Mutational analysis of growth arrest and cellular localization of human immunodeficiency virus type 1 Vpr in the budding yeast, *Saccharomyces cerevisiae*

Junko Nakazawa,1,2 Nobumoto Watanabe,1,* Masaya Imoto,2 and Hiroyuki Osada1

1 Antibiotics Laboratory, Discovery Research Institute, RIKEN, Wako, Saitama 351–0198, Japan
2 Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Kohoku-ku, Yokohama 223–8522, Japan

(Received March 9, 2005; Accepted June 16, 2005)

Viral protein R (Vpr), one of the accessory gene products of human immunodeficiency virus type 1 (HIV-1), is responsible for the incorporation of a viral genome into the nucleus upon infection. Vpr also arrests the cell cycle and induces apoptosis in infected cells. Similarly, in yeast, Vpr localizes in the nucleus and shows growth inhibitory activity; however, the molecular mechanism of growth inhibition remains unknown. To elucidate this mechanism, several point mutations of Vpr, which are known to perturb several phenotypes of Vpr in mammalian cells, were introduced in the budding yeast, *Saccharomyces cerevisiae*. For the first time, we found that growth inhibition by Vpr occurred independently of intracellular localization in yeast, as has previously been reported in mammals. We also identified several amino acid residues, the mutation of which cancels growth inhibitory activity, and/or alters localization, both in yeast and mammalian cells, suggesting the importance of these residues for the phenotypes.

**Key Words**—growth inhibition; *Saccharomyces cerevisiae*; Vpr

**Introduction**

Human immunodeficiency virus type-1 (HIV-1), a member of the lentivirus family, encodes two regulatory genes (tat, rev) and four accessory genes (vif, vpr, vpu, nef) in addition to three common retroviral structural genes (gag, pol, env). Viral protein R (Vpr) is a small 14-kDa protein of 96 amino acids. Vpr is incorporated into the virions through its interaction with the p6 region of HIV-1 Gag precursor (Cohen et al., 1990; Paxton et al., 1993; Yuan et al., 1990) and plays a role in the transportation of the viral preintegration complex (PIC) into the nucleus (Heinzinger et al., 1994). Vpr is nonessential for viral replication in T cell lines and activated peripheral blood lymphocytes in vitro but it is necessary for efficient infection of nondividing cells such as macrophages (Balliet et al., 1994; Balotta et al., 1993; Connor et al., 1995; Hattori et al., 1990; Heinzinger et al., 1994; Ogawa et al., 1989; Westervelt et al., 1992). Vpr is highly conserved among other primate lentiviruses; HIV-1, HIV-2 and the Simian immunodeficiency virus (SIV). In the study of macaques infected with wild-type or Vpr-mutant virus, Vpr appeared to increase both the viral load and the rate at which the animals progressed to AIDS (Lang et al., 1993). In addition, in some of the Vpr-mutant virus-infected animals, viral sequences in which Vpr had reverted to wild-type were isolated, suggesting that the presence of a Vpr open reading frame provided a selective advantage to the virus (Gibbs et al., 1995; Goh et al., 1998). In humans, Vpr variants correlate with
long-term survival in HIV-1-infected individuals (Somasundaran et al., 2002; Zhao et al., 2002); thus Vpr appears to contribute to viral pathogenesis.

The most characteristic function of Vpr is to block the cell cycle at the G2 phase, followed by apoptosis in both HIV-1-infected cells and Vpr-expressing cells (Jowett et al., 1995). The activity of Vpr-mediated arrest of the cell cycle is conserved in HIV-2 and SIV, suggesting an important role for Vpr in the life cycle of such viruses (Planelles et al., 1996). Indeed, arrest in the G2 phase of the cell cycle may create an intra-cellular environment that enhances the transcription of the HIV-1 promoter, the long terminal repeat (LTR) (Goh et al., 1998). In addition, the uncoupling of the centrosome duplication cycle from the cell division cycle due to long G2 arrest induces abnormal M phase spindle formation and subsequent apoptosis (Watannabe et al., 2000).

