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Virology 329 (2004) 199–212

VIROLOGY

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The cowpox virus host range gene, CP77, affects phosphorylation of eIF2 α and vaccinia viral translation in apoptotic HeLa cells

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Received 2 January 2004; returned to author for revision 9 April 2004; accepted 12 July 2004
Available online 11 September 2004

Abstract

Host restriction of vaccinia virus has been previously described in CHO and RK13 cells in which a cowpox virus CP77 gene rescues vaccinia virus growth at the viral protein translation level. Here we investigate the restrictive stage of vaccinia virus in HeLa cells using a vaccinia mutant virus (VV-hr) that contains a deletion of 18-kb genome sequences resulting in no growth in HeLa cells. Insertion of CP77 gene into VV-hr generated a recombinant virus (VV-36hr) that multiplied well in HeLa cells. Both viruses could enter cells, initiate viral DNA replication and intermediate gene transcription. However, translation of viral intermediate gene was only detected in cells infected with VV-36hr, indicating that CP77 relieves host restriction at the intermediate gene translation stage in HeLa cells.

Caspase-2 and -3 activation was observed in HeLa cells infected with VV-hr coupled with dramatic morphological alterations and cleavage of the translation initiation factor eIF4G. Caspase activation was reduced in HeLa cells infected with VV-36hr, indicating that CP77 acts upstream of caspase activation. Enhanced phosphorylation of PKR and eIF2α was also observed in cells infected with VV-hr and was suppressed by CP77. Suppression of eIF4G cleavage with the caspase inhibitor ZVAD did not rescue virus translation, whereas expression of a mutant eIF2α protein with an alanine substitution of serine at amino acid position 51 (eIF2αS51A) partially restored viral translation and moderately increased virus growth in HeLa cells.

Keywords: Vaccinia virus; Host restriction; eIF2α phosphorylation; Apoptosis

Introduction

Poxviruses are known to encode diverse viral molecules that modulate host cell responses to suit the viral needs. However, in some cell types, poxviruses fail to complete their cycle, a phenomenon known as host restriction (Drillien et al., 1981, 1978; Fenner and Sambrook, 1966; Gemmell and Fenner, 1960; Hruby et al., 1980; McClain, 1965; Somogyi et al., 1993; Tagaya et al., 1961; Wyatt et al., 1998). Previous studies of mutant viruses have identified viral host range (hr) genes that rescue virus growth in otherwise restrictive cells. Cowpox virus expresses a hr protein, namely CP77 or CHOhr, that is encoded by the V025 gene, allowing virus growth in CHO cells, whereas a V025 orthologue in vaccinia virus is dysfunctional due to multiple mutations (Goebel et al., 1990; Kotwal and Moss, 1988; Perkus et al., 1990; Shchelkunov et al., 1998; Spehner et al., 1988). Vaccinia virus contains two hr genes, K1L and C7L, which allow virus multiplication in RK13 and human cells (Gillard et al., 1986; Perkus et al., 1990; Sutter et al., 1994). The vaccinia E3L gene supports virus growth in HeLa, Vero, and L929 cells (Beattie et al., 1996; Chang et al., 1995). The SPI-1 genes in rabbitpox and vaccinia virus are required for replication in A549, PK-15, and human keratinocytes (Ali et al., 1994; Shisler et al., 1999). In addition to the orthopoxvirus genes named above, myxoma virus, which belongs to the leporipoxvirus genus, also contains host range genes (M-T2,

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doi:10.1016/j.virol.2004.07.032

M-T4, M-T5, and M11L) required for efficient replication in RL-5 cells and rabbit leukocytes (Barry et al., 1997; Macen et al., 1996; Mossman et al., 1996). The multiplication of fowlpox, penguinpox virus, and even vaccinia virus in heterologous host cells is also known to be restricted under circumstances for which no particular hr gene has yet been identified (Li et al., 1998; Somogyi et al., 1993; Stannard et al., 1998). Nevertheless, poxviruses have clearly evolved an array of hr genes to ensure virus growth in a cell-type-specific or species-specific manner.

Up to now, the cellular mechanism of host restriction remains elusive. Although apoptotic suppression is frequently associated with expression of viral hr genes in the restrictive cells, the nature of such an association is largely unknown (for review, see McFadden and Barry, 1998 and the references therein). One of the exceptions is vaccinia E3L, which binds to double-stranded RNA and down-regulates PKR-mediated apoptosis (Davies et al., 1993; Xiang et al., 2002). Another example is vaccinia K1L, which is required for activation of a host-encoded intermediate transcription factor in RK13 cells although the precise interactions involved remain to be elucidated (Sutter et al., 1994). A third example is myxoma M11L, which was recently shown to prevent the release of mitochondrial cytochrome *c* induced by staurosporine (Everett and McFadden, 2001a, 2001b; Everett et al., 2000, 2002).

Host restriction of vaccinia virus in CHO, RK13, and human MRC-5 cells was thought to be regulated by a conserved cellular pathway, because the cowpox CP77 protein is capable of rescuing host restriction imposed by deletion of vaccinia virus C7L and K1L genes (Perkus et al., 1990; Ramsey-Ewing and Moss, 1996). However, these three genes are not functionally equivalent as neither C7L nor K1L can rescue host restriction in the absence of CP77 expression in CHO cells (Spehner et al., 1988). Similarly, C7L does not complement the K1L gene defect in RK13 cells (Perkus et al., 1990). The nature of the functional hierarchy among CP77, K1L, and C7L genes is not known and there is no obvious sequence homology among these three genes (Goebel et al., 1990; Oguiura et al., 1993; Spehner et al., 1988).

Vaccinia virus growth in CHO, RK13, and human cells has been previously investigated. CHO cells were restrictive for viral intermediate gene translation (Ramsey-Ewing and Moss, 1998). Concurrently, significant DNA laddering and apoptotic bodies were induced in restrictive CHO cells (Bair et al., 1996; Ink et al., 1995). In these cells, expression of viral CP77hr gene partially suppressed apoptosis; however, this may simply reflect an indirect influence of the hr gene on the cellular response, as expression of anti-apoptotic molecules alone, such as E1B and bcl-2, did not rescue virus growth (Chung et al., 1997; Ink et al., 1995). RK13 cells, however, restrict a vaccinia K1L-mutant virus at the early gene translation stage and only minor apoptotic features have been detected in these cells (Chung et al., 1997; Ramsey-Ewing and Moss, 1995, 1996, 1998). Moreover, apoptotic modulation by bcl-2 in RK13 cells did not interfere with virus growth (Chung et al., 1997). In human KB and Hep2 cells, a vaccinia hr mutant was restricted at an early stage of infection before viral DNA replication (Drillien et al., 1981). However, apoptotic events, if triggered, were not investigated in these human cells (Drillien et al., 1981; Gillard et al., 1985, 1986).

In summary, prior studies of host restriction suggested that hr genes possess anti-apoptotic activity that antagonizes host restriction, whereas other studies have implied that apoptosis is the ultimate cellular stress response associated with host restriction. In this study, we used a mutant virus VV-hr to identify its restrictive stage in human HeLa cells. VV-hr, has an 18-kbp deletion of the viral genome that overlaps two hr genes, K1L and C7L, known to be essential for VV multiplication in various human cell lines (Drillien et al., 1981). We also overexpressed CP77 in VV-hr to study the role of CP77 gene in host range regulation. Furthermore, we investigated cellular proteins that are affected by virus infection in HeLa cells to identify cellular molecules that mediate vaccinia virus host restriction.

Results

Without CP77, growth of VV-hr is blocked at the stage of viral intermediate gene translation in HeLa cells

The restrictive stage of VV-hr in HeLa cells was studied as compared to a recombinant virus, VV-36hr, in which the cowpox virus CP77 gene was inserted into the tk locus of VV-hr. HeLa cells were infected with VV-hr or VV-36hr at a MOI of 5 PFU per cell and the cell lysates were harvested for virus titrations. As shown in Fig. 1A, no increase in titer of VV-hr was detected in the one-step growth analysis, whereas the titer of VV-36hr increased up to 100-fold. Thus, as expected, growth of VV-hr was restricted in HeLa cells and expression of CP77 from VV-36hr rescued host restriction.

To determine whether viral proteins are synthesized in HeLa cells infected with VV-hr, we infected HeLa cells with VV-hr and VV-36hr and monitored viral protein synthesis with ^{35}S-methionine labeling at different times post infection (p.i.) (Fig. 1B). In HeLa cells infected with VV-hr, host protein synthesis was completely inhibited within 4–6 h p.i., with only early transient detection of several viral proteins at 1–3 h p.i. but not at 4–8 h p.i. In cells infected with VV-36hr, cellular protein synthesis was also shut off; however, abundant viral protein synthesis was detected and continued into the late stage at 8 h p.i. Therefore, host restriction of VV-hr resulted in a defect in late viral protein synthesis in the infected cells.

