Published in final edited form as:
Virology. 2009 September 1; 391(2): 177–186. doi:10.1016/j.virol.2009.06.012.

Modified vaccinia virus Ankara can activate NF-κB transcription factors through a double-stranded RNA-activated protein kinase (PKR)-dependent pathway during the early phase of virus replication

Heather E. Lynch[a,1], **Caroline A. Ray**[a], **Katrina L. Oie**[a,2], **Justin J. Pollara**[b], **Ian T. D. Petty**[b], **Anthony J. Sadler**[c], **Bryan R. G. Williams**[c], and **David J. Pickup**[a,d,*]

[a] Department of Molecular Genetics and Microbiology, Box 3020, Duke University Medical Center, Durham, NC 27710, USA

[b] Department of Microbiology, North Carolina State University, Raleigh, NC 27695 USA

[c] Monash Institute of Medical Research, Monash Medical Centre, Clayton, 3168 Victoria, Australia

[d] Human Vaccine Institute, Duke University Medical Center, Durham, NC, 27710 USA

Abstract

Modified vaccinia virus Ankara (MVA), which is a promising replication-defective vaccine vector, is unusual among the orthopoxviruses in activating NF-κB transcription factors in cells of several types. In human embryonic kidney (HEK 293T) cells, the MVA-induced depletion of IκBα required to activate NF-κB is inhibited by UV-inactivation of the virus, and begins before viral DNA replication. In HEK 293T, CHO, or RK13 cells, expression of the cowpox virus *CP77* early gene, or the vaccinia virus *K1L* early gene suppresses MVA-induced IκBα depletion. In mouse embryonic fibroblasts (MEFs), MVA induction of IκBα depletion is dependent on the expression of mouse or human double-stranded RNA-activated protein kinase (PKR). These results demonstrate that events during the early phase of MVA replication can induce PKR-mediated processes contributing both to the activation of NF-κB signaling, and to processes that may restrict viral replication. This property may contribute to the efficacy of this vaccine virus.

Keywords

vaccinia; MVA; NF-kappaB; I-kappaB; K1L; CP77; cowpox; PKR; orthopoxvirus; vaccine

*Corresponding author. Tel: 919 684 2480 Fax: 919 684 8735, E-mail addresses: heather.lynch@duke.edu, ray00020@mc.duke.edu, katrina.l.oie@gsk.com, jjpolla2@ncsu.edu, tim_petty@ncsu.edu, anthony.sadler@med.monash.edu.au, bryan.williams@med.monash.edu.au, picku001@mc.duke.edu.
[1]Current address: Duke Human Vaccine Institute, DUMC 103020, Durham, NC 27710. Fax: 919-681-8992.
[2]Current address: Infectious Diseases Medicine Development Center, GlaxoSmithKline Five Moore Drive, 17.1352B, Research Triangle Park, NC 27709. Fax: (704) 625-9200

Introduction

The cellular response to viral infection usually involves major changes in host gene expression to effect the suppression of viral replication by the induction of immune responses. However, the various mechanisms that enable cells to sense viral infections and respond in this way are not completely understood.

Some of the most important effectors of virus-induced changes in host gene expression and immune responses are the NF-κB transcription factors (reviewed by Hayden, West, and Ghosh, 2006). These transcription factors are activated in response to a wide variety of stimuli, including pathogen-associated molecular patterns (PAMPs), as well as other components of immune responses (reviewed by Carmody and Chen, 2007; Doyle and O'Neill, 2006; Meylan and Tschopp, 2006). The NF-κB transcription factors are held inactive by their association with Inhibitor of κB (IκB) proteins. The classical pathway of activation of NF-κB involves the phosphorylation and degradation of the IκB proteins. This allows the released NF-κB transcription factors to bind to DNA, and then induce the transcription of a wide range of genes, including many of critical importance in immune responses, cell proliferation, and apoptosis (reviewed by Hoffmann and Baltimore, 2006; Perkins and Gilmore, 2006).

Viruses of many types have acquired mechanisms to inhibit the antiviral effects of NF-κB activation, though some viruses exploit NF-κB activation for their own replicative purposes (reviewed by Hiscott, Kwon, and Genin, 2001; Hiscott et al., 2006). The orthopoxviruses appear to be primarily in the former group, encoding several proteins that act in various ways to prevent NF-κB activation. These viral proteins include the following: secreted receptors for interleukin (IL)-1β, tumor necrosis factor (TNF), IL-18, and CD30L, each of which are cytokines that can activate NF-κB; an inhibitor of the IL-1β processing enzyme (caspase-1) needed for the processing, activation and secretion of both IL-1 and IL-18; and several inhibitors of components of the IL-1/Toll-like receptor signaling pathways (reviewed by Moss and Shisler, 2001; Seet et al., 2003).

Previously, we showed that orthopoxviruses of several types, namely vaccinia viruses Western Reserve (VV-WR) and Copenhagen (VV-Cop), cowpox virus Brighton red (CPXV-BR), and raccoonpox virus V71-I-85A (RCN), each failed to induce NF-κB signaling upon infection of HEK293 cells. In addition, each of these viruses inhibited TNF-mediated induction of NF-κB signaling, suggesting that these viruses encoded one or more inhibitors of TNF signaling pathways. In marked contrast, the modified vaccinia virus Ankara (MVA) strain induced NF-κB signaling upon infection of HEK 293 cells (Oie and Pickup, 2001). These results suggested that MVA, which lacks functional versions of many of the accessory genes found in other orthopoxviruses (Antoine et al., 1998), might either lack genes encoding inhibitors of NF-κB signaling, or have a greater propensity to induce NF-κB signaling than some of the other orthopoxviruses, or possess a combination of both properties (Oie and Pickup, 2001). Several studies have now shown that MVA lacks several of the inhibitors of NF-κB signaling encoded by other orthopoxviruses. MVA does not encode any of the five inhibitory members of the TNF receptor family encoded by other orthopoxviruses (Hu, Smith, and Pickup, 1994; Loparev et al., 1998; Panus et al., 2002; Saraiva and Alcami, 2001; Smith et al., 1996). It does not encode a version of the vaccinia virus A52R protein that can interact with IL-1 receptor associated kinase (IRAK2) and TNF associated factor 6 (TRAF6) to inhibit IL-1/toll-like receptor induced NF-κB signaling pathways (Bowie et al., 2000; Harte et al., 2003). Shisler and Jin (2004) have shown that repair of the disrupted MVA *K1L* gene can reduce MVA-mediated induction of NF-κB signaling. The N1 protein, which has been found to be capable of associating with components of the IκB kinase complex and suppressing NF-κB signaling (DiPerna et al., 2004) is truncated in MVA in comparison to other orthopoxvirus N1L proteins (Antoine et al.,

1998). MVA also lacks the *M2L* gene, which was recently shown to inhibit viral induction of ERK2-mediated NF-κB activation in HEK 293T cells (Gedey et al., 2006).