Vpr is known to inhibit cell growth, both in the fission yeast, Schizosaccharomyces pombe (Zhao et al., 1996), and in the budding yeast, Saccharomyces cerevisiae (Macreadie et al., 1995). Such inhibition implies that the cellular target of Vpr is a highly conserved protein(s). Previous studies have shown that arrest in the G2 phase is associated with an inactivation of cyclin B/cdc2 kinase, a key regulator of the G2/M transition, through the inhibitory phosphorylation of cdc2 in human cells as well as S. pombe (He et al., 1995; Re et al., 1995; Zhao et al., 1996). Several Vpr interacting proteins have been identified, such as DNA repair-associated proteins (uracil DNA glycosylase (UNG) and a human homolog of the Saccharomyces cerevisiae Rad23 protein (HHR23A)) (Bouhamdan et al., 1996; Withers-Ward et al., 1997), transcription factors (TFIIB, Cyclin T1, and SP-1) (Agostini et al., 1996; Sawaya et al., 2000; Wang et al., 1995), nuclear pore proteins (nucleoporins and envelope proteins) (de Noronha et al., 2001; Fouchier et al., 1998; Le Rouzic et al., 2002; Popov et al., 1998a; Varadarajan et al., 2005), and the nuclear transport factor (importin-α) (Agostini et al., 2000; Popov et al., 1998b; Vodicca et al., 1998). In addition, Vpr expression induces nuclear herniations, resulting in a transient mixing of nuclear and cytoplasmic components (de Noronha et al., 2001). It has also been shown that Vpr induces multiple defects in cell division, including effects on the mitotic spindle, centrosome/spindle pole body (SPB), nuclear envelope, and cytokinesis in human cells and fission yeast (Chang et al., 2004), or induces cytoskeletal defects in budding yeast (Gu et al., 1997). Recently, the activation of DNA damage-checkpoint protein ATR (ATM and Rad3-related) by Vpr expression have been reported (Roshal et al., 2003; Zimmerman et al., 2004). However, the precise molecular mechanism by which Vpr induces cell cycle arrest at the G2 phase remains under investigation.

The Vpr structure, determined by NMR analysis, is characterized by three well-defined α helices: residues 17 to 33, 38 to 50, and 56 to 77, respectively (Morellet et al., 2003). By expressing point-mutated Vpr in mammalian cells, several regions including these helices are shown to be important for virion incorporation, G2 arresting activity, and the nuclear localization of Vpr (Ayyavoo et al., 1997; Di Marzio et al., 1995; Gaynor and Chen, 2001; Mahalingam et al., 1995; Sherman et al., 2001; Singh et al., 2000). However, these regions appear to act independently of each other, since G2 arrest by Vpr is not dependent on nuclear accumulation, nor on virion incorporation in mammalian cells (Mahalingam et al., 1997; Yao et al., 1995).

In this report, we first examined the relationship between nuclear localization and the growth inhibitory activity of Vpr in budding yeast. Although several mutant forms of Vpr were found to have inhibitory activities that differed from those observed in mammalian cells, growth arrest occurred independently of nuclear localization in budding yeast, as well as in mammalian cells.

Materials and Methods

Yeast strains and plasmid. The XhoI-NotI fragment of FLAG-tagged HIV-1NL4-3 Vpr from pME18Neo-FVpr (Nishino et al., 1997) was blunted and inserted into the blunted BamHI site of a yeast expression vector, pYEX-BX (AmRad) containing promoter elements (CUP1) for copper-inducible expression. A resultant vector with reversed orientation of FLAG-tagged Vpr was used as a control through this study. To express N-terminal EGFP-tagged Vpr, the NcoI/EcoRI fragment of EGFP from pEGFP-C1 (Clontech) was blunted and inserted into the N-terminus of the FLAG-tag of each FLAG-tagged (wild/mutant) Vpr vector after XhoI digestion and blunting.

Point mutations in the Vpr ORF were introduced using a Quick Change Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. The sequences of the oligonucleotides used
here are available upon request. Among the candidates for the R73A mutant, we found a clone that has an additional thymidine inserted after the 77th codon. This clone has an artificial termination codon (TAG) at the 79th codon, in addition to the R73A and H78T mutations, and was designated as the CΔ18 (C-terminal, 18 amino acids deletion) mutant in this study.