We next investigated whether the lack of late viral protein synthesis in VV-hr was due to an indirect consequence of an earlier blockage of viral DNA replication because gene expression in poxviruses is regulated in a cascade manner. HeLa cells were infected with VV-hr

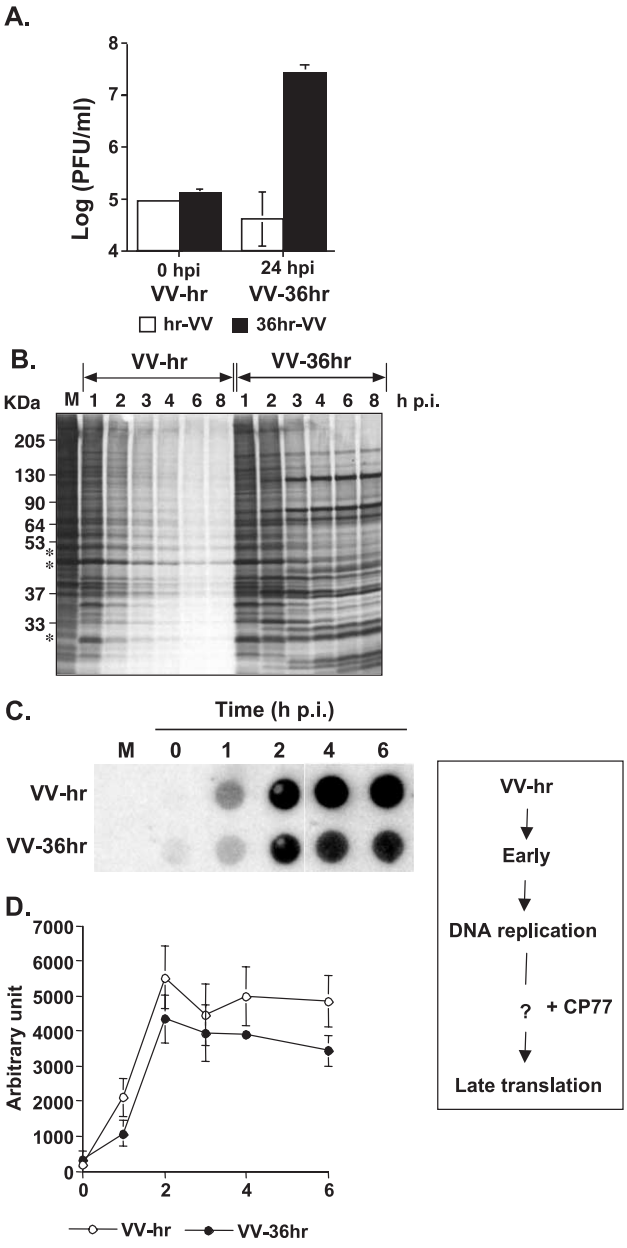

Fig. 1. Host restriction of VV-hr and VV-36hr in HeLa cells. (A) Virus growth analysis of VV-hr and VV-36hr in HeLa cells. HeLa cells were infected with VV-hr or VV-36hr at a MOI of 5 PFU per cell and cell lysates were harvested at 0 and 24 h p.i. as indicated at the bottom of the figure. The experiments were repeated three times and virus titers in the cell lysates were determined by plaque assays on BHK21 cells as described in Materials and methods. (B) ^{35}S-methionine labeling experiments for viral protein synthesis. HeLa cells were infected as described in A and pulsed for 60 min in ^{35}S-methionine-containing medium at various times as indicated. Cells were then harvested immediately and analyzed on 10% SDS-PAGE. The asterisks * mark the diagnostic cellular protein bands for monitoring protein shut-off. (C). Dot blot analysis of viral DNA synthesis in the infected cells. HeLa cells were infected as described in A and harvested at different times p.i. Viral DNA was extracted and aliquots were applied to nitrocellulose filters and hybridized with a ^{32}P-labeled VGF DNA fragment, washed, and autoradiographed. A typical blot is shown here. (D) Quantitation analysis of viral DNA synthesis in the infected cells. The nitrocellulose filter as shown in C was scanned with a PhosphoImager and quantitated by computer program analysis as described by the manufacturer (Molecular Dynamics). The experiments were performed twice, and the resulting blots were scanned, quantitated, and averaged with a phosphoimager (Molecular Dynamics, Inc.). A summary of results shown here is illustrated in the box at the right hand of panel D.

or VV-36hr and harvested for viral DNA isolation at different times p.i. The amounts of viral DNA were monitored by dot blot analyses, as shown in Fig. 1C. Significant increases in viral DNA were detected in cells infected with VV-hr and VV-36hr as early as 1 h p.i., indicating that active viral DNA replication had been initiated at this time. Quantification of the DNA-impregnated filters revealed that the kinetics of VV-hr DNA synthesis is similar to that of VV-36hr (Fig. 1D). As viral DNA replication occurred in HeLa cells infected with VV-hr and VV-36hr, host restriction of VV-hr must have occurred after this step.

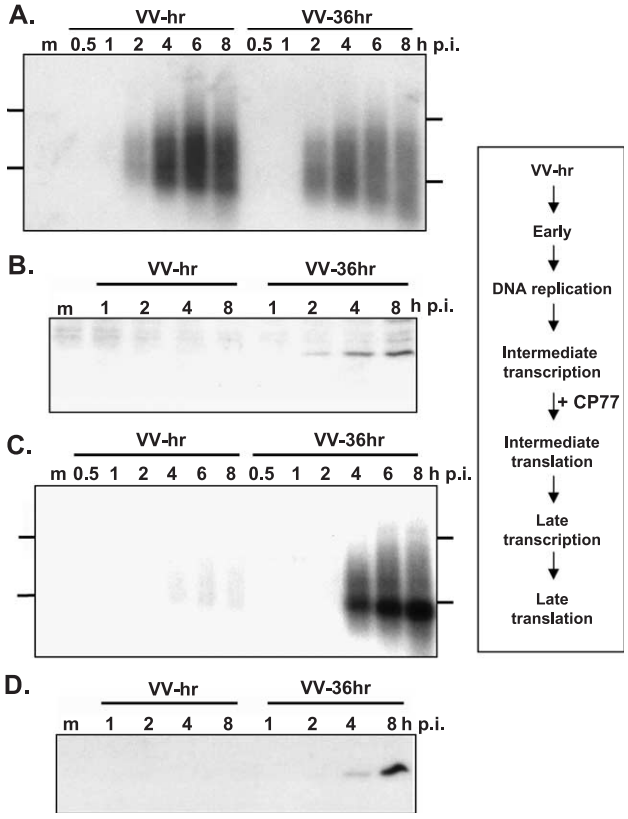

Fig. 2. CP77 regulates translation of the intermediate G8R gene in HeLa cells. (A) Expression of intermediate G8R RNA in the infected cells. HeLa cells were infected as described in Fig. 1, and total RNA was isolated at various times for Northern blot analyses with a ^{32}P-labeled G8R DNA fragment. The blot was washed and autoradiographed. The two black marks next to the blot indicate the positions of 28 S and 18 S rRNA, respectively. (B) Expression of intermediate G8R protein expressed in the infected cells. HeLa cells were infected and cell lysates were harvested at various times, separated on SDS-PAGE, and transferred for immunoblot analysis with an anti-G8R antibody. (C) Expression of late viral F17R RNA in the infected cells. The blot in A was stripped and rehybridized with a ^{32}P-labeled F17R DNA fragment, washed again, and autoradiographed. (D) Expression of late F17R protein in the infected cells. The cell lysates used in B were also used for immunoblot analysis with an Ab recognizing F17R protein. The summary of results shown in Fig. 2 is illustrated in the box on the right hand.

Viral DNA replication is required to activate intermediate gene expression from the viral genome (Keck et al., 1990; Vos and Stunnenberg, 1988). We therefore monitored transcription of the intermediate viral gene, G8L, in cells infected with VV-hr and VV-36hr to determine whether host restriction of VV-hr occurs at this stage. Northern blot analyses were performed with total RNA isolated from HeLa cells infected with VV-hr or VV-36hr and hybridized with a ^{32}P-labeled DNA probe derived from G8R gene sequences (Fig. 2A). The size of G8R transcripts is heterogeneous, ranging from 0.8 to 4.5 kb, due to the lack of a specific transcription termination signal at the 3′ end (Baldick and Moss, 1993; Wright et al., 1991). G8R RNA transcripts were detected in cells infected by both VV-hr and VV-36hr as early as 2 h p.i., revealing that there was no blockage of viral intermediate gene transcription in VV-hr.

To determine if intermediate G8R RNA is translated into protein, cell lysates were prepared from HeLa cells infected with VV-hr or VV-36hr for immunoblot analyses with an antiserum recognizing the G8R protein (Fig. 2B). In contrast to the Northern blot analysis, the G8R protein was detected in cells infected with VV-36hr but not with VV-hr at 2–8 h p.i. These results demonstrated that translation of the viral intermediate gene, G8R, was blocked in HeLa cells infected with VV-hr.

Because intermediate proteins such as A1L, A2L, and G8R serve as transcription factors for late viral genes, it is expected that the lack of the G8R protein in cells infected with VV-hr would not allow transcription or translation of late viral genes to proceed (Keck et al., 1993; Passarelli et al., 1996; Wright et al., 1991). The transcription of a viral late F17R gene, which encodes a major late transcript of 1.5 kb in size, was monitored in cells infected with VV-hr or VV-36hr. Northern blots only detected the F17R transcript in cells infected with VV-36hr but not with VV-hr (Fig. 2C). Consequently, the F17R protein was detected in cells infected with VV-36hr but not in cells infected with VV-hr (Fig. 2D).

In summary, the above results revealed that host cell restriction of VV-hr virus growth in HeLa cells occurs at the stage of intermediate gene translation and that the CP77 gene rescues the viral translation defect.