The conservation of genes encoding these inhibitory proteins among most of the orthopoxviruses suggests that fundamental properties of orthopoxvirus structure or replication may intrinsically provide stimuli that can activate NF-κB signaling pathways. The nature of the responses to infection may then be governed both by the capacity of the cell to sense and transduce these stimuli, and by the presence or absence of viral inhibitory factors. Whilst the activation of NF-κB, and the ensuing immune responses, may be detrimental to poxviral replication *in vivo*, such stimulation of immune responses may be beneficial in humans when induced by a replication-impaired vaccine virus, such as MVA (reviewed by Pickup, 2007). For this reason, a greater understanding of the viral factors contributing to the activation and suppression of NF-κB in response to orthopoxvirus infections should assist the development of more effective poxviral vaccines.

Results

Early events in MVA replication induce NF-κB activation

The object of this study was to identify elements of virus-cell interactions that contribute to orthopoxvirus-induced activation of NF-κB signaling pathways. MVA infection of HEK 293 cells provides a useful experimental system with which to study this process. In this system, we previously demonstrated that MVA infection, (i) induced IκBα degradation, (ii) caused the accumulation of nuclear NF-κB capable of binding DNA fragments containing NF-κB binding sites, and (iii) induced the transcription of reporter genes under the control of NF-κB-regulated promoters (Oie and Pickup, 2001). The degradation of IκBα provides a sensitive and rapid measure of early events in the activation of the classical NF-κB signaling pathway. Therefore, in this study, IκBα degradation was used as the primary indicator of the effects of MVA infection upon the activation of NF-κB.

Analyses of the kinetics of activation of NF-κB signaling after MVA infection of HEK 293T cells showed that IκBα degradation occurs between 1 and 2 hours after inoculation, with degradation continuing through 4 hours post-inoculation (Fig. 1A). Subsequently, the IκBα is partially replenished, and from 8 hours post-inoculation up to 24 hours post-inoculation, IκBα is maintained in amounts less than those in mock-inoculated cells. At this later stage, the IκBα in the infected cells is refractory to further depletion in response to treatment with either additional virus, or exogenous TNFα (data not shown).

To confirm that events during the early phase of virus infection are sufficient to induce IκBα degradation, we examined the effects of MVA infection on IκBα degradation in the presence or absence of cytosine arabinoside (AraC), an inhibitor of viral DNA synthesis. Incubation of mock-inoculated (uninfected) cells in the presence of AraC did not cause detectable IκBα degradation over the time course of the experiment (data not shown). In contrast, MVA-infected cells incubated in the presence of AraC exhibited IκBα degradation that was similar to that in virus-infected cells that were not treated with the drug (Fig. 1B). This result shows that an early event in the MVA infection cycle, preceding viral DNA replication and late gene expression, is sufficient to trigger IκBα degradation.

To determine whether protein synthesis was required for MVA-mediated induction of NF-κB signaling, virus infections were carried out in the presence of cycloheximide. The protein synthesis inhibitor was added to the cells at the same time as the virus. Under these conditions, IκBα degradation was still induced by virus (Fig. 1C). This suggests that neither viral nor cellular protein synthesis is necessary to induce IκBα degradation. However, in the presence of cycloheximide, IκBα degradation occurred with similar kinetics, but to a lesser extent than

occurred in the absence of cycloheximide, suggesting that factors arising from the continued cellular protein synthesis and the progression of virus replication to the late phase contribute to the continued degradation of IκBα during the course of the infection. During this period, as the physiological state of the infected cell changes, viral or cellular protein synthesis may provide additional stimuli or factors that promote or enhance IκBα degradation.

The apparent lack of requirement for viral protein synthesis to initiate virus-induced IκBα degradation raised the possibility that direct interactions between the virus particles and the host cell might be sufficient to induce IκBα degradation. Virus particles can be photochemically inactivated, without affecting their structure, by treatment with the cross-linking agent psoralen and long-wave ultraviolet (UV) irradiation (reviewed by Hanson, 1992). When applied to vaccinia virus, limited UV-psoralen treatment leads to a loss of infectivity, as with increasing duration of the cross-linking treatment there is progressively greater inhibition of viral gene expression (Tsung et al., 1996). Accordingly, MVA was incubated with psoralen, and equal aliquots were subsequently irradiated with long-wave UV light for periods of up to eight minutes. The aliquots of MVA were used to inoculate HEK 293T cells to determine the effect of UV-psoralen treatment on virus-induced IκBα degradation. The amounts of infectious virus remaining in each aliquot were determined by titration on BHK cells. As shown in Fig. 2, the extent of IκBα degradation was directly related to the infectious titer of the inoculum. Although the cell cultures each received equivalent amounts of virus particles, degradation of IκBα occurred only in cells that received infectious virus particles, suggesting that virion components alone delivered to the cells do not activate NF-κB signaling in HEK 293T cells.

Together, these results indicate that events occurring early after the initiation of virus infection are necessary and sufficient to induce NF-κB signaling upon MVA infection of HEK 293T cells. The requirement for infectious virions, together with the ability of MVA to induce IκBα degradation in the presence of cycloheximide, implicate viral early RNAs as prime candidates for stimulating NF-κB activation.

Viral host-range proteins inhibit MVA-induced depletion of IκB

The genome of MVA comprises only a subset of the genes present in other orthopoxviruses (Antoine et al., 1998), suggesting that MVA-mediated activation of NF-κB signaling may at least in part be due to the lack of genes encoding inhibitors of NF-κB activation (Oie and Pickup, 2001). Consistent with this model, an early gene, the *K1L* host-range gene (Perkus, Limbach, and Paoletti, 1989), which is intact in VV-WR, but disrupted in the MVA genome, was found to be capable of inhibiting MVA-induced NF-κB activation in HEK 293T cells (Shisler and Jin, 2004). In addition, analyses of a deletion mutant of cowpox virus (CPXV A549), which lacks an intact copy of the cowpox virus homologue of the vaccinia virus *K1L* gene, demonstrated that cowpox virus encodes proteins other than the K1L protein, which are capable of inhibiting TNF-induced, and possibly virus-induced, degradation of IκBα (data not shown).

One candidate gene that is expressed by cowpox virus, but non-functional in all characterized strains of vaccinia virus is the *CP77* gene. This early gene, which corresponds to the CPXV025 gene in the genome of CPXV-BR (Genbank accession number **AF482758**), has also been referred to as the CHO host-range (*CHO hr*) gene, because it was originally identified as a gene required to permit cowpox virus to replicate in CHO cells (Spehner et al., 1988). Subsequently, it was found that the *CP77* gene could functionally substitute for the *K1L* gene to permit the replication of vaccinia virus in cells of certain types including human cells (Perkus et al., 1990; Ramsey-Ewing and Moss, 1996). Both the K1L protein and the CP77 protein were shown to overcome host-range restriction during the early phase of vaccinia virus replication in RK13 cells, consistent with their function being to prevent the inhibition of translation (Drillien, Spehner, and Kirn, 1978; Ramsey-Ewing and Moss, 1996; Sutter et al., 1994).

