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Viral host-range factor C7 or K1 is essential for modified vaccinia virus Ankara late gene expression in human and murine cells, irrespective of their capacity to inhibit protein kinase R-mediated phosphorylation of eukaryotic translation initiation factor 2α

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Vaccinia virus (VACV) infection induces phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), which inhibits cellular and viral protein synthesis. In turn, VACV has evolved the capacity to antagonize this antiviral response by expressing the viral host-range proteins K3 and E3. This study revealed that the host-range genes K1L and C7L also prevent eIF2α phosphorylation in modified VACV Ankara (MVA) infection of several human and murine cell lines. Moreover, C7L-deleted MVA (MVA-ΔC7L) lacked late gene expression, which could be rescued by the function of host-range factor K1 or C7. It was demonstrated that viral gene expression was blocked after viral DNA replication and that it was independent of apoptosis induction. Furthermore, it was found that eIF2α phosphorylation in MVA-ΔC7L-infected cells is mediated by protein kinase R (PKR) as shown in murine embryonic fibroblasts lacking PKR function, and it was shown that this was not due to reduced E3L gene expression. The block of eIF2α phosphorylation by C7 could be complemented by K1 in cells infected with MVA-ΔC7L encoding a reinserted K1L gene (MVA-ΔC7L-K1L). Importantly, these data illustrated that eIF2α phosphorylation by PKR is not responsible for the block of late viral gene expression. This suggests that other mechanisms targeted by C7 and K1 are essential for completing the MVA gene expression cycle and probably also for VACV replication in a diverse set of cell types.

INTRODUCTION

Modified vaccinia virus Ankara (MVA), a promising candidate replacement vaccine against smallpox, is currently being investigated as a vector vaccine against infectious diseases and cancer (reviewed by Acres & Bonnefoy, 2008; Drexler *et al.*, 2004; Gómez *et al.*, 2008). Its safety has been tested in over 120 000 humans (reviewed by Sutter & Staib, 2003). During the attenuation process, MVA has lost a substantial part of the vaccinia virus (VACV) genome sequence and is unable to replicate productively in most mammalian cells (Antoine *et al.*, 1998; Carroll & Moss, 1997; Meyer *et al.*, 1991). Nevertheless, MVA infection of non-permissive cells induces complete cascade-like gene expression, but is blocked in virion morphogenesis at the late stage of the viral life cycle (Sutter & Moss, 1992). Importantly for use as a vaccine, the inability to form infectious progeny does not affect induction of an efficient immune response against MVA-encoded recombinant and viral antigens (Sutter *et al.*, 1994).

VACV host tropism relies on the expression of certain host-range genes that enable virus replication (Werden *et al.*, 2008). This gene class and the corresponding gene

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products are thought to play critical roles in modulating antiviral responses of the host cells. After identifying the VACV host-range gene K1L, which is truncated in MVA, C7L was the second viral gene shown to regulate productive VACV replication in cells of human origin (Oguiura *et al.*, 1993; Perkus *et al.*, 1990). In human cell lines, but not rabbit kidney cells, C7L expression can compensate for loss of K1L function to support productive virus growth (Perkus *et al.*, 1990; Ramsey-Ewing & Moss, 1995). It was further demonstrated that infection with VACV variants lacking C7L and K1L enhanced phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) (Hsiao *et al.*, 2004; Meng *et al.*, 2008; Nájera *et al.*, 2006). The fact that C7L gene sequences are conserved in the genomes of all orthopoxviruses and that diverse mammalian poxviruses encode functional C7 homologues (Meng *et al.*, 2008) suggest that this host-range protein plays an important regulatory role in the poxvirus life cycle in mammalian cells.

To date, little is known about C7 protein function, confirming the need to study its function in detail. Structurally, C7 contains no protein domains or sequence motifs described as yet that correspond to other orthopoxvirus host-range factors, unlike K1 and CP77 with their multiple ankyrin repeat domains.

Deletion of C7L from MVA is interesting, as K1L is already fragmented in the MVA genome. We hypothesized that the lack of both host-range factors could severely affect the infection phenotype of MVA in mammalian cells. Here, we demonstrated that MVA late gene expression in non-permissive human and murine cells completely depended on C7L gene function. We have provided a detailed analysis of the molecular life cycle of the C7L mutant virus (MVA-ΔC7L) in the context of complementing K1L gene function. In addition, we investigated antiviral mechanisms triggered by the absence of K1 and C7.

RESULTS

Construction of MVA-ΔC7L, MVA-C7Lrev and MVA-ΔC7L-K1L, and C7L expression analysis

To elucidate the relevance of the C7L host-range gene for the intracellular MVA life cycle, we constructed a mutant MVA lacking C7L (MVA-ΔC7L) by using the host-range gene K1L as a transient selection marker, as described previously (Staib *et al.*, 2000). Furthermore, we generated a revertant virus, MVA-C7Lrev, by reinserting the C7L gene under the control of its authentic promoter into the genome of MVA-ΔC7L. Northern blot analysis of viral RNA with C7L-specific riboprobes showed an early transcription profile (Fig. 1a). mRNA levels started at 2 h post-infection (p.i.) and declined again at 5 h p.i., but were stabilized in the presence of the DNA replication inhibitor 1-β-D-arabinofuranosylcytosine (AraC). As expected, C7L transcripts were also detected in MVA-C7Lrev-infected cells, but were absent in MVA-ΔC7L-infected cells. Furthermore, we raised monoclonal antibodies specific for the C7 protein and analysed whole-cell extracts from infected BHK-21 cells by Western blotting (Fig. 1b). Starting at 2 h p.i. with MVA, we detected a specific protein of approximately 18 kDa corresponding in size to the predicted molecular mass of C7. This protein was not detected in lysates from MVA-ΔC7L-infected cells. Addition of AraC at the time of infection did not inhibit synthesis of C7, confirming early expression of C7L, as proposed previously (Assarsson *et al.*, 2008; Oguiura *et al.*, 1993).

For replication-competent VACV, it has been demonstrated that either of the host-range genes C7L or K1L is sufficient to support replication in human cells (Oguiura *et al.*, 1993; Perkus *et al.*, 1990). To study possible overlapping functions of these factors, we decided to construct a variant that lacked C7L but contained K1L (MVA-ΔC7L-K1L). We inserted the coding sequences of K1L together with its authentic promoter into the deletion III site of MVA-ΔC7L. Expression of the C7 and K1 proteins by the respective viruses was verified by Western blotting (Fig. 1c). Additionally, all of the mutants constructed were able to replicate as efficiently as wild-type MVA in permissive chicken embryo fibroblasts (CEFs) (data not shown).

Late viral gene expression of MVA in non-permissive cells requires the host-range factor C7 or K1

Host-restricted VACV deficient for K1L and C7L expression exhibits impaired late gene expression in HeLa cells (Meng *et al.*, 2008). Here, we analysed the gene expression profiles of MVA lacking these two genes (MVA-ΔC7L) in non-permissive human and murine cell lines. As expected, in contrast to wild-type MVA, metabolic labelling of human HeLa and murine NIH 3T3 cells clearly demonstrated the inability of MVA-ΔC7L to synthesize late viral polypeptides (Fig. 2a). Conversely, permissive BHK-21 cells supported full gene expression for MVA-ΔC7L, with typical bands representing viral late proteins detectable at 5 h p.i. (arrows in Fig. 2a). In all cell lines analysed, typical shut-off of cellular protein synthesis became visible over time (Fig. 2a), as shown previously (Sutter & Moss, 1992). Insertion of either K1L or C7L into MVA-ΔC7L restored late viral gene expression in HeLa (shown for MVA-ΔC7L-K1L and MVA-C7Lrev) and NIH 3T3 (shown for MVA-ΔC7L-K1L) cells (Fig. 2a).