Apoptosis occurred in HeLa cells infected with VV-hr after viral DNA replication and was delayed/suppressed by CP77 expression

HeLa cells infected with VV-hr or VV-36hr developed different morphological changes (Fig. 3). VV-36hr infection resulted in cell rounding and slow detachment (Fig. 3C); however, VV-hr infection induced dramatic morphological shrinkage of the infected cells (Fig. 3B). Membrane blebbing and cell shrinkage have been described as indicative of cytoskeleton rearrangement due to apoptosis, implying that apoptosis of HeLa cells occurred after VV-hr infection (Coleman et al., 2001; Mills et al., 1998; Sebbagh et al., 2001). Cell rounding, in contrast, is a typical necrotic response commonly described in the productive infection of vaccinia virus. To determine how far the virus life cycle needs to proceed before such distinct apoptotic morphology was triggered, we treated HeLa cells during virus infection with cordecepin (CP), cycloheximide (CHX), and cytosine arabinoside (araC) to block early viral transcription, early translation, and DNA replication, respectively (Person and Beaud, 1978). All three chemicals inhibited cellular shrinkage and reversed cells to an attached phenotype, indicating that viral DNA replication is necessary to trigger shrinkage of HeLa cells infected with VV-hr (Fig. 3D,F,H). All three chemicals also inhibited cell rounding in cells infected with VV-36hr (Fig. 3E,G,I). Cell shrinkage was inhibited when VV-hr-infected cells were incubated with 50

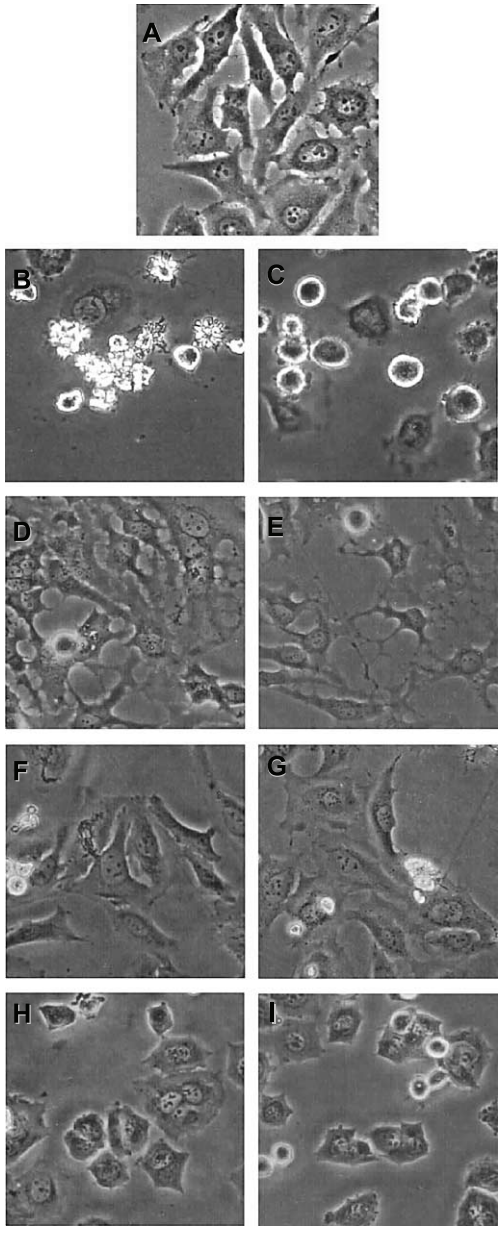

Fig. 3. The apoptotic morphology of HeLa cells infected with VV-hr depends on continuation of the viral life cycle beyond DNA replication. HeLa cells were mock-infected (A) or infected with VV-hr (B, D, F, and H) or VV-36hr (C, E, G, and I) in the absence (B and C) or in the presence of CP (40 μg/ml) (D, E), CHX (100 μg/ml) (F, G) or araC (40 μg/ml) (H, I) and photographed at 8 h p.i.

μM ZVAD, indicating that caspase activation is involved in cell shrinkage (data not shown).

To demonstrate that apoptosis was indeed triggered in HeLa cells infected with VV-hr, we initially isolated cellular genomic DNA from cells infected with VV-hr or VV-36hr to examine whether nuclear DNA is degraded in these cells. However, we did not detect DNA laddering by agarose gel analysis at 8 h p.i. (data not shown). As DNA fragmentation represents a late apoptotic event, we directly measured caspase activity in cell lysates. Increased activities of caspase-2 and -3, but not caspase -1, -8, and

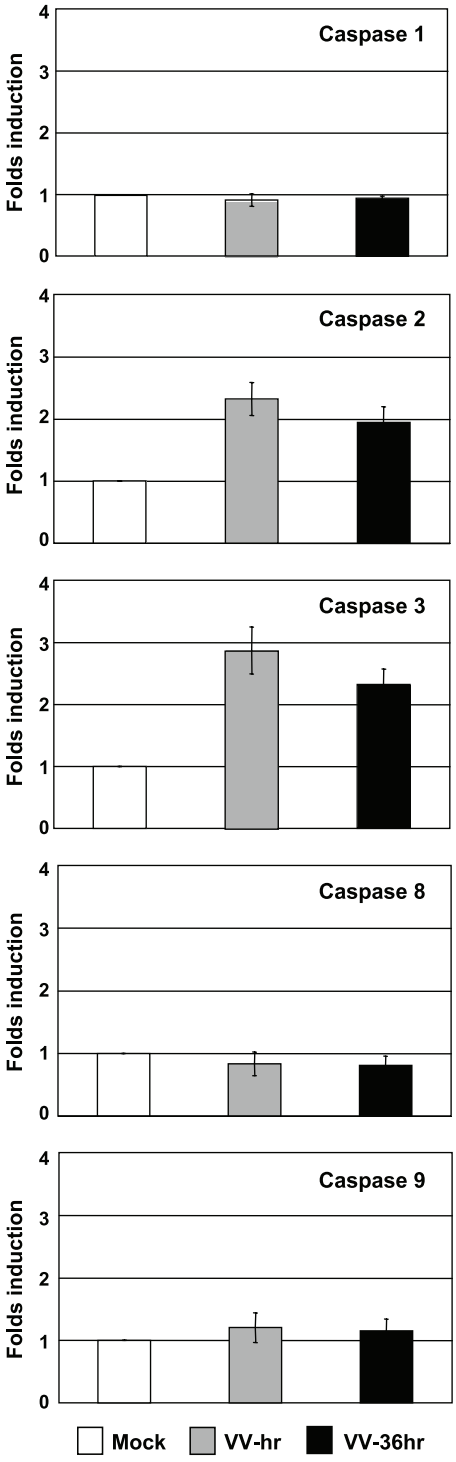

Fig. 4. Caspase activation in HeLa cells infected with VV-hr and VV-36hr. HeLa cells were infected with VV-hr or VV-36hr at a MOI of 5 PFU per cell. Cell lysates were harvested at 8 h p.i. and used in caspase activity assays with fluorogenic substrates for caspase -1, -2, -3, -8, and -9, as described in Materials and methods. The experiments were repeated three times. Fluorescence intensities representing activation of caspases were measured with a spectrofluorometer, using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The folds induction of caspase activity shown in the *Y*-axis represents the ratio of caspase activity detected in cells infected with VV-hr or VV-36hr to the activity detected in the mock cells.

-9, were detected in HeLa cells infected with VV-hr at 8 h p.i. (Fig. 4). Actually, we could detect caspase-2 and -3 activities as early as 4 h p.i. but the activities accumulated higher at 8 h p.i. (data not shown). Both caspase activities were reduced in HeLa cells infected with VV-36hr, indicating that CP77 delayed or suppressed caspase activation (Fig. 4).

Caspase-dependent cleavage of eIF4G and increased phosphorylation of eIF2α and PKR were observed in HeLa cells infected with VV-hr

The above results altogether indicated that shutdown of host protein synthesis occurred in HeLa cells infected with VV-hr and VV-36hr whereas blockage of viral translation only occurred in apoptotic cells infected with VV-hr. We thus investigated if apoptosis has a direct impact on translation factors.

In mammalian cells, translation initiation requires multiple translation initiation factors (eIFs). For example, eIF2α forms a ternary complex with GTP and initiator methionyl-tRNAi (met-tRNAi) and this complex binds to the 40 S ribosomal subunit to form the 43 S preinitiation complex. mRNA binds to the 43 S complex via an eIF4F complex, comprising eIF4A, eIF4E, and eIF4G, to form the 48 S preinitiation complex that initiates translation. Previous studies have shown that the structures of eIF4G, eIF4E, and eIF2α are frequently altered in apoptotic cells (Gray and Wickens, 1998; Marissen and Lloyd, 1998; Polunovsky et al., 2000; Saelens et al., 2001). Besides, viruses are known to modify eIF4G, eIF4E, and eIF2α to facilitate viral translation in virus-infected cells (Feduchi et al., 1995; Gale et al., 2000).

HeLa cells were infected with VV-hr and VV-36hr and cell lysates were harvested at different times for immunoblot analysis with antibodies that recognized eIF4G. As shown in Fig. 5A, VV-hr infection of HeLa cells triggered significant loss of intact eIF4G of 220-kDa size with the concomitant appearance of a truncated eIF4G fragment of 100-kDa size at 6–12 h p.i. Cleavage of eIF4G into the 100-