Further, Hsiao et al. (2004) had noted both that the infection of HeLa cells with a vaccinia virus deletion mutant lacking 18-kbp of the viral genome, including the *K1L* and *C7L* host-range genes, resulted in the rapid induction of phosphorylation of PKR and eIF2α, and that these effects were suppressed by the expression of the *CP77* gene. These observations suggested that the CP77 protein might possess many of the same properties as the K1L protein, including an ability to inhibit virus-induced IκBα degradation.

To test this hypothesis, a recombinant virus (MVA/CP77) was constructed, in which the disrupted *CP77* gene in the MVA genome was replaced by a copy of the functional gene from cowpox virus. Analyses of protein synthesis in CHO cells infected with either MVA or MVA/CP77 confirmed that the substitution of the cowpox virus gene conferred the expected host-range phenotype on the recombinant MVA. The presence of a functional *CP77* gene enabled the recombinant MVA to both suppress the rapid inhibition of protein synthesis induced by the unmodified virus, and permit the synthesis of early and late viral proteins (Fig. 3). Further analysis of MVA/CP77 confirmed that the repair of the *CP77* gene permitted the recombinant MVA virus to replicate both in CHO cells and RK13 cells (data not shown). In contrast to MVA, MVA/CP77 did not induce the depletion of IκBα in virus-infected HEK 293T cells (Fig. 4A). Indeed, the MVA/CP77 virus behaved similarly to recombinant virus MVA/K1L (virus A664) in which the disrupted *K1L* gene has been repaired (Fig. 4B).

These results showed that the functional similarities between *CP77* and *K1L* genes noted previously in the context of viral host-range also extend to their abilities to inhibit MVA-induced depletion of IκBα in HEK 293T cells. This raised the question of possible connections between the host-range functions of these genes and their abilities to interfere with virus-induced depletion of IκBα. However, the *K1L* gene is not sufficient to extend the host range of MVA to HEK 293 cells (Carroll and Moss, 1997). In these and other human cells, the primary restriction on MVA replication appears to be not the expression of viral genes, but the assembly of progeny virus particles (Gallego-Gomez et al., 2003; Sancho et al., 2002; Sutter and Moss, 1992), and this process appears to be impaired irrespective of the expression of the *K1L* gene. In cells of other types that are non-permissive for MVA or certain strains of vaccinia virus, the block in viral replication occurs at an earlier phase of replication, where cell and viral protein synthesis is inhibited prior to the onset of viral late gene expression, as for example in MVA-infected CHO cells (Fig. 3). Expression of the *CP77* gene alleviates this block to viral replication in CHO cells (Spehner et al., 1988). Similarly, expression of the *K1L* gene alleviates this block to MVA replication in RK13 cells (Meyer, Sutter, and Mayr, 1991). The growth phenotypes of MVA/K1L and MVA/CP77 in RK13 cells and CHO cells were similar to those reported previously for MVA/K1L recombinants or equivalent VV-WR recombinants expressing the *K1L* or *CP77* genes (Perkus et al., 1990; Carroll and Moss, 1997). While MVA cannot productively infect either RK13 cells or CHO cells, MVA/K1L was able to productively infect RK13 cells, and MVA/CP77 could productively infect cells of either type. As in cells of other types, MVA infection of RK13 cells or CHO cells induced the depletion of IκBα within two hours post-infection, whereas after infection with MVA/CP77, there was less depletion of IκBα in CHO cells (about 20–30% of original levels remaining at 4 hours post-infection), and no detectable depletion of IκBα in RK13 cells (Fig. 5).

Accordingly, there is a correlation between the expression of the *K1L* and *CP77* host-range genes, and the inhibition of virus-induced depletion of IκBα. However, MVA replicates in BHK cells where virus-induced IκBα depletion occurs (Oie and Pickup, 2001). This suggests that the pathways leading to viral activation of NF-κB signaling, and the pathways leading to host-range restriction may be divergent or separate. The host-range proteins may separately affect these pathways, or they may produce the observed effects on each pathway by targeting components common to each pathway.

The double-stranded RNA activated protein kinase, PKR, is required for MVA-induced IκBα degradation in mouse embryo fibroblasts

There are a number of possible mechanisms through which MVA might induce NF-κB signaling via viral early RNAs. One potential mechanism is via Toll-like receptors (TLRs), which can detect a wide variety of agonists, including components of virus particles and nucleic acids, to induce signaling leading to the activation of NF-κB (reviewed by Barton, 2007; Bowie and Haga, 2005; Finberg and Kurt-Jones, 2004). However, TLR-mediated induction of NF-κB signaling is unlikely both because HEK 293T cells are not responsive to most TLR agonists unless TLRs are adventitiously expressed in these cells, and because orthopoxviruses, including MVA, encode inhibitors of TLR-induced signaling pathways. The main alternative mechanism of signal induction is via intracellular nucleic acid sensors such as the RIG-I related RNA helicases, and PKR (reviewed by Bowie and Fitzgerald, 2007; Garcia, Meurs, and Esteban, 2007; Meylan and Tschopp, 2006). In particular, PKR has long been known to mediate IκBα degradation and NF-κB activation (Kumar et al., 1994), and to be targeted for inhibition by proteins encoded by various orthopoxviruses (Beattie, Tartaglia, and Paoletti, 1991; Chang, Watson, and Jacobs, 1992; Davies et al., 1993; Davies et al., 1992). This prompted us to determine if PKR might be one of the factors involved in the induction of NF-κB signaling by MVA.

Transgenic mice homozygous for a deletion of exons 2 and 3 in the PKR gene ($PKR^{0/0}$ mice) have been generated, and cells from these mice were shown to express no detectable PKR protein (Yang et al., 1995). To determine if PKR is involved in MVA-induced IκBα depletion, embryonic fibroblasts derived from these $PKR^{0/0}$ mice, or PKR wild-type ($PKR^{+/+}$) control mice, were infected with MVA (Fig. 6A). Depletion of IκBα occurred in MVA-infected $PKR^{+/+}$ cells with kinetics similar to those of IκBα depletion in MVA-infected HEK 293T cells, occurring between 1 and 2 hours after infection, and maintained through 4 hours post-infection. In contrast, MVA infection of $PKR^{0/0}$ cells did not result in detectable depletion of IκBα (Fig. 6A), which suggests that functional PKR is required for MVA-induced depletion of IκBα.