The S. cerevisiae strain used in this study was MLC30, which is derived from the W303-1A strain (Miyamoto et al., 2002). Cells transformed with Vpr or control plasmid by the lithium acetate procedure were selected in non-inducible medium (synthetic complete medium lacking uracil with 2% glucose). For Vpr expression, the cells were cultured in inducible medium (synthetic complete medium lacking both uracil and leucine with 0.5 mM CuSO₄ and 2% glucose).

Flow cytometry analysis. FACS analysis was performed as described in Zhang et al. (Zhang et al., 1997) with some modification. Briefly, after cells were grown to the log phase, cells were cultured in the inducible medium for indicated periods and were fixed in 70% ethanol for 12 h at 4°C. After resuspension in 50 mM sodium citrate, RNase A was added to a final concentration to 1 mg/ml and incubated at 37°C for 2 h. Thirty μg/ml propidium iodide were added and sonicated briefly. Samples were analyzed with a Becton-Dickinson FACSort.

Western blotting. The yeast cells were lysed in lysis buffer (CelLytic-Y (SIGMA) with 10 mM 1,4-dithio-DL-threitol and complete protease inhibitor cocktail (Roche)) and clarified by centrifugation. The supernatants were separated by SDS-PAGE (15% acrylamide), transferred to a PVDF membrane (MILLIPORE), and probed with anti-FLAG antibody (M2, SIGMA) or anti-GFP antibody (MBL).

Localization of EGFP-tagged Vpr. Yeast cells were grown to the early log phase at 30°C in the non-inducible medium and then cultured in the inducible medium for 1 h. The cells were fixed in 3.7% formaldehyde in PBS for 10 min, washed, and fixed again in 70% ethanol for 1 h. The cells were then washed twice with PBS, stained with 4,6-diamidino-2-phenylindole (DAPI; 0.4 μg/ml in PBS), and observed under fluorescence and Nomarski microscopy.

Results and Discussion

Growth arrest by Vpr in budding yeast

The effect of Vpr on the growth of budding yeast was examined using a Vpr expression vector with a copper-inducible promoter system. When the expression of FLAG-tagged Vpr was analyzed with anti-FLAG antibody, strong expression of Vpr was detected as early as 1 h after induction, although weak levels of expression were detected even before induction (Fig. 1A). Then, the effects of Vpr on growth were determined by the increase in cell density. In non-inducible medium, the yeast with a Vpr-expression plasmid grew at a similar rate as that of the yeast bearing the control plasmid (data not shown). The induction of Vpr expression inhibited the growth (Fig. 1B, crosses), while yeast without Vpr expression vector grew rapidly (Fig. 1B, open circles) in the same medium. Thus, as has been reported by other groups (Berglez et al., 1999; Macreadie et al., 1995, 1997), Vpr was found to inhibit the growth of budding yeast under the present conditions.

When DNA contents of the growth-arrested cells were analyzed with FACS, only a modest increase of 1C population (DNA contents at G1 phase) was observed in Vpr expressing cells, indicating that cells do not arrest at a specific phase in the cell cycle (Fig. 1C). This result was also confirmed by the morphological observation under microscope. Status of budding, which reflects the cell cycle progression in this type of yeast cells, did not change significantly by the expression of Vpr (Fig. 1D). Although abnormal morphology such as multiple budding caused by Vpr expression for relatively long periods were reported (Gu et al., 1997), we did not observe such abnormality at least for 4 h after the induction, when the growth was completely arrested. Thus it is likely that the previously reported morphological abnormality is a result rather than the cause of the growth arrest by the Vpr expression.