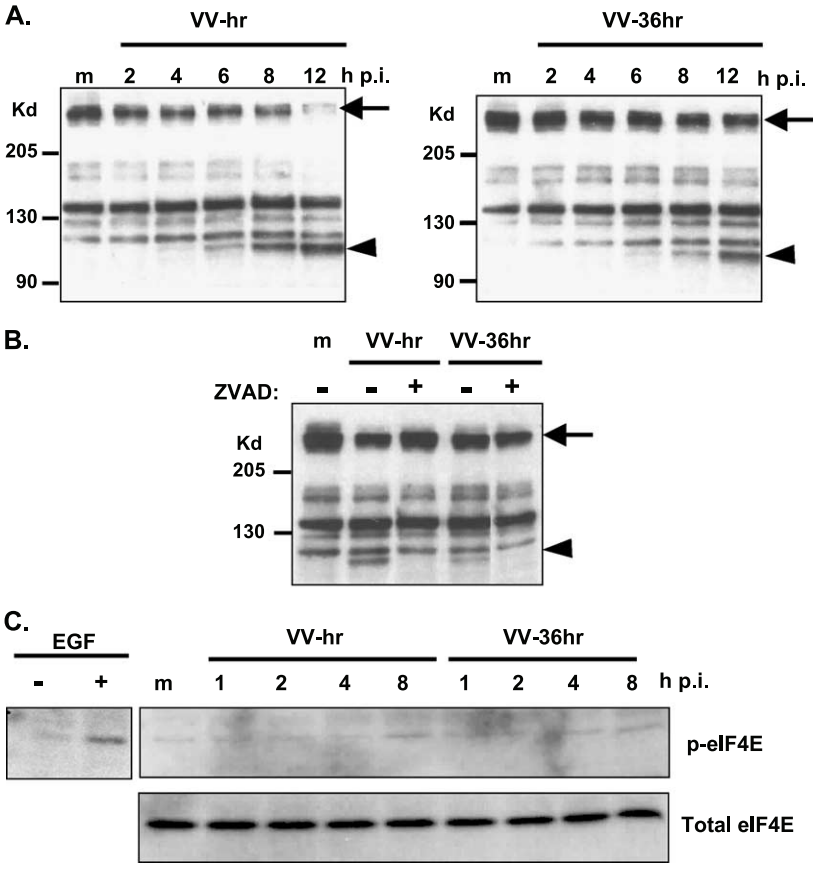

Fig. 5. (A) Cleavage of eIF4G in HeLa cells infected with VV-hr and VV-36hr. HeLa cells were infected with VV-hr and VV-36hr and harvested at various times as indicated at the top of the figure. Lysates were separated on a 7% SDS-PAGE and probed with an antibody that recognized eIF4G (1:1000). The arrows indicate the positions of the intact eIF4G and the arrowheads indicate the 100-kDa cleaved fragments of eIF4G. (B) Caspase-dependent cleavage of eIF4G in the infected cells. HeLa cells were infected with VV-hr or VV-36hr with or without ZVAD (50 μM) and harvested at 10 h p.i. for immunoblot analysis as in (A). When ZVAD (50 μM) was used, it was added to cells 1 h before infection and remained throughout the period of the experiments. (C) HeLa cells were infected with VV-hr and VV-36hr and harvested at various times for immunoblot analysis with Abs recognizing either the phosphorylated form of eIF4E (p-eIF4E) or total eIF4E. As a positive control for p-eIF4E-specific Abs, HeLa cells were serum-starved overnight, treated with hEGF at 100 ng/ml for 30 min, and harvested for immunoblot analysis.

kDa fragment was also observed in HeLa cells infected with VV-36hr with slightly more full-length forms of eIF4G detected at 12 h p.i. Addition of a caspase inhibitor ZVAD blocked generation of truncated eIF4G, confirming that cleavage of eIF4G was caspase-dependent (Fig. 5B). The difference in eIF4G cleavage between VV-hr and VV-36hr was moderate and thus unlikely to be the cause of viral translational restriction.

Another component subject to regulation in response to cellular stress is eIF4E (Gale et al., 2000; Topisirovic et al., 2003). Phosphorylation of eIF-4E at Ser209 has been observed in serum-treated cells to correlate with activation of cellular protein synthesis (Flynn and Proud, 1995). We therefore analyzed the phosphorylation pattern of eIF4E Ser209 in cell lysates at different times p.i. No difference was detected regarding either the phosphorylation status or the total amount of eIF4E among mock-infected, VV-hr-infected, or VV-36hr-infected cells (Fig. 5C).

Finally, we investigated the role of eIF2α in the virus-infected HeLa cells, because the level of phosphorylated eIF2α is often regarded as an integrated stress sensor for the arrest of translation initiation in virus-infected cells (Clemens, 2001; Gale et al., 2000; Kawagishi-Kobayashi et al., 1997; Yan et al., 2002). Significant phosphorylation of eIF2α was detected in HeLa cells infected with VV-hr at 4 and 8 h p.i. (Fig. 6A). Phosphorylation of eIF2α was detected in HeLa cells infected with VV-36hr, but at a reduced level compared to VV-hr. This observation is interesting and implies a possibility of phosphorylation of eIF2α in regulation of viral translation in HeLa cells.

As phosphorylation of eIF2-α by PKR is known to be a major stress response during viral infections, we investigated whether PKR was indeed activated in these infected cells (Fig. 6B) (Gale et al., 1996; Kimball, 1999; Knipe and Howley, 2001; Saelens et al., 2001). Phosphorylation of PKR results in slower migration of PKR protein on SDS-PAGE, i.e., the upper band, whereas unphosphorylated PKR runs slightly faster on gel, i.e., the lower band. Anti-total PKR Ab recognizes both forms so detects PKR as a doublet on SDS-PAGE (in Fig. 6B, bottom panel). Anti-phospho-PKR Ab mainly recognizes the phosphorylated form (upper band) of PKR with minor cross-reactivity to the unphosphorylated form (lower band) (in Fig. 6B, top panel). Mock-infected HeLa cells contained mostly unphosphorylated PKR, as revealed by the predominant lower band in immunoblot analyses with an anti-total PKR Ab (lower panel in Fig. 6B). Phosphorylation of PKR was readily detected in cells infected with VV-hr, concomitant with an increased phosphorylation of eIF2α (compare the upper panels in Fig. 6B with 6A). In HeLa cells infected with VV-36hr, a reduced level of phosphorylated PKR was detected, consistent with less phosphorylation of eIF2α.

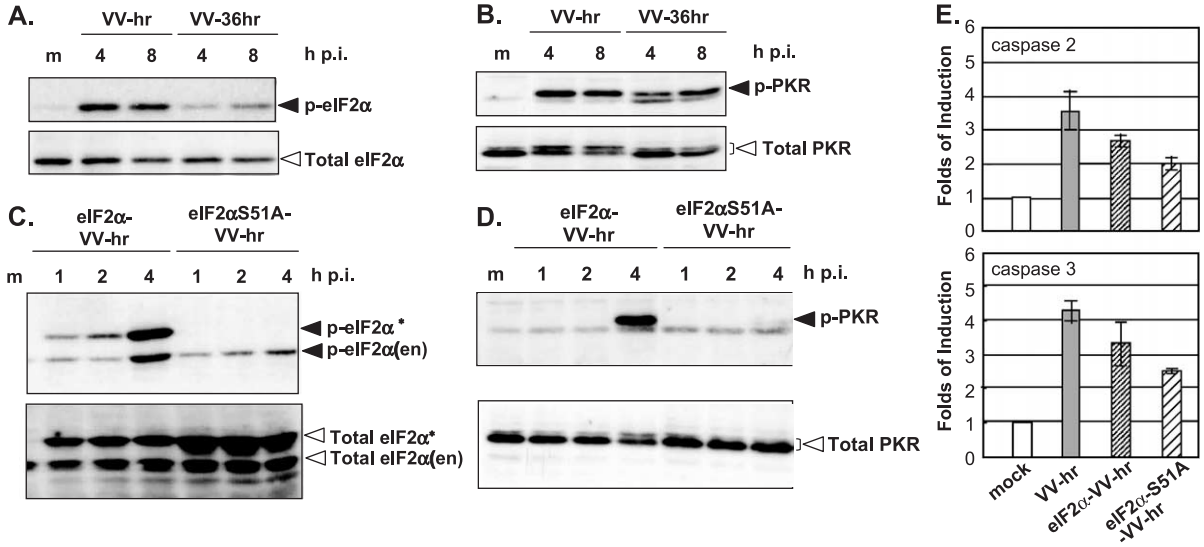

Fig. 6. Investigation of phosphorylation of eIF2α and PKR in HeLa cells infected with various viruses. (A) Increased phosphorylation of eIF2α in HeLa cells infected with VV-hr. HeLa cells were mock-infected (m) or infected with VV-hr or VV-36hr and harvested at various times for immunoblot analyses with Abs recognizing phospho-eIF2α (filled arrow head) or total eIF2α (open arrowhead). (B) Increased phosphorylation of PKR in HeLa cells infected with VV-hr. HeLa cells were infected as described in A and harvested for immunoblot analyses with Abs recognizing phospho-PKR (the upper band, marked by a filed arrowhead) or total PKR (appears as doublets on gels, marked by an open arrowhead). (C) Expression of eIF2αS51A suppresses phosphorylation of endogenous eIF2α in Hela cells infected with VV-hr. HeLa cells were infected with eIF2α-VV or eIF2αS51A-VV-hr and harvested at various times for immunoblot analyses with Abs recognizing phospho-eIF2α (top panel) or total eIF2α (bottom panel). The asterisks (*) indicate the exogenous eIF2α expressed from eIF2α-VV or eIF2αS51A-VV-hr that contain a myc tag and, consequently, migrated slower than the endogenous (en) proteins. (D) Expression of eIF2αS51A suppresses phosphorylation of endogenous PKR in Hela cells infected with VV-hr. HeLa cells were infected as described in C, and analyzed with Abs recognizing phospho-PKR (filled triangle) or total PKR (open triangle) as in B. (E) Caspase-2 and -3 activation in HeLa cells infected with eIF2α-VV or eIF2αS51A-VV-hr. HeLa cells were infected with various viruses at a MOI of 5 PFU per cell. Cell lysates were harvested at 8 h p.i. and used in caspase activity assays with fluorogenic substrates for caspase-2 and -3 as described in legend of Fig. 4. The experiments were repeated three times.

Inhibition of caspase-dependent cleavage of eIF4G had no effect on host restriction whereas inhibition of eIF2α phosphorylation partially rescued viral gene translation

If phosphorylation of eIF2α or less likely caspase-dependent cleavage of eIF4G is the cause of host restriction, reversal of one of these events should mimic the function of the CP77 gene and rescue virus growth. To examine the role of eIF4G on viral translation, cells were infected with VV-hr and incubated with ZVAD to block eIF4G cleavage; however, no translation of G8R protein could be detected and no virus progeny was produced (data not shown). To examine the role of phosphorylation of eIF2α in host restriction, we constructed recombinant viruses derived from VV-hr expressing either wild-type eIF2α or a mutant eIF2α containing single serine to alanine mutation at amino acid position 51 (S51A), which interferes with phosphorylation of eIF2α (Kramer, 1990; Murtha-Riel et al., 1993; Price et al., 1991). Both recombinant viruses expressed abundant levels of eIF2α protein that migrated slower than the endogenous protein, due the presence of a myc tag at the C-terminus (Fig. 6C) (Saelens et al., 2001). Furthermore, expression of the eIF2αS51A mutant, but not wild-type eIF2α, suppressed phosphorylation of endogenous eIF2α in the infected cells. Finally, we also found that expression of the eIF2αS51A mutant, but not wild-type eIF2α suppressed phosphorylation of endogenous PKR in the infected cells at 4 h p.i. (Fig. 6D). This result was unexpected and suggested a feedback regulation of eIF2α phosphorylation on PKR activation.