To study recombinant late gene expression in the absence of C7, we monitored expression of the *Escherichia coli lacZ* gene under the regulation of the specific VACV late promoter P11 after infecting cells with the viruses MVA-ΔC7L-P11LZ and MVA-P11LZ (Sutter & Moss, 1992) (Fig. 2b). Infection with MVA-P11LZ resulted in high levels of reporter enzyme β-galactosidase activity in non-permissive human HeLa and MRC-5 cells, as well as in murine NIH 3T3 and permissive BHK-21 cells. In contrast,

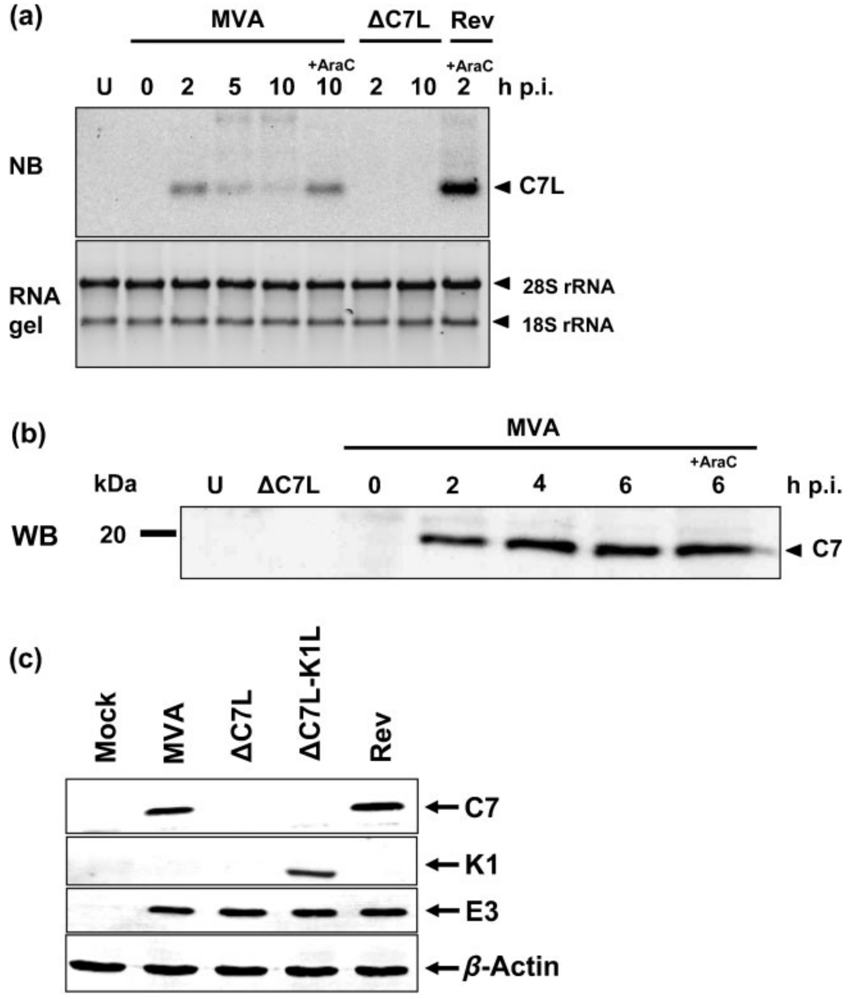


Fig. 1. C7L gene expression profile and protein expression analysis of deleted C7L and inserted K1L in the constructed mutants MVA-ΔC7L (ΔC7L), MVA-ΔC7L-K1L (ΔC7L-K1L) and MVA-C7Lrev (Rev) compared with wild-type MVA (MVA). (a) Northern blot analysis (NB) of total cellular RNA extracted from NIH 3T3 cells infected with the indicated viruses at the indicated time points and probed for the presence of C7L transcripts. Uninfected cells (U) served as a control, and treatment with AraC is indicated. An ethidium bromide-stained RNA gel served as a loading control. RNA transcripts are indicated on the right. (b) Western blot analysis (WB) of BHK-21 cells infected with the indicated viruses and lysed at various time points as indicated. The protein bands detected by a C7-specific antibody are indicated on the right. (c) Western blot analysis of lysates from BHK-21 cells infected with the indicated viruses and harvested at 4 h p.i. C7, K1 and E3 proteins, indicated on the right, were detected with specific antisera. Equal loading was checked by detection of β-actin. Mock indicates uninfected cells and E3 served as an internal infection control.

infection with MVA-ΔC7L-P11LZ failed to support β-galactosidase production in human or murine cells.

To identify the stage in the viral life cycle when the block occurred, we analysed viral DNA replication, a prerequisite for poxvirus intermediate transcription and consequently late gene expression (Keck *et al.*, 1990; Vos & Stunnenberg, 1988). We monitored viral DNA synthesis by isolating total DNA from NIH 3T3 cells infected with MVA, MVA-ΔC7L or MVA-ΔC7L-K1L. The DNA replication of all three viruses proceeded with kinetics similar to those described for MVA infection of non-permissive cells (Sutter & Moss, 1992), revealing that C7 is not required for viral DNA replication (Fig. 2c). Next, we monitored viral transcription by Northern blot analysis, probing for representative early, intermediate and late transcripts, as described previously (Ludwig *et al.*, 2005). In non-permissive NIH 3T3 cells, MVA-ΔC7L-mediated early and intermediate transcription was comparable to wild-type MVA, but late transcription was dramatically reduced. As clearly demonstrated, production of the host-range protein K1 in MVA-ΔC7L-K1L infections fully complemented the lack of C7 and rescued viral late transcription (Fig. 2d).

MVA-ΔC7L infection does not induce apoptosis

Host restriction of some orthopoxvirus mutants in certain cell lines has been associated with induction of apoptosis (Hornemann *et al.*, 2003; Hsiao *et al.*, 2004; Nájera *et al.*, 2006; Turner & Moyer, 2002). Therefore, we monitored MVA-ΔC7L-infected cells for signs of apoptosis, which might explain the lack of late gene expression. As activation of proteases of the caspase family is crucial for apoptosis (Thornberry, 1998), we screened for active caspase-3 in human and murine cells. Treatment of NIH 3T3 cells with staurosporine, a well-characterized control for apoptosis, resulted in about 60 % of cells positive for active caspase-3 within 24 h of exposure. Additionally, infection with a control mutant virus with apoptosis-inducing capacity (MVA-ΔF1L; Fischer *et al.*, 2006) clearly enhanced caspase-3 activation (Fig. 3a). In contrast, infection with MVA-ΔC7L induced only background levels of active caspase-3 comparable to MVA (Fig. 3a). Similarly, we observed no onset of apoptosis upon MVA-ΔC7L infection of HeLa cells when analysing caspase-3-like activity by enzymic cleavage of the fluorogenic caspase-3 substrate DEVD-7-amino-4-methylcoumarin (DEVD-AMC) (Fig. 3b).