To confirm that the phenotype seen in $PKR^{0/0}$ mouse cells was specifically due to the absence of functional PKR, we performed a rescue experiment, using derivatives of these cells that either stably express (Hu PKR^{+}), or do not express (Hu PKR^{-}), the wild-type human PKR gene (Deb et al., 2001; Yang et al., 1995). MVA infection of Hu PKR^{-} cells resulted in no detectable depletion of IκBα (Fig. 6B), whereas MVA infection of Hu PKR^{+} cells resulted in a rapid and sustained depletion of IκBα (Fig. 6B), similar to that seen in MVA-infected $PKR^{+/+}$ cells.

Although MVA infection of $PKR^{+/+}$ cells results in the depletion of IκBα, both early and late viral genes appear to be expressed, and unlike in CHO cells (Fig. 3), in these $PKR^{+/+}$ cells, MVA-mediated expression of the *CP77* gene had no discernible effects on cellular or viral protein synthesis (Fig. 7). These results are consistent with those of Ludwig *et al.*, 2006, who showed that MVA directed expression of both intermediate and late genes in both $PKR^{+/+}$ and $PKR^{0/0}$ cells. Thus, all temporal classes of MVA genes are expressed in these MEFs either in the presence or the absence of PKR, and irrespective of MVA-induced depletion of IκBα.

Collectively, these results show that one mechanism of MVA-induced depletion of IκBα during the early phase of virus replication is dependent upon PKR. The infection of either $PKR^{+/+}$ (wt) cells, or Hu PKR^{+} cells, with either MVA/CP77 or MVA/K1L resulted in the inhibition of MVA-induced depletion of IκBα (Fig. 8), consistent with a role for each of the CP77 and K1L proteins in the inhibition of PKR-mediated IκBα depletion.

Discussion

This study and earlier studies have shown that orthopoxviruses can induce NF-κB signaling upon infection (Gedey et al., 2006; Guerra et al., 2004; Guerra et al., 2006; Oie and Pickup, 2001; Shisler and Jin, 2004). Induction is dependent upon the viral species, viral strain, and the cell-type, suggesting that induction of the NF-κB transcription factors might be a contributory factor to the differences in the pathogenic and immunogenic properties of the various orthopoxviruses. The transient nature of IκBα depletion induced by virus infection of these cells is similar to that induced by TNFα in uninfected cells (reviewed by Hoffmann, Natoli, and Ghosh, 2006; Moss et al., 2008; Scheidereit, 2006), except that in the latter, the IκBα is replenished within about 90 minutes (Oie and Pickup, 2001). The more prolonged period of depletion of IκBα by virus infection may occur because of continued stimulation post-infection, or viral inhibition of host protein synthesis, which would be expected to reduce the capacity of the infected cell to replace the degraded IκBα or synthesize NF-κB to stabilize IκBα (Mathes et al., 2008). Virus infection may also interfere with other processes involved in the cellular suppression of NF-κB signaling, including the nuclear export of NF-κB/IκB complexes (Moss et al., 2008), and the expression or the functions of negative-feedback regulators of NF-κB signaling, such as host cell deubiquitinating enzymes (Enesa et al., 2008; Heyninck and Beyaert, 2005).

The results of this study demonstrate that one mechanism by which MVA can initiate a signaling pathway leading to the activation of NF-κB is through a PKR-dependent process stimulated by an early event during viral replication. This result is consistent with results of several previous studies showing that PKR is both one of the major intracellular sensors of virus replication, and a factor capable of inducing the activation of NF-κB (reviewed by Garcia et al., 2006).

MVA infection is capable of inducing IκBα degradation and NF-κB signaling in HEK 293T cells, whereas several other orthopoxviruses, including vaccinia virus (WR), raccoonpox virus, and cowpox virus (BR) do not cause a similar induction. MVA may have a greater capacity to activate the PKR-mediated pathway of NF-κB signaling than some of the other orthopoxviruses. In addition, it may lack some of the capacities of other orthopoxviruses to inhibit PKR-mediated processes. One indication that MVA may have a greater capacity to initiate NF-κB activation than some of the other orthopoxviruses is that MVA induces IκBα degradation even in the presence of cycloheximide, whereas cowpox virus induces little if any IκBα degradation under similar conditions (Oie and Pickup, 2001). This suggests that irrespective of the presence or absence of viral inhibitors of NF-κB signaling, these two viruses differ in their abilities to stimulate signaling.

Our results are consistent with those of previous studies indicating that vaccinia virus has the capacity to induce PKR activation. In particular, with respect to MVA, recently Ludwig et al. (2006) demonstrated that infection of various human cells, or MEFs, with MVA (MVAΔE3L) lacking the *E3L* gene induced the activation of PKR. Infection with MVAΔE3L resulted in the inhibition of translation of viral intermediate mRNAs. The K3L protein appears to play a secondary role to E3L, because in a variety of human cells and MEFs, in the absence of E3L, the K3L protein does not appear to be sufficient to suppress phosphorylation of eIF2α. Thus, MVA has both the capacity to induce PKR-mediated effects, and, through E3L and K3L proteins, the capacity to suppress at least some of these effects. The observed cell-type specific nature of this mechanism of MVA-mediated activation of PKR-dependent NF-κB signaling may in part reflect differences among the properties of PKR-dependent signaling pathways in cells of different types (Langland and Jacobs, 2002).

The nature of the stimuli responsible for MVA-mediated activation of IκBα degradation has yet to be determined. Double-stranded RNA (dsRNA) is a prime candidate for the stimulus, because this is one of the major inducers of PKR (reviewed by Garcia, Meurs, and Esteban, 2007). Ludwig et al. (2006) suggested that one potential source of the MVA-mediated activation of PKR might be dsRNAs generated by the transcription of viral intermediate genes, because dsRNAs were readily detectable among these mRNAs. Our results show that MVA can activate PKR-mediated signaling pathways even in the absence of viral DNA replication, suggesting that early viral events may provide the first stimuli to induce PKR-mediated host responses to viral infection. Whilst the majority of complementary RNAs capable of forming dsRNAs are produced during the intermediate and late phases of virus replication, there is evidence that vaccinia virus infection may generate smaller amounts of dsRNAs at early times during infection (Boone, Parr, and Moss, 1979; Colby, Jurale, and Kates, 1971). These small quantities of early viral dsRNAs might be capable of triggering host responses to infection. Initial evidence to this effect was provided by Kibler et al. (1997), who noted that although AraC had no effect on apoptosis in mock-infected cells or in cells infected with wild-type virus, apoptosis was induced in HeLa cells infected with VV-Cop (VVΔE3L) lacking the *E3L* gene even in the presence of AraC, albeit at a reduced amount in comparison to that in cells infected in the absence of AraC. They suggested that the reduced apoptotic response in AraC-treated cells might be the result of a reduced amount of dsRNAs in VVΔE3L-infected AraC-treated cells versus untreated cells. Further, early viral RNA synthesis was found to be necessary for vaccinia virus induction of the rapid cessation of protein synthesis in non-permissive CHO cells (Drillien, Spehner, and Kirn, 1978).