To determine whether expression of eIF2αS51A suppresses apoptosis, we measured caspase activation in HeLa cells infected with VV-hr, eIF2αVV-hr, or eIF2αS51A-VV-hr at 8 h p.i. (Fig. 6E). Consistent with Fig. 4, VV-hr infection induced 3–4-fold increase of caspase-2 and -3 activities and expression of WT-eIF2α or eIF2αS51A reduced caspase activities to a similar level with VV-36hr (shown in Fig. 4). Therefore, modulation of eIF2α and its phosphorylation status could influence cellular apoptotic response in HeLa cells infected with VV-hr.

Because eIF2αVV-hr and eIF2αS51A-VV-hr suppressed caspase activation, we then investigated whether viral translation is resumed in these cells. HeLa cells were infected with VV-hr, VV-36hr, eIF2αVV-hr, or eIF2αS51A-VV-hr and synthesis of the viral intermediate G8R protein was monitored by immunoprecipitation of ^{35}S-methionine-labeled cell lysates with an Ab recognizing a synthetic G8R peptide (Fig. 7A). Cells infected with VV-36hr expressed the intermediate G8R protein that was not detected in cells infected with VV-hr. Overexpression of wild-type eIF2α did not induce G8R protein synthesis. In contrast, in cells infected with eIF2αS51A-VV-hr, synthesis of the G8R protein became detectable, suggesting that phosphorylation of eIF2α contributes to suppression of viral intermediate gene translation in HeLa cells infected with VV-hr. In another experimental approach, inhibition of eIF4G cleavage was combined with suppression of phosphorylation of

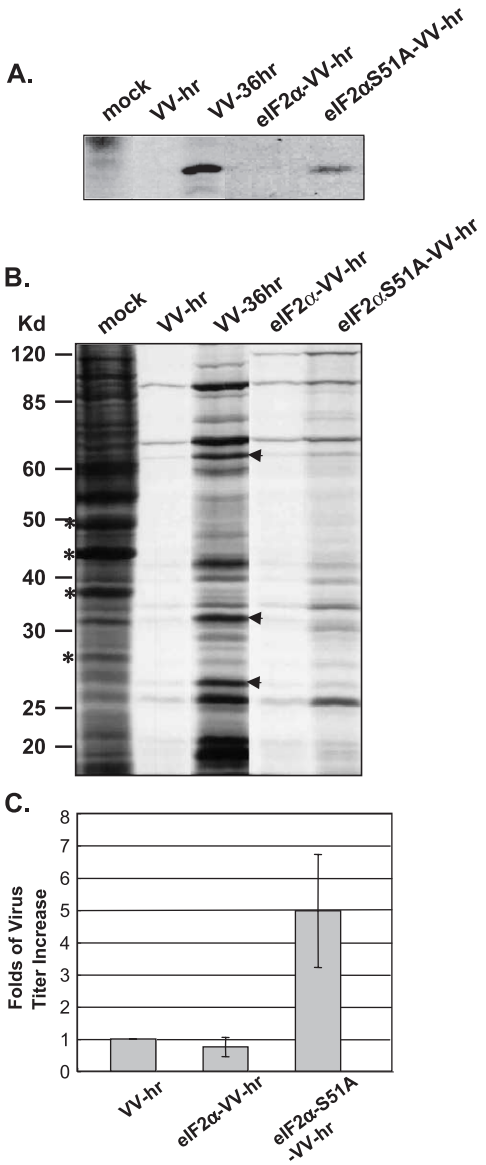

Fig. 7. eIF2αS51A-VV-hr partially rescues viral translation with moderate effect on progeny production. (A) Immunoprecipitation of viral intermediate G8R protein from HeLa cells infected with VV-hr, VV36hr, VV-eIF2α-VV-hr, or VV-eIF2αS51A-VV-hr. HeLa cells were infected with each virus and viral proteins were labeled with ^{35}S-methionine at 4–8 h p.i.. Cell lysates were harvested and immunoprecipitation was performed with an anti-G8R Ab (1:100) and analyzed on a 10% SDS-PAGE. (B) Radioactive labeling for viral late protein synthesis in virus-infected HeLa cells. HeLa cells were either mock-infected (m) or infected with VV-hr, VV-36hr, eIF2α-VV, or eIF2αS51A-VV-hr. At 10 h p.i., infected cells were starved for 30 min in methionine-free medium, labeled with ^{35}S-methionine for 1 h, and harvested for analysis on a 10% SDS-PAGE. Asterisks * mark the diagnostic cellular protein bands. Black arrowheads indicate viral late proteins that are present in cells infected with VV-36hr but not in eIF2αS51A-VV-hr. (C) Virus growth on HeLa cells. HeLa cells were infected with each virus at a MOI of 1 PFU per cell and cells were harvested at 48 h p.i. Virus titers in the cell lysates were determined by plaque formation assays on BHK21 cells. The data were obtained from three independent and the *Y*-axis represents the fold increase in virus production after normalization as described in Materials and methods.

eIF2α by incubating ZVAD with HeLa cells infected with eIF2αS51A-VV. Addition of ZVAD had no additive effect on viral G8R protein synthesis in cells infected with VV-hr-eIF2αS51A-VV (data not shown).

Synthesis of viral late proteins was also examined in HeLa cells infected with VV-hr, VV-36hr, eIF2αVV-hr, or eIF2αS51A-VV by ^{35}S-methionine labeling at 8 h p.i. (Fig. 7B). As expected, abundant late viral protein synthesis was detected in cells infected with VV-36hr but not with VV-hr. Moreover, increased late viral protein synthesis was also detected in cells infected with eIF2αS51A-VV-hr, consistent with the increased level of G8R protein observed in Fig. 7A. It is worth noting that some of the strong viral late proteins remained missing in the presence of eIF2αS51A (arrowheads in Fig. 7B), indicating that phosphorylation of eIF2α may only regulate translation of some but not all viral late protein synthesis in cells.

Finally, we tested whether partial recovery of viral translation in HeLa cells infected with eIF2αS51A-VV-hr could lead to increased production of infectious virions. HeLa cells were infected with each virus at a MOI of 1 PFU per cell and virus multiplication was monitored at 48 p.i. (Fig. 7C). VV-hr did not grow and was used as the negative control. EIF2α-VV-hr also did not grow within 48 h p.i. VV-36hr, on the other hand, grew more than 100-fold (data not shown). Virus titers of eIF2αS51A-VV-hr only showed a moderate increase of 5-fold at 48 h p.i., indicating that a full relief of viral translation restriction in restrictive HeLa cells requires participation of additional cellular factors that are targeted by CP77.

Discussion

In this paper, we have demonstrated that a host range mutant virus, VV-hr, could transcribe, but not translate, viral intermediate genes in HeLa cells. Expression of the cowpox virus CP77 gene from a recombinant VV-36hr suppressed apoptosis and rescued virus translation. Upon infection with VV-hr, HeLa cells display morphological and biochemical features of apoptosis. Furthermore, we found that VV-hr triggered PKR activation and phosphorylation of eIF2α and that CP77 expression suppressed both events. Inhibition of eIF2α phosphorylation reduced both PKR activation and caspase activities prompted us to test out our hypothesis that whether phosphorylation of eIF2α acts as the sensor not only for cellular stress but also for viral translation in cells. Our results with eIF2αS51A mutant showed that viral translation blockage in VV-hr could be partially relieved when phosphorylation of eIF2α in cells was suppressed, indicating a role of eIF2α in viral translation. However, the status of eIF2α may only be important for a subset of viral genes and is not sufficient to rescue viral infectivity from restrictive HeLa cells. Therefore, we rationalize that CP77 must act on upstream of eIF2α, caspase, and PKR activation, to justify its influence on additional molecules involving viral translation and host restriction. Our study is limited in HeLa cells and whether our results may be of use for CHO and RK13 cells remains to be determined.

Identification of caspase-2 and capspase-3 activation in HeLa cells after viral DNA replication raised the possibility that caspases could cleave important cellular or viral factors necessary for viral translation. Currently, there have been no reports of viral proteins acting as caspase substrates except for crmA, which is a direct inhibitor of caspases (Gagliardini et al., 1994; Komiyama et al., 1994; Zhou et al., 1997). In contrast, caspase-dependent cleavage of eIF4G was found in cells infected with VV-hr and VV-36hr. However, such cleavage is unlikely to be the cause of the viral translation defect because the difference was small between VV-hr and VV-36hr as well as inhibition of caspase-dependent cleavage of eIF4G did not restore viral translation. Therefore, we did not find any data arguing an indispensable role of intact eIF4G in viral translation, a conclusion consistent with that proposed by Mulder et al. (1998). Could other caspase substrates be important for host restriction? It is difficult to assess whether cleavage of any specific substrate contributes to host restriction. However, our data with pan-caspase inhibitors, such as ZVAD and Boc-D, suggested that inhibition of caspase activity in HeLa cells did not relieve viral translation (data not shown). Besides, expression of E1B19K has been shown to block FADD-mediated caspase 8 activation, but fails to rescue viral growth in CHO cells (Ink et al., 1995). Likewise, expression of bcl-2, known to block mitochondria-mediated caspase-9 activation, did not relieve host restriction in CHO cells (Chung et al., 1997). It is thus unlikely that activation of caspase cascades per se could explain host restriction but we could not completely exclude this possibility.

We were surprised to detect PKR activation in VV-hr infected HeLa cells because VV-hr was derived from the Copenhagen strain of vaccinia virus and, consequently, should contain E3L and K3L genes, both of which are known to antagonize PKR functions and down-regulate eIF2α phosphorylation (Beattie et al., 1995a, 1995b). We have cloned and sequenced the endogenous E3L and K3L genes from VV-hr and VV-36hr. The sequences proved identical to the ORF of the published WR strain except for two changes at aa 74 and 75, from EA to KP, which are the natural Copenhagen sequences (Goebel et al., 1990). It therefore appeared that these genes are intact in VV-hr and VV-36hr viral genomes and the changes do not account for PKR activation in the infected cells. Another mechanism for PKR activation has been described in apoptotic cells in which caspase-dependent cleavage of PKR at residue D251 leads to phosphorylation of eIF2α and translation inhibition (Saelens et al., 2001). Although it is an attractive hypothesis to explain PKR activation, we found no evidence for such a cleavage form of PKR present in the VV-hr infected HeLa cells. A third possibility could be the deregulation of

phosphatases that normally promote dephosphorylation of PKR and eIF2α (Brush et al., 2003) and this issue will be investigated in the future.