Caspase-3-like activity was detected only in MVA-ΔF1L-infected cells, whereas MVA-, MVA-ΔC7L- and MVA-C7Lrev-infected HeLa cells showed levels of enzymic activity comparable to the mock control. These results suggested that the characteristic deficiency of MVA-ΔC7L to produce late proteins was not associated with programmed cell death.

eIF2α phosphorylation in MVA-ΔC7L-infected cells

Phosphorylation of the α-subunit of eIF2 by protein kinase R (PKR) is a major cellular stress response triggered via different stress stimuli, including viral infection, and leads to downregulation of global translation initiation (Clemens, 2001). It was reported previously that VACV mutants lacking K1L and C7L induce eIF2α phosphorylation (Hsiao *et al.*, 2004; Nájera *et al.*, 2006). Thus, we determined levels of eIF2α phosphorylated at Ser51 after infection with MVA, MVA-ΔC7L, MVA-ΔC7L-K1L and recombinant MVA expressing both K1L and C7L (MVA-K1L) by Western blot analysis. We included MVA-ΔE3L as a control for efficient induction of PKR-mediated eIF2α phosphorylation in human cells (Ludwig *et al.*, 2006). Treatment of cells for 1 h with 1 μM thapsigargin, a pharmacological agent that triggers eIF2α phosphorylation via dsRNA-activated protein kinase-like ER kinase (PERK) (Treiman *et al.*, 1998) served as an additional positive control. Upon MVA-ΔE3L but not wild-type MVA infection of NIH 3T3, MRC-5 or HeLa cells, eIF2α was readily phosphorylated at 4 and 8 h p.i., a pattern mirrored by cells infected with MVA-ΔC7L, although eIF2α phosphorylation induced by MVA-ΔE3L was more prominent than with MVA-ΔC7L infection (Fig. 4a, b). These observations were true for all non-permissive cell lines analysed, whilst, as expected, no eIF2α phorphorylation was observed in permissive BHK-21 cells (data not shown). Interestingly, K1 was also able to compensate for the lack of C7, as we detected no phosphorylation of eIF2α in cells infected with MVA-ΔC7L-K1L. The strongest induction of eIF2α phosphorylation by MVA-ΔC7L was observed in MRC-5 cells.

eIF2α phosphorylation during infection with MVA-ΔC7L is mediated by PKR

Depending on the stress signal, eIF2α can be phosphorylated by different kinases, as shown schematically in Fig. 5(a). Except for haem-regulated inhibitor (HRI) kinase, eIF2α phosphorylation by PKR, PERK and GCN2 has been reported to be associated with viral infections (Berlanga *et al.*, 2006; García *et al.*, 2007; Jordan *et al.*, 2002). Thus, we used infection of MEFs from different knockout mouse strains to try and define which protein kinase was responsible for phosphorylation during MVA-ΔC7L infection. Infection of wild-type MEFs confirmed the results obtained in NIH 3T3 cells. Phosphorylation of eIF2α was evident upon infection with MVA-ΔC7L or MVA-ΔE3L, but not with MVA, MVA-ΔC7L-K1L or MVA-K1L infection (Fig. 5b). Similar observations were made in MEFs lacking either protein kinase GCN2 or PERK. However, in PKR-knockout MEFs, no increase in phosphorylation was detected for any of the viruses (Fig. 5b, c). Taken together, the data clearly demonstrated that C7, as well as K1, can counteract phosphorylation of eIF2α mediated by PKR.

Reduced expression of E3 in MVA-ΔC7L-infected cells is not responsible for eIF2α phosphorylation by PKR

Whilst studying C7 function in VACV, Meng *et al.* (2008) reported that C7 is required to sustain E3L expression in HeLa cells. Moreover, it was suggested that the reduced E3 protein level might be responsible for the activation of PKR. Interestingly, when we analysed samples of the different virus-infected wild-type and knockout MEFs as well as NIH 3T3 cells (see Fig. 5b) for E3 expression, we indeed detected reduced E3 levels in samples from MVA-ΔC7L infection compared with wild-type MVA, MVA-ΔC7L-K1L or MVA-K1L infection (Fig. 6a). To avoid the potential impact of reduced E3 expression on eIF2α phosphorylation, we took advantage of NIH 3T3-E3L cells that stably overexpress functional E3 protein (Budt *et al.*, 2009). We confirmed the functionality of E3 produced by these cells upon infection with MVA-ΔE3L, which failed to induce eIF2α phosphorylation (Fig. 6b). Remarkably, MVA-ΔC7L again induced phosphorylation of eIF2α at levels comparable to wild-type NIH 3T3 cells (compare Fig. 4a, b and Fig. 6b, c). In contrast, MVA, MVA-ΔC7L-K1L and MVA-K1L once more showed no eIF2α phosphorylation (Fig. 6b, c).

Lack of PKR activity is not sufficient to rescue late gene expression of MVA-ΔC7L

The results obtained so far strongly suggested that phosphorylation of eIF2α induced in the absence of C7 in MVA infection leads to a global translational shut-off that also affects translation of viral intermediate and consequently late genes. Moreover, the failure of MVA-ΔE3L to express viral late genes is rescued in PKR-deficient MEFs (Ludwig *et al.*, 2006). Thus, we also monitored late gene expression of MVA-ΔC7L in wild-type and PKR-deficient MEFs. Samples from infected MEFs were probed with a specific antibody recognizing VACV envelope protein B5, a well-characterized late gene product (Engelstad & Smith, 1993). Surprisingly, late gene expression was not rescued in MVA-ΔC7L-infected PKR-deficient MEFs at 8 h p.i., a time point at which B5 was clearly detectable in these cells infected with MVA, MVA-ΔC7L-K1L, MVA-K1L and MVA-ΔE3L (Fig. 7a). The same results were obtained at 24 h p.i., excluding any possible delay in late gene expression (Fig. 7b). The general lack of viral late polypeptide synthesis in MVA-ΔC7L-infected wild-type and knockout MEFs was further confirmed by