Mutational analysis of growth inhibition by Vpr

Following Vpr expression in mammalian cells, several characteristic phenotypes seem to appear independently of each other, as a particular phenotype can be cancelled by certain point mutations, whereas yet another phenotype can be disturbed by still other mutations (see Table 1). To elucidate the molecular mechanism by which Vpr inhibits cell growth in yeast and to determine the relationship of this mechanism to particular phenotypes in mammalian cells, we introduced several point mutations into Vpr that are known to cancel phenotypes in mammals, and we examined
the growth inhibitory effects of them in yeast cells (Fig. 2A). The expression of Vpr in mammalian cells induces cell cycle arrest at the G2 phase (Jowett et al., 1995). The C-terminal region of Vpr has been shown to be important for this activity, because C-terminal deletion, as well as point mutations in the C-terminus (H71R, R73A, I74P, G75A, C76A, R80A, and R90K), have been demonstrated to cancel or strongly reduce such activity (Di Marzio et al., 1995; Gaynor and Chen, 2001; Mahalingam et al., 1997; Mansky et al., 2001; Nishizawa et al., 1999; Selig et al., 1997; Stewart et al., 1997; Vodicka et al., 1998). In addition, a point mutation of the N-terminal region, A30L (Di Marzio et al., 1995; Gaynor and Chen, 2001; Mahalingam et al., 1997), is also known to cancel this activity. For the nuclear localization of Vpr, both the N-terminal region and the C-terminus are considered to be important, because E25K, H71R, and C-terminal deletion mutants have all been shown to change the localization of Vpr from the nucleus to the cytoplasm in mammalian cells (Lu et al., 1993; Mahalingam et al., 1997; Vodicka et al., 1998; Yao et al., 1995).

The expression of Vpr also induces apoptosis in mammalian cells (Stewart et al., 1997). Several domains in Vpr appear to be involved in this activity, because mutations in the N-terminus (Q3R), in the middle region (L67A), and in the C-terminus (I74P, C76A, and R80A) cancel or reduce apoptosis-inducing activity (Gaynor and Chen, 2001; Nishizawa et al., 2000; Somasundaran et al., 2002). Vpr is known to bind UNG, which is involved in DNA repair and in the DNA damage checkpoint activation process (Bouhamdan et al., 1996). By deletion or point mutation analysis, at
least three domains of Vpr appear to be important; it has been shown that three separately located point mutations (E25K, W54R, and H71R) can inhibit the binding between Vpr and UNG (Bouhamdan et al., 1996; Mansky et al., 2001; Selig et al., 1997).

Expression vectors for all of these mutants were constructed and introduced into yeast cells. The expression of mutant forms of Vpr protein was examined by immunoblotting using anti-FLAG antibody. Although there were some differences in the expression levels of each mutant, expression was detected in the case of all of the Vpr mutants studied here (Fig. 2B). Then, the growth inhibitory activity of each of these mutants was analyzed. On the non-inducible agar plate (Vpr-off), no significant growth retardation was detected in yeast cells expressing any form of Vpr, including the wild-type Vpr (wt). However, on the inducible agar plate (Vpr-on), strong growth inhibition was observed in association with several mutant forms (Q3R, E25K, W54R, R73A, R80A, and R90K) of Vpr, in addition to being observed in cells expressing wild-type Vpr (the inhibitory activities are designated as “+” at the bottom of Fig. 2C). In contrast, the growth inhibitory activity of Vpr was almost cancelled (−) by C-terminal deletion (CΔ18; 18 amino acids were deleted at the C-terminus) or mutations in the C-terminal region (H71R, I74P, and C76A). In some of the other mutants (A30L, L67A, and G75A), a modest level of growth inhibition (±) was observed. These differences in growth inhibitory activity did not appear to be correlated with differences in the levels of expression of Vpr protein (Fig. 2, B and C).

As shown above, and as summarized in Table 1, the C-terminal domain of Vpr is important for growth inhibitory activities in mammalian cells, including the induction of cell cycle arrest and apoptosis. We have

Table 1. Summary of effects of mutant Vpr in S. cerevisiae and mammalian cells.

<table>
<thead>
<tr>
<th>S. cerevisiae</th>
<th>Mammalian cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth arrest</td>
</tr>
<tr>
<td>wt</td>
<td>+</td>
</tr>
<tr>
<td>Q 3 R</td>
<td>+</td>
</tr>
<tr>
<td>E 25 K</td>
<td>+</td>
</tr>
<tr>
<td>A 30 L</td>
<td>±</td>
</tr>
<tr>
<td>W 54 R</td>
<td>+</td>
</tr>
<tr>
<td>L 67 A</td>
<td>±</td>
</tr>
<tr>
<td>H 71 R</td>
<td>−</td>
</tr>
<tr>
<td>R 73 A</td>
<td>+</td>
</tr>
<tr>
<td>I 74 P</td>
<td>−</td>
</tr>
<tr>
<td>G 75 A</td>
<td>±</td>
</tr>
<tr>
<td>C 76 A</td>
<td>−</td>
</tr>
<tr>
<td>R 80 A</td>
<td>+</td>
</tr>
<tr>
<td>R 90 K</td>
<td>+</td>
</tr>
<tr>
<td>CΔ18</td>
<td>−</td>
</tr>
</tbody>
</table>

+, wild-type level; ±, intermediate activities; −, low activity or none.