Translation of vaccinia virus genes appears complicated due to several issues: First, vaccinia virus shuts off host protein synthesis in the infected cells. In some cases, shut off is mediated through a virion-associated protein but in other cases viral early gene expression is required (Drillien et al., 1978; Moss, 1968; Person and Beaud, 1978). Although inhibition of the formation of 40 S-Met-tRNA^{Met} initiation complex and disintegration of polyribosomes in vaccinia virus-infected cells have been described, up to now, a detailed mechanism for host protein shut off is not established (Damaso and Moussatche, 1992; Jefferts and Holowczak, 1971; Person et al., 1980). Unlike picornaviruses, which transcribe RNA containing internal ribosome entry site (IRES) elements to trap ribosomes for translation initiation, vaccinia virus RNA does not contain any elaborate stem-and-loop RNA structure and little is known about the *cis*-acting viral RNA elements that are important for viral translation in cells. It is also not known whether translation complexes for viral early, intermediate and late genes are identical or not. There are two modes that CP77 may use to rescue viral translation in cells. CP77 could either function as a component of a viral translation complex in these cells. Alternatively, CP77 may regulate upstream signals to influence translation indirectly. We have tentatively assigned CP77 as being upstream of caspase and PKR activation since our data support such a regulatory role for CP77.

From our results, we also expect that there should be additional cellular targets that are regulated by CP77 to relieve translational restriction in these cells. A number of reports suggest that during rapid apoptosis, shutdown of protein synthesis occurs at multiple levels (Marissen et al., 2000; Nadano and Sato, 2000; Polunovsky et al., 2000). Future studies may reveal to us that control of translation plays a major role in the cell death processes, at the same time providing cells with an antiviral strategy. Identification of additional cellular targets regulated by CP77 will be important for our understanding of poxvirus host restriction.

Materials and methods

Cell cultures, viruses, and reagents

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). BSC40 cells cultured in DMEM supplemented with 10% calf serum and BHK21 cells were cultured in RPMI with 10% FBS. Cordecepin (CP), cycloheximide (CHX), and cytosine arabinoside (araC) were purchased from Sigma Inc. Fluorogenic substrates for caspase-1, -2, -3, -8, and -9 were purchased from AnaSpec Inc. VV-Isolation of VV-hr has been described previously (Drillien et al., 1981). VV-hr has an 18-kbp deletion of the viral genome that overlaps two genes designated K1L and C7L, known to be essential for VV multiplication in various human cell lines (Gillard et al., 1986; Goebel et al., 1990; Oguiura et al., 1993). VV-36hr was obtained from Dr. R. Drillien and constructed from VV-hr through an intermediate construct, VV-hr ts38, by insertion into its thymidine kinase locus of a cowpox virus gene, designated CP77, which enables virus multiplication in CHO cells. One recombinant isolate designated VV-36hr was plaque-purified several times and used for further studies as described (Drillien et al., 1982; Spehner et al., 1988). Antibodies against PKR and eIF2α were purchased from Santa Cruz, Inc. Antibodies against the phospho-PKR (Thr 446) and phospho-eIF2α (Ser 51) were from Cell Signaling and Stressgen Inc., respectively. Antibodies against F17R protein were a gift from Dr. Jacomine Krise-Locker. Rabbit anti-G8R antiserum was generated from a synthetic peptide "QKKSYVFNFHKYEEK" derived from G8R sequences (Bravo Inc., Taiwan).

Generation of eIF2α-VV-hr and S51A-eIF2α-VV-hr

Plasmids containing human wt-eIF2α (pEF6-eIF2-A-myc-His), S51A-eIF2α (pEF6-eIF2-A-S51A-Myc-His) DNA were previously described and obtained from the public BCCM/LMBP collection (LMBP collection no. 4506/4505) (Saelens et al., 2001). The plasmids were digested with *Bam*HI, blunt-ended, and then digested with *Pme*l. The cDNA inserts were subsequently cloned into a *Sma*I-digested pSC11-360 vector that contained synthetic early promoter sequences from vMJ360 (AAAAATTGAAAAATTAGCT), replacing the original p7.5K promoter for foreign gene expression (Davison and Moss, 1989). These plasmids were designated pSC11-360-wt-eIF2α and pSC11-360-S51A-eIF2α, respectively. BHK21 cells (1×10^6 cells/100 mm) were infected with VV-hr at a MOI of 0.1 PFU/cell and subsequently transfected with the aforementioned plasmids (3 μg in 60 μl lipofectamine) for 2.5 h. Cells were washed and cultured in normal medium and harvested at 3 days p.i. The cell lysates were collected and titrated for recombinant virus plaque isolation on BHK21 cells under 1% top agarose with X-gal (150 μg/ml) staining for lacZ expression. Blue plaques were picked and recombinant viruses were subsequently plaque purified three times until reaching 100% purity.

One-step growth curve analysis

HeLa cells were infected with VV-hr or VV-36hr at a MOI of 5 PFU per cell at 37°C for 1 h. Cells were incubated in normal medium and harvested at 0 and 24 h p.i. Virus titers were determined on BHK21 cells using plaque assays as described above.

Dot blot analysis for viral DNA replication

Viral DNA replication was detected using a modification from a previously published method (Seto et al., 1987). In brief, HeLa cells were infected with WT-VV, VV-hr, and VV-36hr at a MOI of 10 PFU per cell and harvested at various times (0, 1, 2, 3, 4, and 6 h) p.i. Cells were washed in PBS and lysed with digestion buffer (100 mM NaCl, 10 mM Tris–Cl pH 8, 25 mM EDTA pH 8, 0.5% SDS). The lysates were digested with proteinase K (100 μg/ml), extracted with phenol–chloroform, and total DNA was isolated by isopropanol precipitation. After RNase treatment, DNA samples were applied to nitrocellulose paper using a microsample filtration manifold (Schleicher & Schuell, Inc.). The filters were hybridized with a ^{32}P-labeled VGF DNA fragment, washed, and autoradiographed. The experiments were repeated twice, and the resulting blots were scanned, quantitated and averaged with a phosphoimager (Molecular Dynamics, Inc.).

Radioactive amino acid labeling for viral protein synthesis

HeLa cells were mock-infected or infected with VV-hr or VV-36hr at a MOI of 10 PFU per cell and cultured in DMEM + 10% FBS. At various times (0, 1, 2, 3, 4, 6, and 8 h) p.i., cells were starved in methionine-free DMEM medium for 30 min and labeled with ^{35}S-methionine-containing medium (100 μCi/ml) for 1 h. Cells were subsequently washed with PBS and lysed with SDS-containing sample buffer. Lysates were separated on 10% SDS-PAGE and the levels of viral protein synthesis were determined by the presence of diagnostic late protein bands on autoradiograms. Alternatively, HeLa cells were infected with VV-hr, VV-36hr, eIF2α-VV-hr, or eIF2αS51A-VV-hr at a MOI of 10 PFU per cells, starved in methionine-free DMEM medium for 30 min, pulse-labeled for 1 h at 10 h p.i., and immediately harvested. The cell lysates were separated on 10% SDS-PAGE, dried, and autographed.

To immunoprecipitate viral intermediate G8R protein, HeLa cells were infected with VV-hr, VV36hr, VV-eIF2α-VV-hr, or VV-eIF2αS51A-VV-hr at a MOI of 10 PFU per cell. Cells were starved for 30 min and subsequently labeled with ^{35}S-methionine (100 μCi/ml) at 4–8 h p.i. Cell lysates were harvested and immunoprecipitation was performed with an anti-G8R Ab (1:100) at 4°C for 60 min and protein A beads were added for an additional 60-min incubation, washed, and analyzed on a 10% SDS-PAGE.

Northern blot analysis

HeLa cells were infected as described and harvested at 0.5, 1, 2, 4, 6, and 8 h p.i. Total RNA was isolated with a Qiagene RNeasy minikit, separated on a 1% formaldehyde agarose gel, transferred to nitrocellulose paper and hybridized with a ^{32}P-labeled G8R or F17R PCR DNA fragment, washed, and autoradiographed as described (Baldick and Moss, 1993).

Immunoblot analysis

HeLa cells were infected with VV-hr or VV-36hr at a MOI of 5 PFU per cell and cell lysates were harvested at various time points after infection. Whole cell extracts were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane using a transfer apparatus according to the manufacturer's protocol (BioRad). After incubation with 0.2% I-block (Applied Biosystems Inc.) in PBST (1× PBS/0.1% Tween-20) for 60 min, the membrane was incubated with each primary antibody at room temperature for 60 min (for G8R-, F17R-, PKR-specific Abs) or at 4°C overnight (for phospho-PKR, phospho-eIF2α, phospho eIF4E, eIF2α-, eIF4E-, eIF4G-specific Abs). Membranes were washed five times for 5 min with PBST and incubated with a 1:2000 dilution of alkaline phosphatase-conjugated goat anti-mouse (Pierce Inc.) or 1:2000 goat anti-rabbit (NEB) secondary antibody for 120 min. Blots were washed with PBST for five times and developed with CDP-Star (Applied Biosystems Inc.) as described by the manufacturer.