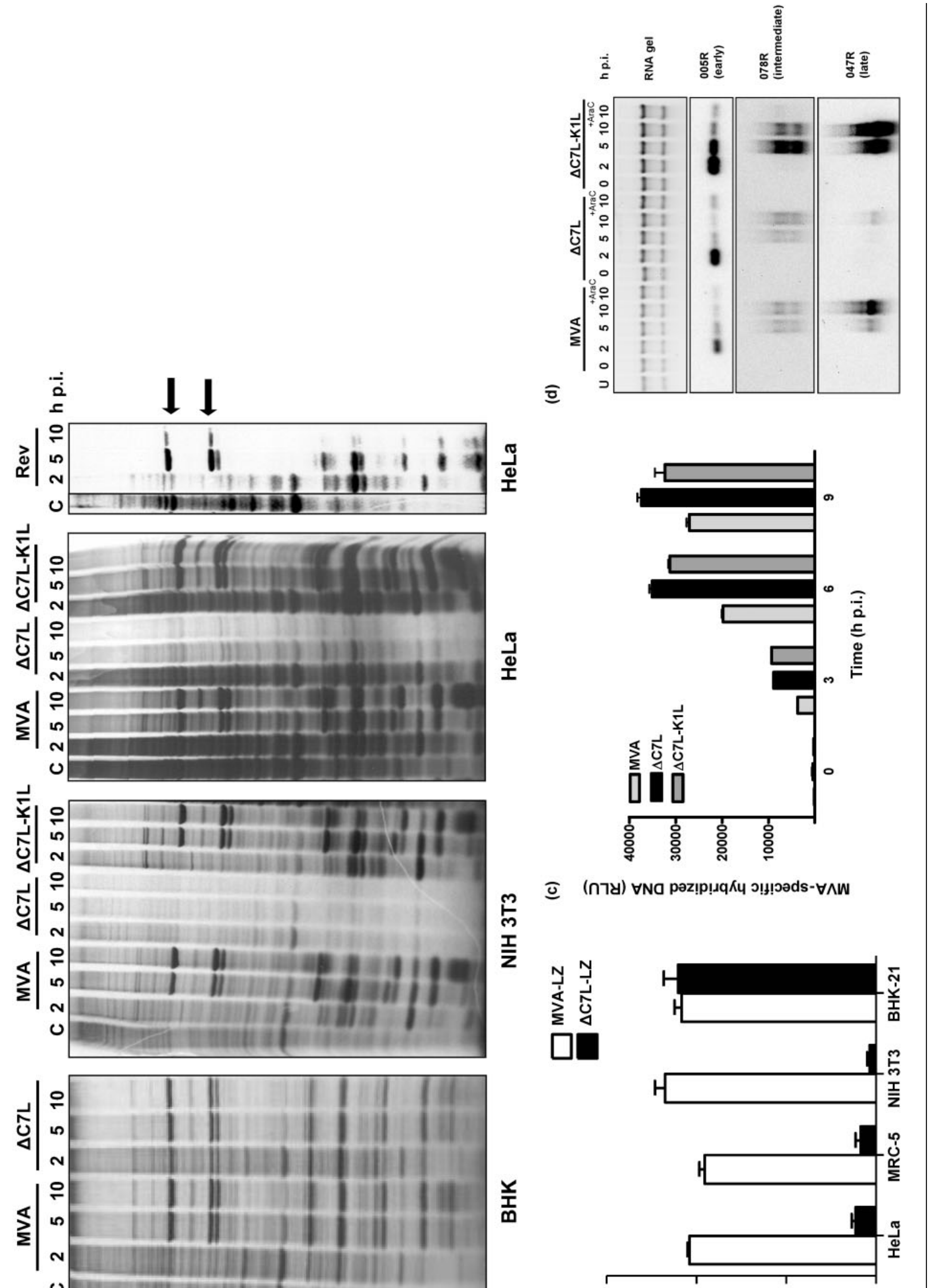

(a)
MVA
ΔC7L
ΔC7L-K1L
Rev
C 2 5 10
h p.i.
BHK
NIH 3T3
HeLa
HeLa
(b)
β-Galactosidase activity (A_{570})
3
2
1
MVA-LZ
ΔC7L-LZ
HeLa
MRC-5
NIH 3T3
BHK-21
(c)
MVA-specific hybridized DNA (RLU)
40000
30000
20000
10000
0
3
6
9
Time (h p.i.)
MVA
ΔC7L
ΔC7L-K1L
(d)
MVA
ΔC7L
ΔC7L-K1L
U 0 2 5 10 10
+AraC
h p.i.
RNA gel
005R (early)
078R (intermediate)
047R (late)

Fig. 2. Molecular analyses of the intracellular life cycle of MVA-ΔC7L and MVA-ΔC7L-K1L. (a) Autoradiography of a metabolic [^{35}S]methionine labelling from BHK-21, HeLa and NIH 3T3 cells infected with wild-type MVA (MVA), MVA-ΔC7L (ΔC7L), MVA-ΔC7L-K1L (ΔC7L-K1L) or MVA-C7Lrev (Rev) at the indicated time points. Uninfected cells (C) served as a control. Representative late viral proteins are indicated by arrows. (b) β-Galactosidase activity in lysates of HeLa, MRC-5, NIH 3T3 and BHK-21 cells infected with MVA-P11-LZ (MVA-LZ) or MVA-ΔC7L-P11-LZ (ΔC7L-LZ) for 18 h, measured as A_{570} and normalized to background levels. (c) Analysis of viral DNA replication by hybridization of DNA isolated from NIH 3T3 cells infected with the indicated viruses using a DIG-labelled MVA-specific DNA probe. Values are given as relative light units (RLU) measured by chemiluminescent detection with an anti-DIG alkaline phosphatase-conjugated antibody. Means ± SD of two independent experiments are shown. (d) Northern blot analysis of total cellular RNA extracted from infected NIH 3T3 cells harvested at the indicated time points. Treatment with AraC is indicated. Probes specific for the viral genes 005R (early), 078R (intermediate) and 047R (late) were used. An ethidium bromide-stained RNA gel served as a loading control.

metabolic labelling (data not shown). These results suggested that PKR-mediated phosphorylation of eIF2α induced by the mutant virus does not account for defective late gene expression observed in the absence of C7L gene function.

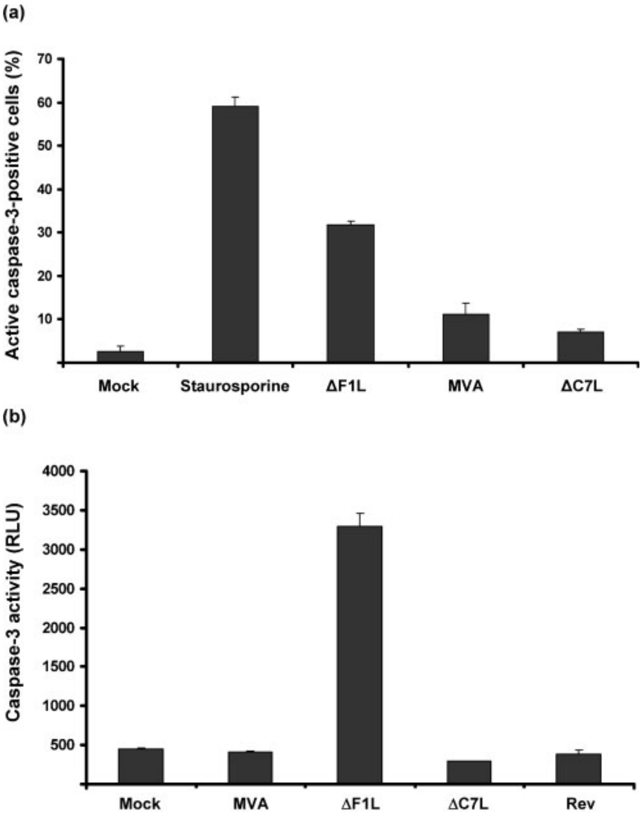


Fig. 3. Infection with MVA-ΔC7L does not induce apoptosis. (a) FACS analysis of NIH 3T3 cells stained for active caspase-3, 24 h after infection with MVA-ΔF1L (ΔF1L), wild-type MVA (MVA) or MVA-ΔC7L (ΔC7L). As a positive control, cells were treated with 1 μM staurosporine. Means ± SD of FACS analysis data from three independent experiments are displayed. (b) Caspase-3-like activity in HeLa cell extracts infected with the above viruses plus MVA-C7Lrev (Rev), measured by a fluorogenic enzyme assay in relative light units (RLU). Means ± SD of three independent experiments are shown.

DISCUSSION

To discover whether the characteristic phenotype of MVA infection in non-permissive mammalian cells depends crucially on the activity of the VACV host-range factor C7, we aimed to elucidate the function of the C7L gene conserved in the MVA genome.

We constructed an MVA C7L knockout mutant (MVA-ΔC7L) and confirmed that C7L belongs to the early gene class as verified by mRNA and protein analyses. This agrees with earlier reports (Assarsson *et al.*, 2008; Oguiura *et al.*, 1993). Infection of non-permissive human and murine cells exhibited impaired gene expression compared with wild-type MVA. Detailed analyses of crucial steps in the intracellular virus life cycle pointed to a failure subsequent to viral intermediate gene expression and intact DNA replication.

Late transcripts were absent in our analyses, also suggesting insufficient intermediate translation, as this is a prerequisite for late gene expression (Keck *et al.*, 1990; Vos & Stunnenberg, 1988). This notion is supported by the finding that the viral intermediate gene product A1 (viral late gene transcription factor 2) was not synthesized upon C7L mutant virus infection (data not shown). Largely, the mutant phenotype we identified is similar to the host-range phenotype described previously for the non-permissive infection of HeLa cells with a C7L- and K1L-deficient VACV (VV-hr) (Perkus *et al.*, 1990). Similar to the observation that C7 and K1 can complement each other in human cells, we also demonstrated that these factors are interchangeable for MVA to complete gene expression in human as well as in murine cells.