One possible contributory factor that might differentiate MVA from many other orthopoxviruses with respect to its ability to induce PKR-mediated signaling at early times might be its capacity to generate complementary early RNAs. Through its extended passage *in vitro*, MVA may have acquired mutations either in genome structure, or transcriptional control elements, permitting more extended early transcription of complementary regions than other orthopoxviruses.

Another major difference between MVA and other orthopoxviruses that do not induce the early activation of PKR signaling may be the lack of a full complement of genes in MVA encoding viral inhibitors of cellular sensors of viral infection. Although MVA encodes both the E3L and K3L inhibitors of PKR, it has been shown to lack a number of orthopoxvirus genes known to encode inhibitors of various pathways of NF-κB activation. It lacks the vaccinia virus *A52R* gene, which encodes a protein that can disrupt signaling mediated by IRAK2 and TRAF6 (Harte et al., 2003). It lacks a functional version of the vaccinia virus *K1L* gene, which encodes a protein capable of suppressing MVA-induced activation of NF-κB (Shisler and Jin, 2004). It lacks a functional version of the vaccinia virus *M2L* gene, which encodes a protein capable of suppressing MVA-mediated activation of NF-κB via an ERK2-dependent pathway (Gedey et al., 2006). And, although it contains on open reading frame capable of encoding a protein similar (141/149 residues identical) to the vaccinia virus B14 protein that can interfere with IkappaB kinase Beta activity (Chen et al., 2008), it is not known if this variant protein is functional in MVA. In this study, we have confirmed a role for the *K1L* gene in suppressing MVA-mediated activation of NF-κB. In addition, we have shown that the cowpox virus *CP77* gene, which can functionally substitute for the *K1L* gene to permit replication of MVA in RK13 cells, also can suppress MVA-induced degradation of IκBα. Our results suggest a role for each of these host-range proteins in the suppression of virus-induced activation of PKR-mediated signaling pathways. Interestingly, such a role also provides a potential explanation for one mechanism of action of these host-range proteins in extending viral host range, because PKR is one of the primary effectors of the virus-induced inhibition of protein synthesis that can render cells refractory to viral replication. Therefore, the results suggest a model whereby through the inhibition of PKR-mediated functions, the K1L and CP77 proteins may each

contribute not only to the suppression of viral NF-κB activation, but also to the suppression of PKR-mediated processes contributing to host-range restriction of virus replication. Whether or not the K1L and CP77 proteins interfere with the activation of other PKR-mediated antiviral processes, such as the activation of p38 mitogen-activated protein kinase (Goh, deVeer, and Williams, 2000), and the induction of interferon regulatory factors 1 and 3 (Kirchhoff et al., 1995; Kumar et al., 1997; Zhang and Samuel, 2008) remains to be determined.

In most natural orthopoxvirus infections, the early induction of PKR-mediated responses leading to the activation of NF-κB-mediated transcription, and cell-type specific inhibition of protein synthesis would be expected to adversely affect viral replication *in vivo*, as suggested by the altered properties and attenuation of vaccinia viruses lacking the *E3L* gene (Brandt et al., 2005; Brandt and Jacobs, 2001; Deng et al., 2006; Langland et al., 2006; Ludwig et al., 2006; Zhang and Samuel, 2008). However, these same properties may be highly advantageous for a vaccine virus (Jentarra et al., 2008; Vijaysri et al., 2008). The unusual ability of MVA to induce early-phase PKR-mediated responses leading potentially both to the stimulation of immune responses, and to the impairment of virus replication, may prove to be one of the principal factors contributing to the efficacy and the safety of this vaccine virus.

Materials and Methods

Cells and viruses

Syrian baby hamster kidney 21 (BHK21) cells were maintained in minimum essential media (MEM) alpha (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS; Sigma, St. Louis, MO). HEK 293T cells and BSC40 cells were maintained in MEM (Gibco) with 10% FBS. Human osteosarcoma (143B) cells were maintained in MEM supplemented with 5% FBS. Chinese hamster ovary K1 (CHO) cells were maintained in F-12 Kaighn's media (F-12 K; Gibco) supplemented with 10% FBS. Rabbit kidney (RK13) cells were maintained in MEM supplemented with non-essential amino acids (Gibco) and 10% FBS. Mouse embryonic fibroblast (MEF) cells derived from $PKR^{+/+}$ and $PKR^{0/0}$ mice as described by Yang et al. (995) were grown in HGDMEM with 10% FBS. Bac7 and Bac10 cells (Deb et al., 2001), which are $PKR^{-/-}$ MEF cells containing bacterial artificial chromosomes (BAC) harboring the human PKR gene that is expressed or not expressed, respectively, were maintained in HGDMEM supplemented with 200 μg/ml of Zeocin (Invitrogen, Carlsbad, CA) and 10% FBS.

Viruses employed in this study were: A660, a plaque-purified isolate derived from MVA obtained from Dr. B Moss, (NIH); A392, a plaque-purified isolate of the VV-WR strain; CPXV-BR, cowpox virus Brighton red strain; A665, the MVA/CP77 virus, which is MVA containing the CPXV-BR *CP77* gene in place of the disrupted MVA counterpart to this gene; A664, the MVA/K1L virus that contains a functional VV-WR *K1L* gene in place of the disrupted MVA *K1L* gene.

Antisera and other reagents

IκBα was detected using a rabbit, polyclonal, anti-IκB-α (C-21) IgG from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Donkey, anti-rabbit-horseradish peroxidase (HRP) conjugate was from Amersham (Piscataway, NJ). GAPDH was detected with HRP-conjugated mouse monoclonal antibody to human GAPDH (Abcam, Cambridge, MA). Rabbit, polyclonal, anti-vaccinia virus (strain Lister) antibody was obtained from Biodesign International (Saco, ME). Recombinant, human, TNFα (R&D Systems Inc., Minneapolis, MN) was reconstituted at a concentration of 10 μg/ml in sterile phosphate buffered saline (PBS) with 0.1% bovine serum albumin (BSA). Cycloheximide and cytosine arabinoside (AraC) were obtained from

Sigma. Psoralen (trioxsalen, 4′-aminomethyl-, hydrochloride; Calbiochem, San Diego, CA) was reconstituted in dimethyl sulfoxide at a concentration of 2.5 mg/ml, and stored at 4°C.

Virus stock preparation and virus purification

MVA was purified through three rounds of plaque purification in BHK21 cells, cultured, purified, and quantified in these cells as described (Carroll and Moss, 1997; Earl, 1991). MVA/CP77 and MVA/K1L (A664) viruses were plaque-purified and cultured as described (Staib, Drexler, and Sutter, 2004). CPXV-BR was cultured in human 143B cells.

