J. Bartek, unpublished). As shown in Fig. 1(a), co-expression of cdk6KN completely ablated activation of the cyclin A promoter by HHV8-Vcyc, indicating that cdk6 kinase
HHV-8-Vcyc activates cyclin A transcription

Fig. 1. Transactivation of the human cyclin A promoter by HHV8-Vcyc in NIH 3T3 cells. (a) Reporter gene constructs were tested in co-transfection experiments with expression vectors for HHV8-Vcyc, cdk6, cdk6KN and p16INK4A. Co-transfection of pMo-E7 was included as control. After transfection, cells were placed in 0–5% NBCS and luciferase activity was determined. Luciferase activity was normalized to the activity of a co-transfected β-galactosidase control plasmid; relative induction is shown. (b) Detection of HHV8-Vcyc protein after transfection. C33A cells were transfected with expression vectors pX and pX-HHV-8cyc, as indicated. Expression of Myc-tagged HHV8-Vcyc was detected by immunoblot analysis with an anti-Myc tag antibody.

activity is required. This conclusion was confirmed by additional experiments, in which the cdk inhibitor p16INK4A was co-expressed (Fig. 1a).

In order to identify elements in the cyclin A promoter that respond to increased HHV8-Vcyc–cdk6 expression, we analysed a mutated promoter (pCAΔE2F) lacking an E2F-binding site that was shown previously to mediate transactivation of the promoter through D-type cyclins in collaboration with cdk4 (Schulze et al., 1995). As shown in the right half of Fig. 1(a), activity of the mutated promoter was only marginally induced by HHV8-Vcyc, and co-expression of cdk6 induced promoter activity only weakly. Thus, we conclude that the E2F-binding site of the cyclin A promoter is a critical target for HHV8-Vcyc–cdk6.

HHV8-Vcyc fails to induce p107 phosphorylation

It has been shown before that expression of the cyclin A gene is negatively controlled in untransformed cells (Schulze et al., 1995) and that repression is mediated by E2F–DP–p107 complexes that bind to the cyclin A promoter via the E2F-binding site (Spitkovsky et al., 1997; Zerfaß et al., 1996). Expression of cyclin D1 results in phosphorylation, and hence inactivation, of p107 (Beijersbergen et al., 1995). To test whether HHV8-Vcyc can induce p107 phosphorylation, we co-transfected C33A cells, a human cervical carcinoma cell line that can be transfected with very high efficiency, with expression vectors for p107, E2F-4 and DP-1 along with various cyclin–cdk complexes. Extracts were prepared from transfected cells and analysed by Western blot experiments with antibodies to p107. This experiment revealed that co-expression of cyclin D1 and cdk4 resulted in a significant conversion of p107 into the hyperphosphorylated form, while expression of HHV8-Vcyc and cdk6 failed to induce any alteration in the electrophoretic mobility of p107 (Fig. 2). These results indicate that HHV8-Vcyc, unlike cyclin D1, is not able to induce phosphorylation of p107.

We next analysed the stability of E2F–p107 complexes upon co-expression of HHV8-Vcyc and cdk6. U2O-S cells, a human osteosarcoma cell line, were used for these experiments, since it has been shown before that phosphorylation of p107 by D-type cyclins triggers the dissociation of E2F–p107 complexes in this cell type (Beijersbergen et al., 1995). U2O-S cells were transfected with expression vectors for p107, E2F-4 and DP-1 and either HHV8-Vcyc and cdk6 or cyclin D1 and cdk4. Extracts were prepared from transfected cells and subjected to immunoprecipitation experiments with antibodies to p107. Immunoprecipitates were then probed with antibodies to E2F-4 and DP-1. This experiment revealed that, on co-expression of p107, both E2F-4 and DP-1 were found in a complex with p107. As expected, the association of p107 with E2F-4 and DP-1 was strongly reduced on co-expression of...
cyclin D1 and cdk4, reflecting the inability of hyperphosphorylated p107 (see Fig. 2) to interact with E2F–DP-1 heterodimers (Fig. 3, upper panel; see also Zerfaß-Thome et al., 1997). As shown in Fig. 3, co-expression of HHV8-Vcyc and cdk6 did not affect the association between p107 and E2F–DP-1 heterodimers significantly in U2-OS cells, and similar results were obtained with C33A cells (data not shown). From the results shown in Fig. 3, it appears that the effect of cyclin D1 and cdk4 on p107–E2F complexes was more pronounced than that on p107–DP-1 complexes. This could mean that other, as yet unidentified, E2F family members bind to p107 and that these complexes are less sensitive to the action of cyclin D1 and cdk4. This possibility was not further investigated.