Host-cell restriction of VV-hr was reported to be associated with induction of programmed cell death (Hsiao *et al.*, 2004), thus prompting us to search for signs of apoptosis in MVA-ΔC7L-infected cells. By monitoring caspase-3 activity, we clearly demonstrated that the host-range defect of MVA-ΔC7L was not linked to an early apoptotic event. This observation seems to be in contrast to the report by Nájera *et al.* (2006) that inserting the C7L gene sequence from MVA into the genome of VACV NYVAC strain suppresses induction of caspase-dependent apoptosis in infected HeLa cells. However, this discrepancy may be explained by the different genetic backgrounds of the two

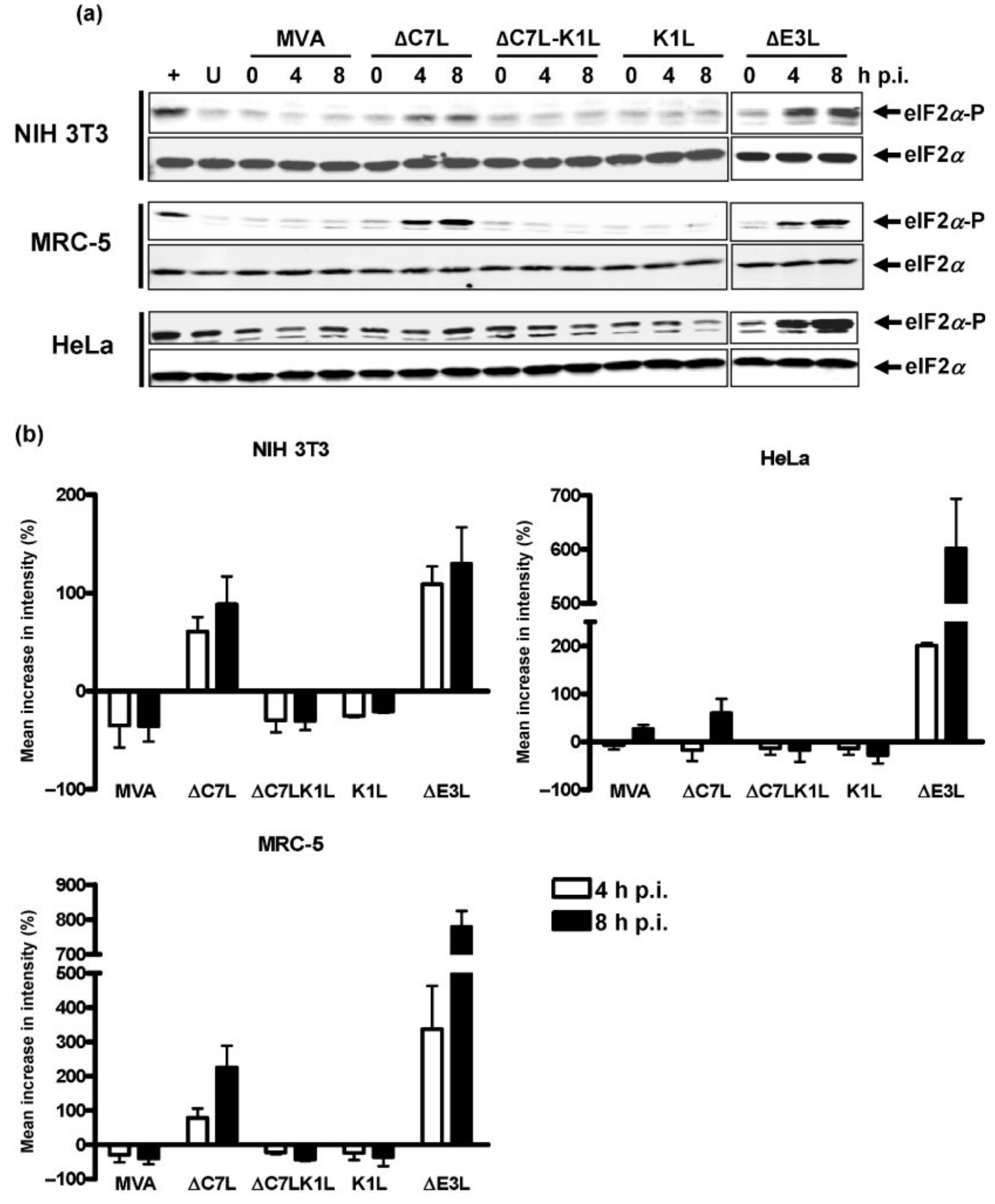
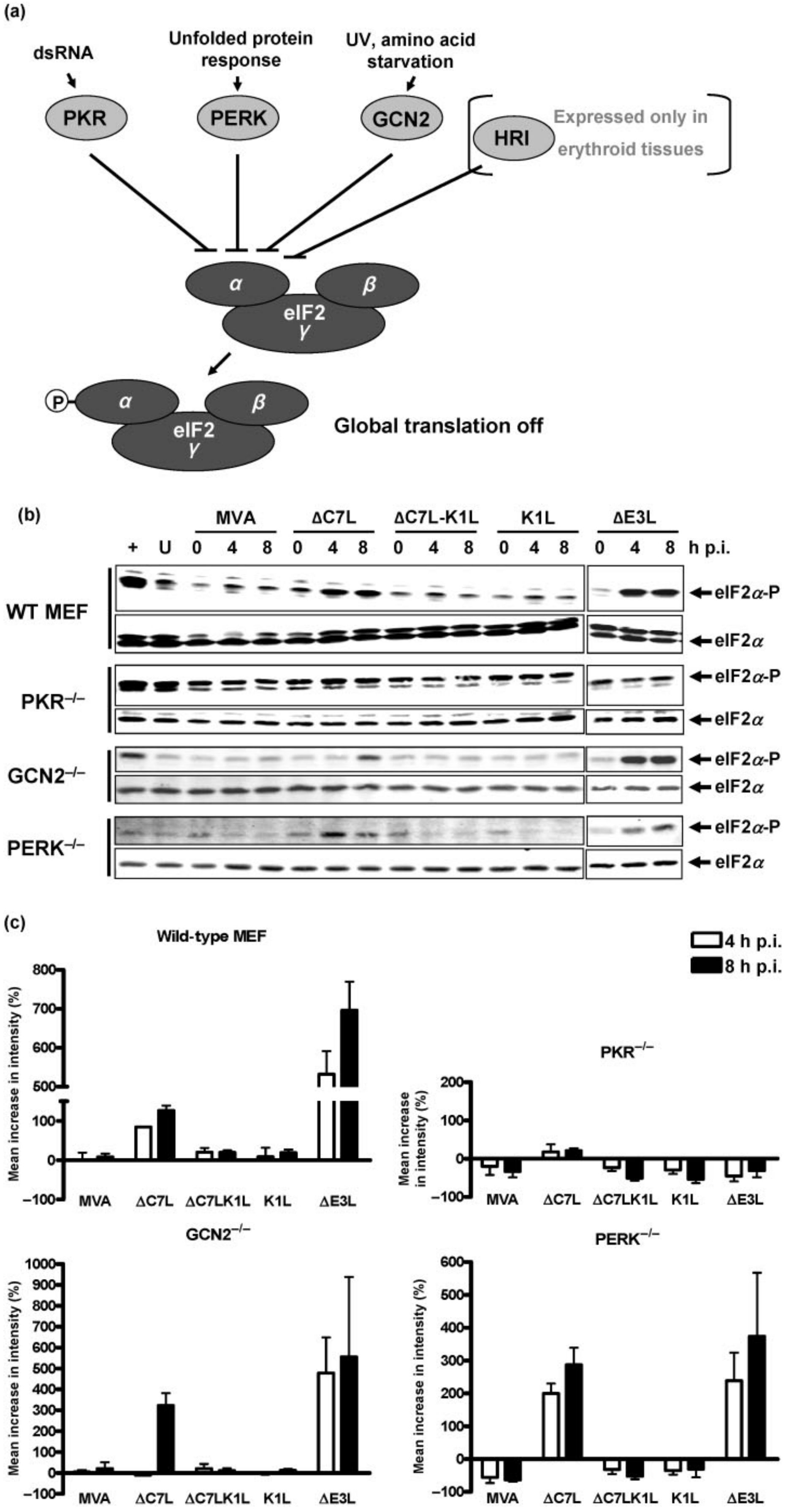