A shadow box indicates the relieved effect of wild-type function.

Eighteen amino acids at the C-terminus were deleted with R73A and H78T substitutions.

Nineteen amino acids at the C-terminus were deleted.

also shown in yeast cells that C-terminal deletion (CΔ18), as well as certain point mutations at the C-terminus (H71R, I74P, G75A, and C76A), cancelled growth inhibitory activity. However, we also found that several other mutations of the C-terminus (R73A, R80A, and R90K), which are known to cancel such inhibitory activity in mammalian cells, were shown in yeast cells to have almost the same activity as that of the wild-type form. During the preparation of this manuscript, similar negative effect of R80A in budding yeast was reported by another group (Yao et al., 2004). In their study, negative effects of two other mutations (Q3R, E25K) were also reported in keeping with our results. In addition, the same effects of R73A and G75A were also reported by another group previously (Berglez et al., 1999). However, in their report, the effect of C76A mutation was negative, which is inconsistent with our results. Future studies will be needed to resolve this discrepancy.

These results suggest that the C-terminal region of
Vpr is fundamentally important for the growth inhibitory effect of Vpr in yeast cells as well as in mammalian cells. However, it remains unclear why some mutations in this region failed to cancel the growth inhibitory activity only in yeast cells. It is possible that the observed differences between mammalian and yeast cells with regard to Vpr mutations may have been due to the sequence diversity of yet unknown target protein(s) that interact(s) with the C-terminus of Vpr. Amino acids that are important for growth arrest only in mammalian cells, but not in yeast cells, may be required for interaction with mammalian target protein(s), but not with the yeast counterpart(s). In this regard, a protein involved in DNA repair, UNG (uracil DNA glycosylase), is known to bind Vpr; however, the ability to bind to UNG was not correlated with growth inhibitory activities in mammalian cells (Selig et al., 1997). It is conceivable that the target protein of Vpr other than UNG might exist also in yeast, which would be very important for growth arrest in both yeast and mammalian cells.

**Mutational analysis of the localization of Vpr**

Vpr is localized in the nucleus, primarily on the nuclear envelope (Lu et al., 1993; Vodicka et al., 1998). Extensive studies of the relationship between this localization and the growth inhibitory effects of Vpr have revealed that these two phenotypes appear independently in mammalian cells. However, in budding yeast, the relationship between these phenotypes has not been examined to date. Thus, we analyzed the intracellular localization of our point mutants in order to examine the possible relationship between these two phenotypes in yeast.

To examine the localization of Vpr in yeast, expression vectors were constructed that encoded wild-type protein or each mutant form of Vpr with EGFP (enhanced green fluorescent protein) at the N-terminus; these vectors were then introduced into yeast cells. One hour after induction, all the EGFP-tagged Vpr were expressed at the predicted size when examined by western blotting with anti-GFP antibody (Fig. 3A). The abilities of mutations (H71R, C76A, and CΔ18) to cancel the growth inhibitory activity of FLAG-tagged Vpr were also confirmed in EGFP-tagged Vpr (Fig. 3B). Thus the EGFP-tag does not seem to alter the effect of point mutations in Vpr sequence although the growth inhibitory activity of EGFP-tagged Vpr was slightly weaker than that of FLAG-tagged Vpr (Fig. 3B). Then the localization of Vpr was analyzed under fluorescent microscopy after fixation and DAPI staining. In addition, as a control, an expression construct for EGFP alone (EGFP-vec) was also introduced into yeast cells.

When the yeast cells were cultured in non-inducible medium, no significant EGFP signal was detected in any of these yeast transformants. However, after 1 h of culture in the inducible medium, a strong EGFP signal was detected throughout the entire cell expressing EGFP alone (Fig. 3C, EGFP-vec). In contrast, wild-type EGFP-Vpr was detected in a dotted pattern at the periphery of the nucleus (possibly at the nuclear envelope) (Fig. 3C, wt). In addition, abnormally bright dots were observed even in cytoplasm of some cells. The origin of these dots was not clear but we think these are aggregates of EGFP-Vpr fusion proteins. The localization pattern of wild-type (designated as Nuc in Table 1) was very similar to that observed by another group using anti-Vpr antibody in budding yeast (Vodicka et al., 1998), thus suggesting that the EGFP signal from our EGFP-Vpr fusion protein represents the actual localization of Vpr.