To visualize p107 specifically from transfected cells, an epitope-tagged version of p107 was used. U2O-S cells were transfected with expression vectors for E2F-4, DP-1 and HA-tagged p107 (HA-p107; Beijersbergen et al., 1995; Zerfaß-Thome et al., 1997). After immunoprecipitation of DP-1 from transfected cells, we found that HA-p107 was co-precipitated, indicating an association of DP-1 with HA-p107 in the transfected cells. On co-expression of cyclin D1 and cdk4, the amount of co-precipitated HA-p107 was drastically reduced; in contrast, co-expression of HHV8-Vcyc and cdk6 had no detectable influence on the amount of DP-1-bound HA-p107 (Fig. 3, lower panel). This finding confirms the previous conclusion that, unlike cyclin D1 and cdk4, HHV8-Vcyc and cdk6 fail to trigger dissociation of E2F–p107 complexes on the cyclin A promoter.

HHV8-Vcyc fails to interact physically with the cyclin A promoter

Our finding that HHV8-Vcyc triggers activation of the cyclin A promoter yet fails to disrupt E2F–p107 complexes is reminiscent of our previous finding that trans-activation of the cyclin A promoter by cyclin E–cdk2 complexes does not involve disruption of E2F–p107 complexes (Zerfaß-Thome et al., 1997). Instead, it was found that cyclin E–cdk2 complexes bound to the cyclin A promoter. Apparently, in this case activation of the cyclin A gene is triggered through the tethering of an active cyclin E–cdk2 complex to the promoter region via the E2F-binding site; the available evidence suggests that this complex can no longer act as a transcriptional repressor (Zerfaß-Thome et al., 1997; for review, see La Thangue, 1996). In order to investigate whether activation of the cyclin A promoter by HHV8-Vcyc and cdk6 may involve a similar mechanism, we analysed the ability of HHV8-Vcyc to promote conversion of the p107-containing form of E2F into the S-phase-specific form, which is characterized by the association of a cyclin–cdk2 complex with E2F–p107 (Cao et al., 1992; Devoto et al., 1992; Pagano et al., 1992a; Zerfaß-Thome et al., 1997).

C33A cells were transfected with expression vectors for p107, E2F-4 and DP-1. Extracts were prepared from transfected cells and analysed in band-shift experiments by using the E2F-binding site of the human cyclin A promoter as the labelled probe. As shown in Fig. 4, co-expression of E2F-4 and DP-1 with p107 resulted in the appearance of a new band (designated B in Fig. 4). This band contained E2F-4–DP-1 heterodimers in complex with p107, as revealed by antibody supershift experiments (Fig. 4b and data not shown). Apparently, this band also contained a second, non-specific complex that was not shifted by any of our antibodies; unlike the other complexes, the abundance of this non-specific complex varied considerably with experimental conditions (data not shown; compare lane 3 in Fig. 4a with lane 2 in Fig. 4b).

The E2F complex in band B was devoid of any cyclin or cdk subunit, as demonstrated by antibody supershift experiments (Fig. 4b and data not shown). On co-expression of cyclin E and cdk2, the E2F–p107 complex was replaced by a new complex of lower mobility; this new complex (band A in Fig. 4a) contained cyclin E and cdk2 in addition to E2F–4–DP-1 and p107 (Fig. 4b; see also Zerfaß-Thome et al., 1997). When we analysed E2F complexes in extracts from cells that had been transfected with expression vectors for HHV8-Vcyc and cdk6, we failed to detect the appearance of any slower-migrating complexes (Fig. 4a). Furthermore, antibodies to cdk6 did not recognize any of the complexes. Similarly, the 9E10 antibody, directed against the Myc tag attached to HHV8-Vcyc, did not
affect the band-shift pattern (data not shown). Taken together, these results suggest that, unlike cyclin E–cdk2, HHV8-Vcyc–cdk6 complexes are unable to associate stably with the E2F element in the cyclin A promoter.

**HHV8-Vcyc induces phosphorylation of E2F-4**

It has been reported that the E2F-4 protein is phosphorylated at several sites (Ginsberg et al., 1994) and that phosphorylation of E2F-4 varies as cells progress through the cell cycle (Moberg et al., 1996), suggesting that the ability of E2F-4 to regulate gene expression may be modulated by phosphorylation. These findings raise the possibility that activation of E2F-4 target genes may involve phosphorylation of E2F-4. As a first step towards answering this question, we investigated whether expression of HHV8-Vcyc induced detectable changes in the phosphorylation pattern of E2F-4. To this end, U2O-S cells were transfected with expression vectors for E2F-4, DP-1 and p107, as described above. Extracts were prepared from these cells and subjected to immunoprecipitation using antibodies to E2F-4 and DP-1. Immunoprecipitates were then analysed by direct immunoblotting with antibodies to E2F-4. We found that immunoprecipitation by anti-E2F-4 antibodies yielded two distinct forms of E2F-4 and that both bands were also detected after immunoprecipitation with antibodies to DP-1. These results suggest that the transfected E2F-4 protein is present in two distinct species, both of which bind DP-1 efficiently.