Fig. 4. C7 and K1 inhibit eIF2α phosphorylation. (a) Western blot analysis of eIF2α phosphorylation in NIH 3T3, MRC-5 and HeLa cells infected with wild-type MVA (MVA), MVA-ΔC7L (ΔC7L), MVA-ΔC7L-K1L (ΔC7L-K1L), MVA-K1L (K1L) or MVA-ΔE3L (ΔE3L). Samples at 0, 4 and 8 h p.i. were probed with antibodies specific for eIF2α or phosphorylated eIF2α (eIF2α-P). Uninfected cells (U) served as a control. +, Treatment with 1 μM thapsigargin. (b) Mean increases in signal intensity of phosphorylated eIF2α at 4 and 8 h p.i. relative to 0 h p.i. Data represent means ± SD of two independent experiments.

Fig. 5. Phosphorylation of eIF2α during MVA-ΔC7L infection is mediated by PKR. (a) Schematic illustration of the eIF2α kinases. HRI, Haem-regulated inhibitor. (b) Western blot analysis of phosphorylated (eIF2α-P) and total eIF2α (eIF2α) in $PKR^{-/-}$, $PERK^{-/-}$, $GCN2^{-/-}$ and wild-type (WT) MEFs infected with wild-type MVA (MVA), MVA-ΔC7L (ΔC7L), MVA-ΔC7L-K1L (ΔC7L-K1L), MVA-K1L (K1L) or MVA-ΔE3L (ΔE3L) at 0, 4 and 8 h p.i. Uninfected cells (U) served as a control. +, Treatment with 1 μM thapsigargin. (c) Mean increase in signal intensity of phosphorylated eIF2α at 4 and 8 h p.i. relative to 0 h p.i. Data represent means ± SD of two independent experiments.


(a)
dsRNA
Unfolded protein response
UV, amino acid starvation
PKR
PERK
GCN2
HRI
Expressed only in erythroid tissues
α
β
eIF2
γ
P
Global translation off
(b)
MVA
ΔC7L
ΔC7L-K1L
K1L
ΔE3L
+ U 0 4 8 0 4 8 0 4 8 0 4 8 0 4 8 h p.i.
WT MEF
PKR–/–
GCN2–/–
PERK–/–
eIF2α-P
eIF2α
(c)
Wild-type MEF
4 h p.i.
8 h p.i.
Mean increase in intensity (%)
800
700
600
500
100
0
–100
MVA
ΔC7L
ΔC7LK1L
K1L
ΔE3L
PKR–/–
200
GCN2–/–
1000
900
400
300
PERK–/–

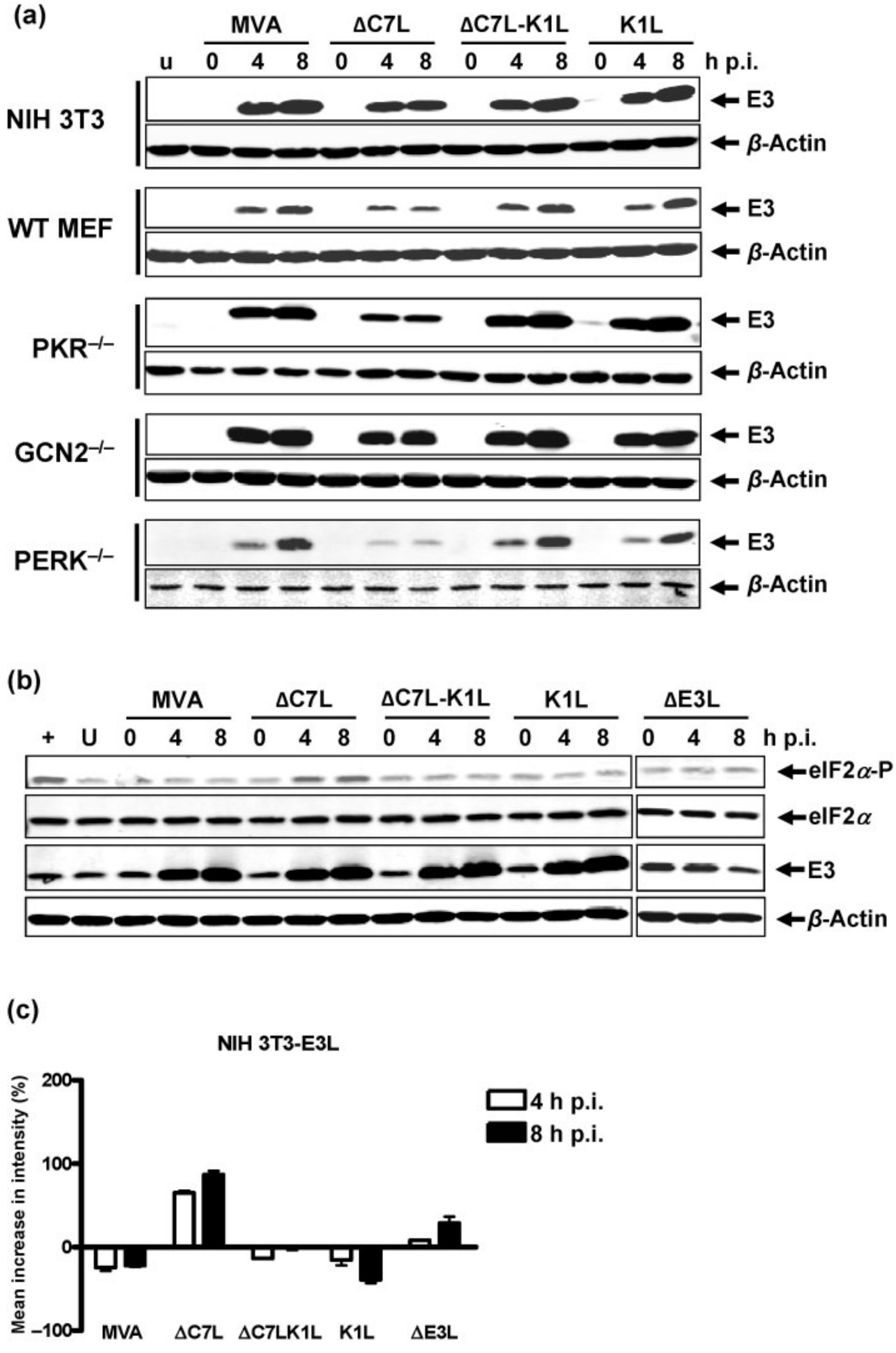


Fig. 6. Reduced E3 expression during MVA-ΔC7L infection is not associated with eIF2α phosphorylation. (a) Western blot analysis of E3 expression in NIH 3T3, PKR$^{-/-}$, PERK$^{-/-}$, GCN2$^{-/-}$ and wild-type MEF cells infected with wild-type MVA (MVA), MVA-ΔC7L (ΔC7L), MVA-ΔC7L-K1L (ΔC7L-K1L) or MVA-K1L (K1L) at 0, 4 and 8 h p.i. Detection of β-actin served as a loading control. (b) Western blot analysis of phosphorylated eIF2α (eIF2α-P), total eIF2α (eIF2α) and E3 expression in NIH 3T3-E3L cells infected with the viruses described in (a) or with MVA-ΔE3L (ΔE3L) at 0, 4 and 8 h p.i. Uninfected cells (U) served as a control. +, Treatment with 1 μM thapsigargin. Equal loading was checked by detection of β-actin. (c) Mean change in signal intensity of phosphorylated eIF2α at 4 and 8 h p.i. relative to 0 h p.i. Data represent means ± SD of two independent experiments.