Ultraviolet (UV) inactivation of MVA

MVA was inactivated by UV irradiation as described (Humlova et al., 2002). Briefly, aliquots of 1.5×10^8 pfu from a lysate MVA stock were each brought to a volume of 1ml in Hank's Balanced Salt solution with 0.1% BSA and psoralen at a final concentration of 2 μg/ml. The aliquots were incubated at room temperature for 10 minutes in 3.5 cm^2 dishes. After this time, one aliquot was transferred to a 1.5 ml tube and put on ice while the other aliquots were put under 365 nm UV light from a UV lamp (Model UVGL-58, UVP, San Gabriel, CA) held at a height of three inches. After various (2–8) minutes of exposure to UV, each aliquot was removed. The aliquots of virus were used either for titration in BHK21 cells, or for the infection of HEK 293T cells.

Preparation of whole-cell and cytoplasmic extracts

Extracts were prepared by methods similar to those described (Dignam, Lebovitz, and Roeder, 1983; Laegreid et al., 1994). Whole-cell lysates were made by lysing cells for 1–5 minutes on ice in 50–100 μl of sample buffer containing 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT, 0.01% bromophenol blue (Laemmli, 1970), supplemented with Roche protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), 20 mM β-glycerophosphate, 10 mM NaF, and 1 mM Na_3VO_4. The whole-cell extracts were then boiled for 5 minutes, vortexed, and stored at −20°C. Cytoplasmic extracts were prepared as described (Oie and Pickup, 2001).

Western blot analysis

Proteins from equal amounts of cells were separated by SDS-PAGE (Laemmli, 1970) in 8–16% polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS; pH 8.2). To confirm equal protein loading and transfer among samples, the proteins were visualized by staining with SYPRO Ruby Total Protein Stain (Sigma) as follows. PAGE-resolved proteins were fixed to the membranes for 15 minutes in a 10% methanol, 7% acetic acid solution, and then washed four times with double-distilled H_2O (ddH_2O) for 5 minutes. The membranes were then stained for 15–20 minutes with SYPRO Ruby Total Protein Stain. After five 1-minute washings with ddH_2O, the proteins on the membranes were visualized by UV illumination. To visualize specific proteins of interest, the membranes were then blocked with 5% milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for 30–60 minutes at room temperature. The membranes were incubated in 5% milk/TBST at a 1:5000 dilution for 2 hours to overnight with primary rabbit antibodies against proteins of interest. Membranes were washed three times for five minutes with TBST before incubation with the secondary antibody, a donkey anti-rabbit-HRP conjugate, at a dilution of 1:5000 in TBST for 1–1.5 hours. After four ten-minute washes with TBST, the proteins were detected using enhanced chemiluminescence (ECL Plus kit; Amersham, Pittsburgh, PA). Alternatively, for detection of GAPDH as a gel loading control, after protein transfer to the membranes, the membranes were blocked with Pierce Superblock plus 0.05% Tween (Superblock-T) for 1 hr at room temperature. The IκBα was detected with a 1: 5000 dilution of rabbit anti-IκBα antibodies incubated in 20% Superblock –T solution at

4°C overnight. Bound antibodies were detected with anti-rabbit-HRP conjugate, at a dilution of 1:10000 in 20% Superblock-T after development with Pierce SuperSignal West Pico chemiluminescent substrate. When probing the same blot for GAPDH, the HRP-conjugated to antibodies on the blot was inactivated by incubating the membrane at 65°C in TBS for 1 hour, then reblocking for 30 min, followed by incubation with 1:40,000 anti-GAPDH mAB in Superblock-T solution for 1 hour at room temperature.

Pulse-labeling experiments

Cells were labeled by adding Promix (Amersham), containing [^{35}S] radiolabeled methionine and cysteine, directly to the growth medium (15 μCi/ml) for 30 minutes before harvesting the cells. Before adding the Promix, growth media was removed, and methionine- and cysteine-free media (Gibco) was added. To harvest, the media containing radiolabel was removed, and the cells were lysed in whole-cell lysis buffer.

Plasmid and recombinant virus constructs

Plasmid constructs were generated using standard techniques. Restriction enzymes were obtained from New England Biolabs, Inc. (Beverly, MA). DNA fragments were amplified using the polymerase chain reaction (PCR) in the Applied Biosystems GeneAmp PCR System 2400 (Applied Biosystems, Branchburg, NJ). PCR was done using 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute 30 seconds at 72°C. The Advantage HF-2 Polymerase kit (BD Biosciences, Palo Alto, CA) was used in high-fidelity PCR amplification for cloning purposes. PCR for construct screening was done using the AmpliTaq DNA Polymerase kit from Applied Biosystems. DNA fragments were isolated from agarose gels using the QIAEX II Gel Extraction Kit (QIAGEN, Germantown, MD). For ligations, the Rapid DNA Ligation Kit (Roche Diagnostics, Indianapolis, IN) was used. Construction of plasmids was verified using sequence analysis, which was performed by the Duke University DNA Analysis Facility. Oligonucleotide primers were generated by IDT (Coralville, IA) or Operon Biotechnologies, Inc. (Huntsville AL).

The MVA/K1L (A664) virus was constructed as follows. PCR was used to generate a 1403-bp DNA fragment containing VV-WR *K1L* gene flanked by an upstream sequence corresponding to 522-bp at the 3′-end of N2L ORF in the MVA genome. An insertion vector containing this construct was used to replace the disrupted *K1L* gene present in MVA, with selection for the recombinant MVA/K1L virus in RK13 cells as described (Staib, Drexler, and Sutter, 2004). The purity of the final plaque-purified isolate was confirmed by PCR using primers that flanked the *K1L* gene. Subsequently, the virus was cultured in RK13 cells.

The MVA/CP77 virus was constructed as follows. The 2287-bp NdeI-HpaI fragment of CPXV-BR DNA (present in plasmid p62) containing the *CP77* gene was subcloned into plasmid vector pYes2, to make plasmid pYes2-25. This NdeI-HpaI fragment was transfected into MVA-infected BHK cells, and recombinant MVA capable of replicating in CHO cells were isolated and plaque-purified in RK13 cells. The purity of the final plaque-purified isolate was confirmed both by PCR using primers that allowed us to distinguish between the intact *CP77* gene and the disrupted MVA homolog of this gene, and by DNA hybridization analyses, as described (Southern, 1975). A probe was used that corresponded to the *CP77* gene and about 100 bases of flanking sequence. The radiolabeled probe was generated using the NEBlot kit (New England Biolabs) according to manufacturer's instructions. The probes were isolated from free label using the Promega Wizard DNA Clean-up kit, according to manufacturer's instructions. The probes were denatured by boiling for 5 minutes, then quenched by adding to the 5x SSC/5X Denhardt's/0.5% SDS hybridization buffer. Hybridizations of the probes to the immobilized DNAs were done at 65°C overnight. The membranes were washed four times for

10 minutes at 65°C with 500 ml of 2x SSC/0.1% SDS, and then wrapped in plastic film. The annealed probes were visualized by autoradiography.