Caspase activity assays

The caspase assays were performed according to the fluorometric CaspACE™ Assay system (Promega, Inc.). In brief, HeLa cells were seeded at 1.2×10^6/100 mm and infected with VV-hr or VV-36hr at a MOI of 5 PFU per cell. Cell lysates were harvested at 8 h p.i., freeze–thawed four times, and centrifuged to remove nuclei. Soluble cell lysates were saved and equal amounts of cellular proteins were used in caspase activity assays with fluorogenic substrates for caspase-1, -2, -3, -8, and -9 in caspase assay buffer [312.5 mM HEPES (pH 7.5)/31.25% sucrose/0.3125% CHAPS] as described in the manufacturer's manuals. Fluorescence was measured with a spectrofluorometer, using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Growth of eIF2α-VV-hr and eIF2αS51A-VV-hr on HeLa cells

HeLa cells were infected with VV-hr, VV-36hr, eIF2α-VV-hr, or eIF2αS51A-VV-hr at a MOI of 1 PFU per cell and cells were harvested at 48 p.i. Virus titers in the cell lysates were determined by plaque formation assays on BHK21 cells. The data were obtained from three independent experiments with standard deviations. The *Y*-axis represents the fold increase of virus production, as calculated using the following equation = [Titers of virus of interest at 48 h p.i. / Titers of virus of interest at 0 h p.i.] / [Titers of VV-hr at 48 h p.i. / Titers of VV-hr at 0 h p.i.].

Acknowledgments

We thank Dr. Jacomine Krise-Locker for Anti-F17R Ab. This work is supported by grants from Academia Sinica and the National Science Council (NSC91-2311-B-001-059) of R.O.C.

References

Ali, A.N., Turner, P.C., Brooks, M.A., Moyer, R.W., 1994. The SP-1 gene of rabbitpox virus determines host range and is required for hemorrhagic pock formation. Virology 202, 305–314.

Bair, C.-H., Chung, C.-S., Vasilevskaya, I.A., Chang, W., 1996. Isolation and characterization of a Chinese hamster ovary mutant cell line with altered sensitivity to vaccinia virus killing. J. Virol. 70, 4655–4666.

Baldick Jr., C.J., Moss, B., 1993. Characterization and temporal regulation of mRNAs encoded by vaccinia virus intermediate-stage genes. J. Virol. 67 (6), 3515–3527.

Barry, M., Hnatiuk, S., Mossman, K., Lee, S.F., Boshkov, L., McFadden, G., 1997. The myxoma virus M-T4 gene encodes a novel RDEL-containing protein that is retained within the endoplasmic reticulum and is important for the productive infection of lymphocytes. Virology 239 (2), 360–377.

Beattie, E., Denzler, K.L., Tartaglia, J., Perkus, M.E., Paoletti, E., Jacobs, B.L., 1995a. Reversal of the interferon-sensitive phenotype of a vaccinia virus lacking E3L by expression of the reovirus S4 gene. J. Virol. 69 (1), 499–505.

Beattie, E., Paoletti, E., Tartaglia, J., 1995b. Distinct patterns of IFN sensitivity observed in cells infected with vaccinia K3L- and E3L-mutant viruses. Virology 210 (2), 254–263.

Beattie, E., Kauffman, E.B., Martinez, H., Perkus, M.E., Jacobs, B.L., Paoletti, E., Tartaglia, J., 1996. Host-range restriction of vaccinia virus E3L-specific deletion mutants. Virus Genes 12 (1), 89–94.

Brush, M.H., Weiser, D.C., Shenolikar, S., 2003. Growth arrest and DNA damage-inducible protein GADD34 targets protein phosphatase 1 alpha to the endoplasmic reticulum and promotes dephosphorylation of the alpha subunit of eukaryotic translation initiation factor 2. Mol. Cell. Biol. 23 (4), 1292–1303.

Chang, H.W., Uribe, L.H., Jacobs, B.L., 1995. Rescue of vaccinia virus lacking the E3L gene by mutants of E3L. J. Virol. 69, 6605–6608.

Chung, C.S., Vasilevskaya, I.A., Wang, S.C., Bair, C.H., Chang, W., 1997. Apoptosis and host restriction of vaccinia virus in RK13 cells. Virus Res. 52 (2), 121–132.

Clemens, M.J., 2001. Initiation factor eIF2 alpha phosphorylation in stress responses and apoptosis. Prog. Mol. Subcell. Biol. 27, 57–89.

Coleman, M.L., Sahai, E.A., Yeo, M., Bosch, M., Dewar, A., Olson, M.F., 2001. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat. Cell Biol. 3 (4), 339–345.

Damaso, C.R., Moussatche, N., 1992. Protein synthesis in vaccinia virus-infected cells. I Effect of hypertonic shock recovery. Arch. Virol. 123 (3–4), 295–308.

Davies, M.V., Chang, H.W., Jacobs, B.L., Kaufman, R.J., 1993. The E3L and K3L vaccinia virus gene products stimulate translation through inhibition of the double-stranded RNA-dependent protein kinase by different mechanisms. J. Virol. 67 (3), 1688–1692.

Davison, A.J., Moss, B., 1989. Structure of vaccinia virus early promoters. J. Mol. Biol. 210 (4), 749–769.

Drillien, R., Spehner, D., Kirn, A., 1978. Host range restriction of vaccinia virus in Chinese hamster ovary cells: relationship to shutoff of protein synthesis. J. Virol. 28 (3), 843–850.

Drillien, R., Koehren, F., Kirn, A., 1981. Host range deletion mutant of vaccinia virus defective in human cells. Virology 111 (2), 488–499.

Drillien, R., Spehner, D., Kirn, A., 1982. Complementation and genetic linkage between vaccinia virus temperature- sensitive mutants. Virology 119 (2), 372–381.

Everett, H., McFadden, G., 2001a. Viral proteins and the mitochondrial apoptotic checkpoint. Cytokine Growth Factor Rev. 12 (2–3), 181–188.

Everett, H., McFadden, G., 2001b. Viruses and apoptosis: meddling with mitochondria. Virology 288 (1), 1–7.

Everett, H., Barry, M., Lee, S.F., Sun, X., Graham, K., Stone, J., Bleackley, R.C., McFadden, G., 2000. M11L: a novel mitochondria-localized protein of myxoma virus that blocks apoptosis of infected leukocytes. J. Exp. Med. 191 (9), 1487–1498.

Everett, H., Barry, M., Sun, X., Lee, S.F., Frantz, C., Berthiaume, L.G., McFadden, G., Bleackley, R.C., 2002. The myxoma poxvirus protein, M11L, prevents apoptosis by direct interaction with the mitochondrial permeability transition pore. J. Exp. Med. 196 (9), 1127–1139.

Feduchi, E., Aldabe, R., Novoa, I., Carrasco, L., 1995. Effects of poliovirus 2A (pro) on vaccinia virus gene expression. Eur. J. Biochem. 234 (3), 849–854.

Fenner, F., Sambrook, J.F., 1966. Conditional lethal mutants of rabbitpox virus. II. Mutants (p) that fail to multiply in PK-2a cells. Virology 28 (4), 600–609.

Flynn, A., Proud, C.G., 1995. Serine 209, not serine 53, is the major site of phosphorylation in initiation factor eIF-4E in serum-treated Chinese hamster ovary cells. J. Biol. Chem. 270 (37), 21684–21688.

Gagliardini, V., Fernandez, P.A., Lee, R.K., Drexler, H.C., Rotello, R.J., Fishman, M.C., Yuan, J., 1994. Prevention of vertebrate neuronal death by the crmA gene. Science 263 (5148), 826–828.

Gale Jr., M., Tan, S.L., Wambach, M., Katze, M.G., 1996. Interaction of the interferon-induced PKR protein kinase with inhibitory proteins P58IPK and vaccinia virus K3L is mediated by unique domains: implications for kinase regulation. Mol. Cell. Biol. 16 (8), 4172–4181.

Gale Jr., M., Tan, S.L., Katze, M.G., 2000. Translational control of viral gene expression in eukaryotes. Microbiol. Mol. Biol. Rev. 64 (2), 239–280.

Gemmell, A., Fenner, F., 1960. Genetic studies with mammalian poxviruses III White (u) mutants of rabbitpox virus. Virology 11, 219–235.

Gillard, S., Spehner, D., Drillien, R., 1985. Mapping of a vaccinia host range sequence by insertion into the viral thymidine kinase gene. J. Virol. 53 (1), 316–318.

Gillard, S., Spehner, D., Drillien, R., Kirn, A., 1986. Localization and sequence of a vaccinia virus gene required for multiplication in human cells. Proc. Natl. Acad. Sci. 83, 5573–5577.

Goebel, S.J., Johnson, G.P., Perkus, M.E., Davis, S.W., Winslow, J.P., Paoletti, E., 1990. The complete DNA sequence of vaccinia virus. Virology 179, 247–266.

Gray, N.K., Wickens, M., 1998. Control of translation initiation in animals. Annu. Rev. Cell Dev. Biol. 14, 399–458.

Hruby, D.E., Lynn, D.L., Condit, R.C., Kates, J.R., 1980. Cellular differences in the molecular mechanisms of vaccinia virus host range restriction. J. Gen. Virol. 47 (2), 485–488.

Ink, B.S., Gilbert, C.S., Evan, G.I., 1995. Delay of vaccinia virus-induced apoptosis in nonpermissive Chinese hamster ovary cells by the cowpox virus CHOhr and adenovirus E1B 19K genes. J. Virol. 69 (2), 661–668.

Jefferts, E.R., Holowczak, J.A., 1971. RNA synthesis in vaccinia-infected L cells: inhibition of ribosome formation and maturation. Virology 46 (3), 730–744.

Kawagishi-Kobayashi, M., Silverman, J.B., Ung, T.L., Dever, T.E., 1997. Regulation of the protein kinase PKR by the vaccinia virus pseudosubstrate inhibitor K3L is dependent on residues conserved between the K3L protein and the PKR substrate eIF2alpha. Mol. Cell. Biol. 17 (7), 4146–4158.

Keck, J.G., Baldick Jr., C.J., Moss, B., 1990. Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late *trans*-activator genes. Cell 61 (5), 801–809.

Keck, J.G., Kovacs, G.R., Moss, B., 1993. Overexpression, purification, and late transcription factor activity of the 17-kilodalton protein encoded by the vaccinia virus A1L gene. J. Virol. 67 (10), 5740–5748.