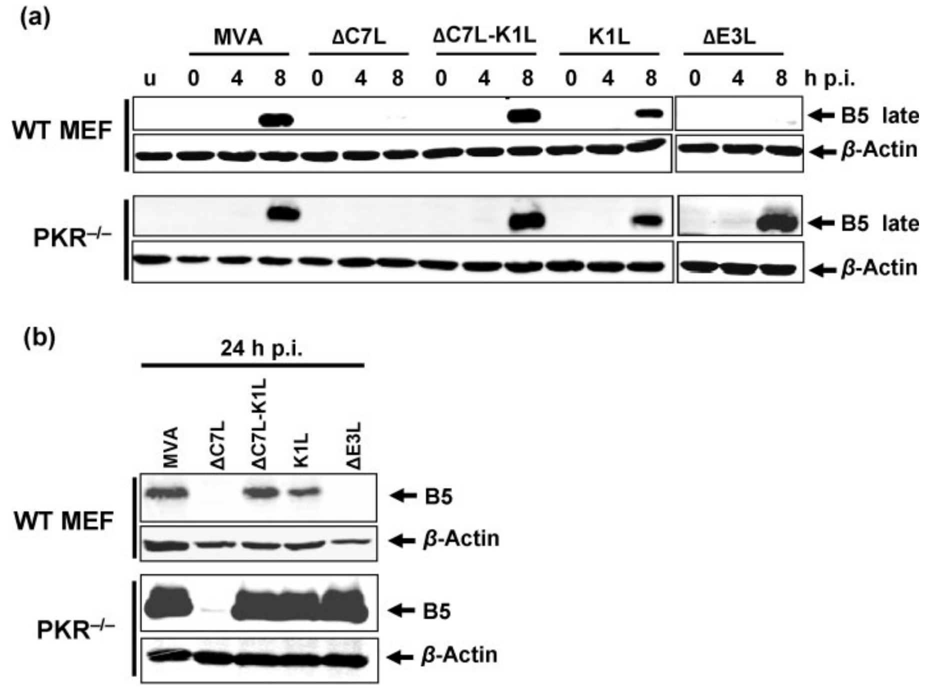


Fig. 7. MVA-ΔC7L late gene expression is not rescued in PKR$^{-/-}$ MEFs. Western blot analysis of B5 expression in wild-type and PKR$^{-/-}$ MEFs infected with wild-type MVA (MVA), MVA-ΔC7L (ΔC7L), MVA-ΔC7L-K1L (ΔC7L-K1L), MVA-K1L (K1L) or MVA-ΔE3L (ΔE3L) at 0, 4 and 8 h p.i. (a) and 24 h p.i. (b). Uninfected cells (U) served as a control.

VACV strains. NYVAC was derived from the VACV strain Copenhagen by targeted deletion of 18 genes, including K1L and C7L. In addition to K1L and C7L, there are 13 other inactivated genes common to both MVA-ΔC7L and VACV NYVAC, with four more open reading frames (ORFs) specifically inactivated in the genome of VACV NYVAC compared with a further 24 inactivated in MVA (Gómez *et al.*, 2008). Such genetic differences may account for the disparate C7L-dependent effects on apoptosis inhibition. Similarly, insertion of C7L into NYVAC allows fully productive infection of human HeLa cells, whereas even co-insertion of the second human host-range gene K1L into MVA is insufficient to result in permissive infection of human or murine cells (Sutter *et al.*, 1994).

Phosphorylation of the α-subunit of eIF2α by PKR represents a well-known cellular pathway, playing a critical role in the antiviral response mediated by interferons (García *et al.*, 2007). The MVA genome encodes two conserved inhibitors of PKR, the viral regulatory proteins E3 and K3 (Davies *et al.*, 1993). Despite the presence of these factors, we observed eIF2α phosphorylation mediated by PKR upon infection of different cell lines with MVA-ΔC7L. Similarly, VV-hr shows enhanced levels of phosphorylated eIF2α during infection, which is inhibited by expressing cowpox host-range factor CP77 as well as C7 and its homologues, myxoma virus M62R and Yaba-like disease virus 67R (Hsiao *et al.*, 2004; Meng *et al.*, 2008). Equally, NYVAC infection of HeLa cells results in elevated phosphorylation of eIF2α, which is decreased by insertion of C7L. Notably, as the genome of MVA is greatly minimized compared with VACV and NYVAC, our observation that MVA-ΔC7L induced phosphorylation of eIF2α points more specifically to C7 being a PKR-inhibiting factor. In addition, we demonstrated for the first time that K1 can also efficiently antagonize eIF2α phosphorylation.

Infection with conventional VACV deleted for K1L and C7L has been observed to diminish E3 protein expression. Consequently, the authors assumed that these reduced E3 protein levels caused by the absence of K1 and C7 were insufficient to inhibit PKR and subsequent phosphorylation of eIF2α (Meng *et al.*, 2008). In contrast, our data on eIF2α phosphorylation in cells stably overproducing functional E3 argue that the somewhat reduced E3 protein levels during MVA-ΔC7L infection are not responsible for eIF2α phosphorylation induced in the absence of C7.

Assuming that eIF2α phosphorylation accounts for global translational inhibition, one would expect that the viral life cycle block observed after MVA-ΔC7L infection of PKR$^{+/+}$ cells should be relieved in cells deficient for PKR. However, PKR-deficient MEFs infected with MVA-ΔC7L revealed no rescue of viral late gene expression. In contrast, infection with MVA-ΔE3L resulted in full rescue of late protein synthesis, in agreement with previous observations (Ludwig *et al.*, 2006).

Interestingly, very recent data from infection of human Huh7 cells with a VACV K1L/C7L double knockout mutant virus suggest that a so-far-unidentified type I interferon-induced antiviral pathway can block VACV protein translation. This antiviral interferon response is PKR-independent and can be inhibited by K1L and C7L (Meng *et al.*, 2009). In addition, upon functional analysis of cowpox virus host-range factor CP77, which can also complement C7L function in VACV, inhibition of eIF2α phosphorylation was also suggested as one, although not the only, activity of this host-range protein (Hsiao *et al.*, 2004). Taken together, these and our findings argue that the host-range factors CP77, K1 and C7 interfere with the host cell to establish an environment favourable for viral gene expression. Thus, as a side effect, their absence could allow signalling events that eventually elicit activation of PKR and subsequent phosphorylation of eIF2α. Which antiviral signalling components/cascades are additionally targeted by these poxvirus host-range proteins remains to be investigated further.

METHODS

Viruses and cells. VACV strain MVA was originally obtained from Anton Mayr (University of Munich, Germany) and the 584th passage on CEFs (cloned isolate F6) was used for this study. Recombinant MVA-P11LZ, MVA-ΔE3L and MVA-ΔF1L have been described elsewhere (Fischer *et al.*, 2006; Hornemann *et al.*, 2003; Sutter & Moss, 1992). All viruses were propagated and titrated following standard methods (Staib & Sutter, 2003). Primary CEFs and rabbit kidney RK13 (ATCC CCL-37) cells were grown in minimal essential medium (MEM) supplemented with 10 % fetal bovine serum (FBS). Baby hamster kidney BHK-21 (ATCC CCL-10), HeLa (ATCC CCL-2), MRC-5 (ATCC CCL-171) and PKR$^{-/-}$ MEF (provided by R. H. Silverman, Cleveland Clinic, OH, USA; Harding *et al.*, 2000a, b) cells were grown in RPMI 1640 supplemented with 10 % FBS. NIH 3T3 (ATCC CRL-1658) cells were grown in Dulbecco's MEM containing 10 % FBS, as were wild-type, GCN2$^{-/-}$ and PERK$^{-/-}$ MEF cells (provided by D. Ron, New York University, NY, USA; Khabar *et al.*, 2000) with the addition of 55 μM β-mercaptoethanol. All cells were maintained at 37 °C with 5 % CO_2.