Acknowledgments

This work was supported by grant 1U54AI057157 from the National Institutes of Health to the Southeastern Regional Center of Excellence in Emerging Infections and Biodefense (SERCEB). HEL and KLO were partially supported by U.S. Public Health Service Grants 5P30-AI-051445, AI32982, and T32CA09111. DJP is a member of the Duke University Comprehensive Cancer Center, and the Duke Human Vaccine Institute, whose shared core facilities were used in this study.

References

Antoine G, Scheiflinger F, Dorner F, Falkner FG. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. Virology 1998;244 (2):365–96. [PubMed: 9601507]

Barton GM. Viral recognition by Toll-like receptors. Seminars in immunology 2007;19 (1):33–40. [PubMed: 17336545]

Beattie E, Tartaglia J, Paoletti E. Vaccinia virus-encoded eIF-2 alpha homolog abrogates the antiviral effect of interferon. Virology 1991;183 (1):419–22. [PubMed: 1711259]

Boone RF, Parr RP, Moss B. Intermolecular duplexes formed from polyadenylylated vaccinia virus RNA. J Virol 1979;30 (1):365–74. [PubMed: 480457]

Bowie A, Kiss-Toth E, Symons JA, Smith GL, Dower SK, O'Neill LA. A46R and A52R from vaccinia virus are antagonists of host IL-1 and toll-like receptor signaling. Proc Natl Acad Sci U S A 2000;97 (18):10162–7. [PubMed: 10920188]

Bowie AG, Fitzgerald KA. RIG-I: tri-ing to discriminate between self and non-self RNA. Trends Immunol 2007;28 (4):147–50. [PubMed: 17307033]

Bowie AG, Haga IR. The role of Toll-like receptors in the host response to viruses. Mol Immunol 2005;42 (8):859–67. [PubMed: 15829275]

Brandt T, Heck MC, Vijaysri S, Jentarra GM, Cameron JM, Jacobs BL. The N-terminal domain of the vaccinia virus E3L-protein is required for neurovirulence, but not induction of a protective immune response. Virology 2005;333(2):263–70. [PubMed: 15721360]

Brandt TA, Jacobs BL. Both carboxy- and amino-terminal domains of the vaccinia virus interferon resistance gene, E3L, are required for pathogenesis in a mouse model. J Virol 2001;75 (2):850–6. [PubMed: 11134298]

Carmody RJ, Chen YH. Nuclear factor-kappaB: activation and regulation during toll-like receptor signaling. Cellular & molecular immunology 2007;4 (1):31–41. [PubMed: 17349209]

Carroll MW, Moss B. Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a nonhuman mammalian cell line. Virology 1997;238 (2):198–211. [PubMed: 9400593]

Chang HW, Watson JC, Jacobs BL. The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase. Proc Natl Acad Sci U S A 1992;89 (11): 4825–9. [PubMed: 1350676]

Chen RA, Ryzhakov G, Cooray S, Randow F, Smith GL. Inhibition of IkappaB kinase by vaccinia virus virulence factor B14. PLoS Pathog 2008;4 (2):e22. [PubMed: 18266467]

Colby C, Jurale C, Kates JR. Mechanism of synthesis of vaccinia virus double-stranded ribonucleic acid in vivo and in vitro. Journal of virology 1971;7 (1):71–6. [PubMed: 5543434]

Davies MV, Chang HW, Jacobs BL, Kaufman RJ. 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 1993;67 (3):1688–92. [PubMed: 8094759]

Davies MV, Elroy-Stein O, Jagus R, Moss B, Kaufman RJ. The vaccinia virus K3L gene product potentiates translation by inhibiting double-stranded-RNA-activated protein kinase and phosphorylation of the alpha subunit of eukaryotic initiation factor 2. J Virol 1992;66 (4):1943–50. [PubMed: 1347793]

Deb A, Zamanian-Daryoush M, Xu Z, Kadereit S, Williams BR. Protein kinase PKR is required for platelet-derived growth factor signaling of c-fos gene expression via Erks and Stat3. Embo J 2001;20 (10):2487–96. [PubMed: 11350938]

Deng L, Dai P, Ding W, Granstein RD, Shuman S. Vaccinia virus infection attenuates innate immune responses and antigen presentation by epidermal dendritic cells. J Virol 2006;80 (20):9977–87. [PubMed: 17005676]

Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic acids research 1983;11 (5):1475–89. [PubMed: 6828386]

DiPerna G, Stack J, Bowie AG, Boyd A, Kotwal G, Zhang Z, Arvikar S, Latz E, Fitzgerald KA, Marshall WL. Poxvirus protein N1L targets the I-kappaB kinase complex, inhibits signaling to NF-kappaB by the tumor necrosis factor superfamily of receptors, and inhibits NF-kappaB and IRF3 signaling by toll-like receptors. J Biol Chem 2004;279 (35):36570–8. [PubMed: 15215253]

Doyle SL, O'Neill LA. Toll-like receptors: from the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity. Biochemical pharmacology 2006;72 (9):1102–13. [PubMed: 16930560]

Drillien R, Spehner D, Kirn A. Host range restriction of vaccinia virus in Chinese hamster ovary cells: relationship to shutoff of protein synthesis. J Virol 1978;28 (3):843–50. [PubMed: 310474]

Earl, PL.; Cooper, N.; Moss, B. Preparation of cell cultures and vaccinia virus stocks. In: Ausubel, REBFM.; Kingston, DD.; Moore, JD.; Seidman, JA.; Smith, JA.; Struhl, K., editors. Current Protocols in Molecular Biology. Vol. 2. Greene Publishing Associates, Wiley-Interscience; New York: 1991. p. 16.16.1-16.16.7.

Enesa K, Zakkar M, Chaudhury H, Luong le A, Rawlinson L, Mason JC, Haskard DO, Dean JL, Evans PC. NF-kappaB suppression by the deubiquitinating enzyme Cezanne: a novel negative feedback loop in pro-inflammatory signaling. J Biol Chem 2008;283 (11):7036–45. [PubMed: 18178551]

Finberg RW, Kurt-Jones EA. Viruses and Toll-like receptors. Microbes Infect 2004;6 (15):1356–60. [PubMed: 15596120]

Gallego-Gomez JC, Risco C, Rodriguez D, Cabezas P, Guerra S, Carrascosa JL, Esteban M. Differences in virus-induced cell morphology and in virus maturation between MVA and other strains (WR, Ankara, and NYCBH) of vaccinia virus in infected human cells. J Virol 2003;77 (19):10606–22. [PubMed: 12970445]

Garcia MA, Gil J, Ventoso I, Guerra S, Domingo E, Rivas C, Esteban M. Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. Microbiol Mol Biol Rev 2006;70(4):1032–60. [PubMed: 17158706]

Garcia MA, Meurs EF, Esteban M. The dsRNA protein kinase PKR: Virus and cell control. Biochimie 2007;89 (6–7):799–811. [PubMed: 17451862]