Kimball, S.R., 1999. Eukaryotic initiation factor eIF2. Int. J. Biochem. Cell Biol. 31 (1), 25–29.

Knipe, D.M., Howley, P.M., 2001. Fields' Virology, fourth ed. Lippincott Williams & Wilkins, Philadelphia.

Komiyama, T., Ray, C.A., Pickup, D.J., Howard, A.D., Thornberry, N.A., Peterson, E.P., Salvesen, G., 1994. Inhibition of interleukin-1 beta converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. J. Biol. Chem. 269 (30), 19331–19337.

Kotwal, G., Moss, B., 1988. Analysis of a large cluster of nonessential genes deleted from a vaccinia virus terminal transposition mutant. Virology 167, 524–537.

Kramer, G., 1990. Two phosphorylation sites on eIF-2 alpha. FEBS Lett. 267 (2), 181–182.

Li, Y., Yuan, S., Moyer, R.W., 1998. The non-permissive infection of insect (gypsy moth) LD-652 cells by Vaccinia virus. Virology 248 (1), 74–82.

Macen, J.L., Graham, K.A., Lee, S.F., Schreiber, M., Boshkov, L.K., McFadden, G., 1996. Expression of the myxoma virus tumor necrosis factor receptor homologue and M11L genes is required to prevent virus-induced apoptosis in infected rabbit T lymphocytes. Virology 218 (1), 232–237.

Marissen, W.E., Lloyd, R.E., 1998. Eukaryotic translation initiation factor 4G is targeted for proteolytic cleavage by caspase 3 during inhibition of translation in apoptotic cells. Mol. Cell. Biol. 18 (12), 7565–7574.

Marissen, W.E., Guo, Y., Thomas, A.A., Matts, R.L., Lloyd, R.E., 2000. Identification of caspase 3-mediated cleavage and functional alteration of eukaryotic initiation factor 2alpha in apoptosis. J. Biol. Chem. 275 (13), 9314–9323.

McClain, M.E., 1965. The host range and plaque morphology of rabbitpox virus (RPu +) and its u mutants on chick fibroblasts PK 2a and L929 cells. Aust. J. Exp. Biol. Med. Sci. 43, 31–44.

McFadden, G., Barry, M., 1998. How poxviruses oppose apoptosis. Semin. Virol. 8, 429–442.

Mills, J.C., Stone, N.L., Erhardt, J., Pittman, R.N., 1998. Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. J. Cell Biol. 140 (3), 627–636.

Moss, B., 1968. Inhibition of HeLa cell protein synthesis by the vaccinia virion. J. Virol. 2 (10), 1028–1037.

Mossman, K., Lee, S.F., Barry, M., Boshkov, L., McFadden, G., 1996. Disruption of M T5, a novel myxoma virus gene member of poxvirus host range superfamily, results in dramatic attenuation of myxomatosis in infected European rabbits. J. Virol. 70 (7), 4394–4410.

Mulder, J., Robertson, M.E., Seamons, R.A., Belsham, G.J., 1998. Vaccinia virus protein synthesis has a low requirement for the intact translation initiation factor eIF4F, the cap binding complex, within infected cells. J. Virol. 72 (11), 8813–8819.

Murtha-Riel, P., Davies, M.V., Scherer, B.J., Choi, S.Y., Hershey, J.W., Kaufman, R.J., 1993. Expression of a phosphorylation-resistant eukaryotic initiation factor 2 alpha-subunit mitigates heat shock inhibition of protein synthesis. J. Biol. Chem. 268 (17), 12946–12951.

Nadano, D., Sato, T.A., 2000. Caspase-3-dependent and -independent degradation of 28 S ribosomal RNA may be involved in the inhibition of protein synthesis during apoptosis initiated by death receptor engagement. J. Biol. Chem. 275 (18), 13967–13973.

Oguiura, N., Spehner, D., Drillien, R., 1993. Detection of a protein encoded by the vaccinia virus C7L open reading frame and tudy of its effect on virus multiplication in different cell lines. J. Gen. Virol. 74 (Pt. 7), 1409–1413.

Passarelli, A.L., Kovacs, G.R., Moss, B., 1996. Transcription of a vaccinia virus late promoter template: requirement for the product of the A2L intermediate-stage gene. J. Virol. 70 (7), 4444–4450.

Perkus, M.E., Goebel, S.J., Davis, S.W., Johnson, G.P., Limbach, K., Norton, E.K., Paoletti, E., 1990. Vaccinia virus host range genes. Virology 179 (1), 276–286.

Person, A., Beaud, G., 1978. Inhibition of host protein synthesis in vaccinia virus-infected cells in the presence of cordycepin (3′-deoxyadenosine). J. Virol. 25 (1), 11–18.

Person, A., Ben-Hamida, F., Beaud, G., 1980. Inhibition of 40S-Met-tRNAfMet ribosomal initiation complex formation by vaccinia virus. Nature 287 (5780), 355–357.

Polunovsky, V.A., Gingras, A.C., Sonenberg, N., Peterson, M., Tan, A., Rubins, J.B., Manivel, J.C., Bitterman, P.B., 2000. Translational control of the antiapoptotic function of Ras. J. Biol. Chem. 275 (32), 24776–24780.

Price, N.T., Welsh, G.I., Proud, C.G., 1991. Phosphorylation of only serine-51 in protein synthesis initiation factor-2 is associated with inhibition of peptide-chain initiation in reticulocyte lysates. Biochem. Biophys. Res. Commun. 176 (3), 993–999.

Ramsey-Ewing, A., Moss, B., 1995. Restriction of vaccinia virus replication in CHO cells occurs at the stage of viral intermediate protein synthesis. Virology 206 (2), 984–993.

Ramsey-Ewing, A.L., Moss, B., 1996. Complementation of a vaccinia virus host-range K1L gene deletion by the nonhomologous CP77 gene. Virology 222 (1), 75–86.

Ramsey-Ewing, A., Moss, B., 1998. Apoptosis induced by a postbinding step of vaccinia virus entry into Chinese hamster ovary cells. Virology 242 (1), 138–149.

Saelens, X., Kalai, M., Vandenabeele, P., 2001. Translation inhibition in apoptosis: caspase-dependent PKR activation and eIF2-alpha phosphorylation. J. Biol. Chem. 276 (45), 41620–41628.

Sebbagh, M., Renvoize, C., Hamelin, J., Riche, N., Bertoglio, J., Breard, J., 2001. Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. Nat. Cell Biol. 3 (4), 346–352.

Seto, J., Celenza, L.M., Condit, R.C., Niles, E.G., 1987. Genetic map of the vaccinia virus HindIII D Fragment. Virology 160 (1), 110–119.

Shchelkunov, S.N., Safronov, P.F., Totmenin, A.V., Petrov, N.A., Ryazankina, O.I., Gutorov, V.V., Kotwal, G.J., 1998. The genomic sequence analysis of the left and right species-specific terminal region of a cowpox virus strain reveals unique sequences and a cluster of intact ORFs for immunomodulatory and host range proteins. Virology 243 (2), 432–460.

Shisler, J.L., Isaacs, S.N., Moss, B., 1999. Vaccinia virus serpin-1 deletion mutant exhibits a host range defect characterized by low levels of intermediate and late mRNAs. Virology 262 (2), 298–311.

Somogyi, P., Frazier, J., Skinner, M.A., 1993. Fowlpox virus host range restriction: gene expression, DNA replication, and morphogenesis in nonpermissive mammalian cells. Virology 197, 439–444.

Spehner, D., Gillard, S., Drillien, R., Kirn, A., 1988. A cowpox virus gene required for multiplication in Chinese hamster ovary cells. J. Virol. 62 (4), 1297–1304.

Stannard, L.M., Marais, D., Kow, D., Dumbell, K.R., 1998. Evidence for incomplete replication of a penguin poxvirus in cells of mammalian origin. J. Gen. Virol. 79 (Pt. 7), 1637–1646.

Sutter, G., Ramsey-Ewing, A., Rosales, R., Moss, B., 1994. Stable expression of the vaccinia virus K1L gene in rabbit cells complements the host range defect of a vaccinia virus mutant. J. Virol. 68, 4109–4116.

Tagaya, I., Kitamura, T., Sano, Y., 1961. A new mutant of dermovaccinia virus. Nature 192, 381–382.

Topisirovic, I., Culjkovic, B., Cohen, N., Perez, J.M., Skrabanek, L., Borden, K.L., 2003. The proline-rich homeodomain protein, PRH, is a tissue-specific inhibitor of eIF4E-dependent cyclin D1 mRNA transport and growth. EMBO J. 22 (3), 689–703.

Vos, J.C., Stunnenberg, H.G., 1988. Derepression of a novel class of vaccinia virus genes upon DNA replication. EMBO J. 7 (11), 3487–3492.

Wright, C.F., Keck, J.G., Tsai, M.M., Moss, B., 1991. A transcription factor for expression of vaccinia virus late genes is encoded by an intermediate gene. J. Virol. 65 (7), 3715–3720.

Wyatt, L.S., Carroll, M.W., Czerny, C.P., Merchlinsky, M., Sisler, J.R., Moss, B., 1998. Marker rescue of the host range restriction defects of modified vaccinia virus Ankara. Virology 251 (2), 334–342.

Xiang, Y., Condit, R.C., Vijaysri, S., Jacobs, B., Williams, B.R., Silverman, R.H., 2002. Blockade of interferon induction and action by the E3L double-stranded RNA binding proteins of vaccinia virus. J. Virol. 76 (10), 5251–5259.

Yan, W., Frank, C.L., Korth, M.J., Sopher, B.L., Novoa, I., Ron, D., Katze, M.G., 2002. Control of PERK eIF2alpha kinase activity by the endoplasmic reticulum stress-induced molecular chaperone P58IPK. Proc. Natl. Acad. Sci. U.S.A. 99 (25), 15920–15925.

Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V.M., Salvesen, G.S., 1997. Target protease specificity of the viral serpin CrmA. Analysis of five caspases. J. Biol. Chem. 272 (12), 7797–7800.