Plasmids. The C7L deletion plasmid pΔK1L-C7L carries two fragments of sequences flanking the MVA C7L ORF (nt 18859–18407, GenBank accession no. U94848), which were inserted into multiple cloning sites 1 and 2 of plasmid pΔK1L (Staib *et al.*, 2000, 2003). The upstream flanking sequence starts in the 5′ intergenic region and ends at the start codon of the C7L ORF; the downstream flanking sequence extends from the translation termination codon into the 3′ intergenic region. To generate the revertant virus MVA-C7Lrev, the transfer plasmid p018L-Rev was used. This vector contains the C7L gene under its authentic promoter, in between flanking sequences allowing insertion into the MVA-ΔC7L genome at the site of the C7L gene. The transfer plasmid pIIILZ-P11 (Sutter & Moss, 1992) was used to generate MVA-ΔC7L-P11LZ. The transfer plasmid pIII-K1L used to generate MVA-ΔC7L-K1L as well as MVA-K1L carries the K1L ORF under the control of its authentic promoter located between flanking sequences of MVA deletion III.

Genetic modification of MVA. Mutants MVA-ΔC7L, MVA-C7Lrev, MVA-ΔC7L-K1L and MVA-K1L were obtained by following the transient K1L-based host-range selection protocol as described previously (Staib & Sutter, 2003; Staib *et al.*, 2000). To generate MVA-ΔC7L-P11LZ, CEFs were infected with MVA-ΔC7L and transfected with transfer plasmid pIIILZ-P11. Recombinant MVA virus expressing β-galactosidase was selected by plaque purification in CEFs stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (300 μg ml^{-1}) by screening for blue virus plaques (Chakrabarti *et al.*, 1985).

Metabolic labelling. Metabolic labelling of cells mock infected or infected with MVA, MVA-ΔC7L, MVA-C7Lrev or MVA-ΔC7L-K1L at an m.o.i. of 20 was performed as described previously (Sperling *et al.*, 2009). Briefly, cells were labelled at various time points after infection with 50 μCi [^{35}S]methionine (Perkin-Elmer), and the cell lysates were resolved by SDS-PAGE and subsequently analysed by autoradiography.

Northern blot analysis. Northern blot analysis was performed as described previously, including the primer sequences for riboprobes specific for ORFs 005R, 078R and 047R (Ludwig *et al.*, 2005). For C7L, the following primer pair was used: 5′-ATGGGTATACAGCACGAATTC-3′ and 5′-CTAATACGACTCACTATAGGGAGAGACATGGACTCATAATCTCTATAC-3′. The reverse primer contained a T7 RNA polymerase promoter recognition sequence (underlined). *In vitro* RNA labelling, hybridization and signal detection were carried out according to the manufacturer's instructions (DIG RNA labelling kit and anti-DIG detection chemicals; Roche Diagnostics), using 68 °C for hybridization, and a high stringency wash in 0.1 × SSC containing 0.1 % SDS buffer.

Analysis of β-galactosidase reporter gene expression. Cell monolayers in 96-well plates were mock infected or infected with MVA-P11LZ or MVA-ΔC7L-P11LZ at an m.o.i. of 10 and incubated at 37 °C for 18 h. Cells were lysed with 200 μl β-galactosidase lysis buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 10 mM $MgSO_4$, 1 mM EDTA, 0.5 % NP-40, 0.5 % β-mercaptoethanol) per well for 5 min at room temperature. Cell lysate (100 μl) was mixed with 100 μl β-galactosidase reaction buffer [80 mM phosphate buffer (pH 7.4), 10 mM $MgCl_2$, 0.07 % β-mercaptoethanol, 6 mM chlorophenol red-β-D-galactopyranoside; Roche Diagnostics] and after 5–30 min, the β-galactosidase activity was quantified at 570 nm using a microplate reader (Bio-Rad). Absorbance values were normalized to mock infection.

Analysis of viral DNA replication. Genomic viral DNA was isolated from infected cells as described previously (Earl & Moss, 1991). To assess viral DNA replication, total DNA was transferred with a dot blot apparatus to a positively charged nylon membrane (Roche Diagnostics) and hybridized to a randomly digoxigenin (DIG)-labelled MVA-specific probe, generated as described above, with hybridization at 42 °C. Buffers including 2 × SSC with 0.1 % SDS (at room temperature) and 0.5 × SSC with 0.1 % SDS (at 65 °C) were used for low- and high-stringency washes, respectively.

Analysis of apoptosis induction. Induction of apoptosis in NIH 3T3 cells was analysed by measuring active caspase-3 in a fluorescence activated cell sorting (FACS)-based assay, as described previously (Sperling *et al.*, 2009). Briefly, NIH 3T3 cells were mock infected or infected with the respective viruses at an m.o.i. of 5. As a positive control, cells were stimulated with 1 μM staurosporine (Sigma-Aldrich). At 24 h p.i., active caspase-3 was stained with a specific phycoerythrin-labelled antibody (BD Biosciences) and analysed with a BD LSR II flow cytometer (BD Biosciences).

Alternatively, analysis of caspase-3-like activity in HeLa cells was performed by enzymic cleavage assay of the fluorogenic caspase-3 substrate DEVD-AMC as described previously (Fischer *et al.*, 2006). Values are presented as arbitrary relative light units (means ± SD of triplicate reactions).

Western blot analysis. Cells were mock infected or infected with the respective viruses at an m.o.i. of 5. At various time points, lysates were prepared with 1 × SDS lysis buffer [62.5 mM Tris/HCl (pH 6.8), 2 % SDS, 3.2 % β-mercaptoethanol, 0.01 % bromophenol blue, 10 % glycerol] supplemented with protease and phosphatase inhibitor cocktails (Roche Diagnostics) and subsequently centrifuged in Qiashredder columns (Qiagen). After separation by SDS-PAGE and transfer onto PVDF membranes, the membranes were blocked and incubated with the respective antibodies overnight at 4 °C. Primary antibodies were detected with secondary antibodies conjugated to IRDye Infrared Dyes (Li-Cor Biosciences) using an Odyssey Infrared Imaging System (Li-Cor Biosciences).

Anti-eIF2α antibody (Cell Signaling Technology), anti-eIF2α phosphorylated at Ser51 antibody (Sigma Aldrich) and polyclonal antisera from rabbits specific for VACV E3, K1 and B5 protein were applied at 1 : 1000 dilutions. Monoclonal antibodies directed against β-actin (Sigma-Aldrich) and VACV C7 protein were used at 1 : 10 000 and 1 : 10 dilutions, respectively.

Quantification of eIF2α phosphorylation. The signal intensities of phosphorylated and non-phosphorylated eIF2α were quantified using Odyssey application software 2.1 (Li-Cor Biosciences). The intensities of phosphorylated eIF2α were normalized to the intensities of non-phosphorylated eIF2α in the respective samples. An increase or decrease in phosphorylation was calculated at 4 and 8 h p.i. relative to the time point 0 h p.i. Each measurement represents the results from two independent experiments.

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