On co-expression of HHV8-Vcyc and cdk6, the faster-migrating band was not observed and a new E2F-4 species with reduced gel mobility was observed. This newly apparent form of E2F-4 was equally well represented in DP-1 immunoprecipitates, indicating that the modification of E2F-4 induced by HHV8-Vcyc and cdk6 did not affect the ability of E2F-4 to interact with DP-1. In control experiments, we found that a similar modification of E2F-4 was induced by two other cdks, cdk2 and cdk4 (Fig. 5a). These results strongly suggest that the slower-migrating bands represent hyperphosphorylated forms of E2F-4. This was confirmed by our findings that the HHV8-Vcyc–cdk6-induced modification of E2F-4 could be reversed by phosphatase treatment and that the latter effect was abolished when a phosphatase inhibitor was included (Fig. 5b). Expression of HHV8-Vcyc therefore induces phosphorylation of E2F-4, the major E2F species interacting with the cyclin A promoter in all cell lines tested so far, suggesting that phosphorylation of E2F-4 may contribute to transactivation of the cyclin A gene by HHV8-Vcyc. It should be

**Fig. 4.** Analysis of E2F complexes by EMSA. (a) C33A cells were transfected with plasmids encoding E2F-4, DP-1 and p107. As indicated, cells were co-transfected with expression vectors for either Myc-tagged HHV8-Vcyc and cdk6 or cyclin E and cdk2. Cell extracts were analysed by EMSA with the E2F-binding site of the human cyclin A promoter as the labelled probe. (b) The composition of the E2F complexes shown in (a) was analysed by incubation of the band-shift reaction products with specific antibodies to cdk2 and p107, as indicated.
noted that our data do not formally exclude the possibility that phosphorylation of E2F-2 in Vcyc-expressing cells may be mediated by kinases distinct from Vcyc–cdk6. Furthermore, it is possible that phosphorylation of pRb by HHV8-Vcyc (Godden-Kent et al., 1997) contributes to the activation of cyclin A gene expression, although indirectly. While pRb does not bind to the cyclin A promoter (Schulze et al., 1995), inactivation of pRb induces expression of the cyclin E gene (Herrera et al., 1996), which encodes a known upstream activator of cyclin A gene expression (Zerfaß-Thome et al., 1997).

The results presented in this communication indicate that HHV8-Vcyc shares with cyclin E and cyclin D1 the ability to activate expression of the cyclin A gene in serum-starved cells. Activation of cyclin A gene expression in the presence of antiproliferative signals is a characteristic property of transforming oncoproteins. Thus, both adenovirus E1A and HPV-16 E7 can activate cyclin A gene expression in growth factor-deprived cells (Zerfaß et al., 1995, 1996); more recently, the ability of HPV-16 E7 to induce anchorage-independent growth has been shown to correlate with the ability of E7 to prevent down-regulation of cyclin A transcription in non-adherent rodent fibroblasts (Schulze et al., 1996, 1998). While these data suggest that the ability of HHV8-Vcyc to induce expression of the cyclin A gene may contribute to the transformed phenotype of HHV-8-infected cells, it is not known whether HHV8-Vcyc alone can transform cells or induce anchorage-independent growth. While this question is only indirectly addressed by our data, accumulating evidence suggests that the viral cyclin, which is expressed in KS (Reed et al., 1998), plays a role in HHV-8-associated oncogenesis (for recent review, see Moore & Chang, 1998).

Another interesting aspect of the data presented here is the fact that HHV8-Vcyc, which according to current hypotheses has evolved from a cellular gene taken up by an HHV-8 ancestor (reviewed by Neipel et al., 1997), differs in its biochemical properties from both D-type and E-type cyclins. This conclusion is supported by the recent finding that the kinase activity of HHV8-Vcyc–cdk6 complexes is much less susceptible to repression by cdk inhibitors like p27KIP1 (Swanton et al., 1997). The precise biochemical nature of these differences and their relationship to deviations in the primary amino acid sequence remain to be clarified.

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References

HHV-8 cyclin activates cyclin A transcription


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