Among the mutants we used in the growth inhibitory study described above, only the E25K, H71R, and C-terminal deletion mutants are known to localize both in the cytosol and in the nucleus in mammalian cells (Mahalingam et al., 1997; Vodicka et al., 1998; Yao et al., 1995). In yeast, the localization of E25K, H71R, and CΔ18 differed from that of the wild-type protein as well (Fig. 3C). These mutants of Vpr were located throughout the entire cell, stronger in nuclei, and no significant localization at the nuclear periphery was observed, nor was the dotted pattern (designated as Nuc+Cyto in Table 1). In addition to these mutants, three other mutants (L67A, C76A, and R73A) showed similar localization (Fig. 3C). Thus, in yeast, both the C-terminal region and the N-terminus are important for Nuc-type localization.

Among the mutants that localized as the Nuc+Cyto-type in yeast, R73A and C76A are known to localize in the nucleus in mammalian cells (Table 1). Again, the reason for this difference between yeast and mammalian cells is not clear, but it may be caused by the sequence diversity of protein(s) responsible for the Nuc-type localization of Vpr. Given that the binding of Vpr to a common molecule is required for Nuc-type localization, mutations such as R73A or C76A may disturb the process of binding only in yeast, but not in
Fig. 3

A

B

C

Fig. 3
mammals. As a candidate molecule that might be responsible for the Nuc-type localization, importin-α is of interest, because it is known to bind to Vpr and to be responsible for its localization both in mammalian and yeast cells (Popov et al., 1998b; Vodicka et al., 1998). A mutation in R73A or C76A may be sufficient to perturb the binding of Vpr to importin-α of yeast, but not to that of mammalian cells.

As summarized in Table 1, we found that mutations affecting the growth inhibitory activity of Vpr are independent from those that affect the cellular localization of Vpr in S. cerevisiae for the first time. In addition, we found several point mutations that exerted an influence on growth inhibitory activity or on the nuclear localization of Vpr in both yeast and mammalian cells. These conserved amino acid residues of Vpr are thought to be critical for interactions with certain molecules required for the phenotypes of Vpr. Since the nuclear localization and growth inhibition of Vpr may be related to the nuclear entry of PIC upon HIV-1 infection and may be involved in the decrease in the number of CD4 positive cells in AIDS patients, respectively, inhibitors of these functions might eventually help in the development of efficient medical treatments for AIDS. Our identification of particular amino acids in Vpr that are critical for these processes are expected to provide useful information for the development for such drugs.

Acknowledgments

We thank Tokichi Miyakawa (Hiroshima Univ., Hiroshima, Japan) for MLC30 strain. Chihiro Tsutsui for Vpr mutants; Yasue Ichikawa and Rie Nakazawa (Bioarchitect Research Group, RIKEN) for DNA sequencing; members of RIKEN Antibiotics Laboratory for discussions. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) and by the Chemical Biology Research Project (RIKEN).

References


Fig. 3. Mutational analysis of the cellular localization of Vpr.
A: The expression of EGFP-tagged wt/mutant Vpr was examined by the western blotting (total protein from approximately 0.05 OD600 unit of yeast cells was loaded in each lane) with anti-EGFP antibody. B: The effect of EGFP-tagged wt/mutant Vpr on the yeast cell growth compared to FLAG-tagged wt/mutant Vpr was determined as described in Fig. 2C. C: Yeast cells transformed with EGFP or EGFP-Vpr expression plasmids (indicated in the figure) were grown in non-inducible medium. After 1 h of incubation in the inducible medium, cells were fixed and stained with DAPI for nuclear visualization. Signals of EGFP and DAPI were observed under fluorescence microscopy. Cells were viewed with Nomarski optics.


the Vpr protein of human immunodeficiency virus type 1 regulates cell proliferation independently of G(2) arrest of the cell cycle. *Virology*, **263**, 313–322.