Gedey R, Jin XL, Hinthong O, Shisler JL. Poxviral regulation of the host NF-kappaB response: the vaccinia virus M2L protein inhibits induction of NF-kappaB activation via an ERK2 pathway in virus-infected human embryonic kidney cells. Journal of virology 2006;80 (17):8676–85. [PubMed: 16912315]

Goh KC, deVeer MJ, Williams BR. The protein kinase PKR is required for p38 MAPK activation and the innate immune response to bacterial endotoxin. Embo J 2000;19 (16):4292–7. [PubMed: 10944112]

Guerra S, Lopez-Fernandez LA, Conde R, Pascual-Montano A, Harshman K, Esteban M. Microarray Analysis Reveals Characteristic Changes of Host Cell Gene Expression in Response to Attenuated Modified Vaccinia Virus Ankara Infection of Human HeLa Cells. J Virol 2004;78 (11):5820–34. [PubMed: 15140980]

Guerra S, Lopez-Fernandez LA, Pascual-Montano A, Najera JL, Zaballos A, Esteban M. Host response to the attenuated poxvirus vector NYVAC: upregulation of apoptotic genes and NF-kappaB-responsive genes in infected HeLa cells. J Virol 2006;80 (2):985–98. [PubMed: 16379000]

Hanson CV. Photochemical inactivation of viruses with psoralens: an overview. Blood Cells 1992;18 (1):7–25. [PubMed: 1617194]

Harte MT, Haga IR, Maloney G, Gray P, Reading PC, Bartlett NW, Smith GL, Bowie A, O'Neill LA. The poxvirus protein A52R targets Toll-like receptor signaling complexes to suppress host defense. J Exp Med 2003;197(3):343–51. [PubMed: 12566418]

Hayden MS, West AP, Ghosh S. NF-kappaB and the immune response. Oncogene 2006;25 (51):6758–80. [PubMed: 17072327]

Heyninck K, Beyaert R. A20 inhibits NF-kappaB activation by dual ubiquitin-editing functions. Trends Biochem Sci 2005;30 (1):1–4. [PubMed: 15653317]

Hiscott J, Kwon H, Genin P. Hostile takeovers: viral appropriation of the NF-kappaB pathway. The Journal of clinical investigation 2001;107(2):143–51. [PubMed: 11160127]

Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S. Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene 2006;25 (51):6844–67. [PubMed: 17072332]

Hoffmann A, Baltimore D. Circuitry of nuclear factor kappaB signaling. Immunological reviews 2006;210:171–86. [PubMed: 16623771]

Hoffmann A, Natoli G, Ghosh G. Transcriptional regulation via the NF-kappaB signaling module. Oncogene 2006;25 (51):6706–16. [PubMed: 17072323]

Hsiao JC, Chung CS, Drillien R, Chang W. The cowpox virus host range gene, CP77, affects phosphorylation of eIF2 alpha and vaccinia viral translation in apoptotic HeLa cells. Virology 2004;329 (1):199–212. [PubMed: 15476887]

Hu FQ, Smith CA, Pickup DJ. Cowpox virus contains two copies of an early gene encoding a soluble secreted form of the type II TNF receptor. Virology 1994;204 (1):343–56. [PubMed: 8091665]

Humlova Z, Vokurka M, Esteban M, Melkova Z. Vaccinia virus induces apoptosis of infected macrophages. J Gen Virol 2002;83 (Pt 11):2821–32. [PubMed: 12388819]

Jentarra GM, Heck MC, Youn JW, Kibler K, Langland JO, Baskin CR, Ananieva O, Chang Y, Jacobs BL. Vaccinia viruses with mutations in the E3L gene as potential replication-competent, attenuated vaccines: scarification vaccination. Vaccine 2008;26 (23):2860–72. [PubMed: 18455281]

Kibler KV, Shors T, Perkins KB, Zeman CC, Banaszak MP, Biesterfeldt J, Langland JO, Jacobs BL. Double-stranded RNA is a trigger for apoptosis in vaccinia virus-infected cells. J Virol 1997;71 (3): 1992–2003. [PubMed: 9032331]

Kirchhoff S, Koromilas AE, Schaper F, Grashoff M, Sonenberg N, Hauser H. IRF-1 induced cell growth inhibition and interferon induction requires the activity of the protein kinase PKR. Oncogene 1995;11 (3):439–45. [PubMed: 7543195]

Kumar A, Haque J, Lacoste J, Hiscott J, Williams BR. Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B. Proc Natl Acad Sci U S A 1994;91 (14):6288–92. [PubMed: 7912826]

Kumar A, Yang YL, Flati V, Der S, Kadereit S, Deb A, Haque J, Reis L, Weissmann C, Williams BR. Deficient cytokine signaling in mouse embryo fibroblasts with a targeted deletion in the PKR gene: role of IRF-1 and NF-kappaB. Embo J 1997;16 (2):406–16. [PubMed: 9029159]

Laegreid A, Medvedev A, Nonstad U, Bombara MP, Ranges G, Sundan A, Espevik T. Tumor necrosis factor receptor p75 mediates cell-specific activation of nuclear factor kappa B and induction of human cytomegalovirus enhancer. The Journal of biological chemistry 1994;269 (10):7785–91. [PubMed: 8126005]

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227 (5259):680–5. [PubMed: 5432063]

Langland JO, Jacobs BL. The role of the PKR-inhibitory genes, E3L and K3L, in determining vaccinia virus host range. Virology 2002;299 (1):133–41. [PubMed: 12167348]

Langland JO, Kash JC, Carter V, Thomas MJ, Katze MG, Jacobs BL. Suppression of proinflammatory signal transduction and gene expression by the dual nucleic acid binding domains of the vaccinia virus E3L proteins. J Virol 2006;80 (20):10083–95. [PubMed: 17005686]

Loparev VN, Parsons JM, Knight JC, Panus JF, Ray CA, Buller RM, Pickup DJ, Esposito JJ. A third distinct tumor necrosis factor receptor of orthopoxviruses. Proc Natl Acad Sci U S A 1998;95 (7): 3786–91. [PubMed: 9520445]

Ludwig H, Suezer Y, Waibler Z, Kalinke U, Schnierle BS, Sutter G. Double-stranded RNA-binding protein E3 controls translation of viral intermediate RNA, marking an essential step in the life cycle of modified vaccinia virus Ankara. J Gen Virol 2006;87 (Pt 5):1145–55. [PubMed: 16603515]

Mathes E, O'Dea EL, Hoffmann A, Ghosh G. NF-kappaB dictates the degradation pathway of IkappaBalpha. EMBO J 2008;27 (9):1357–67. [PubMed: 18401342]

Meyer H, Sutter G, Mayr A. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. J Gen Virol 1991;72 (Pt 5):1031–8. [PubMed: 2033387]

Meylan E, Tschopp J. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. Molecular cell 2006;22 (5):561–9. [PubMed: 16762830]

Moss B, Shisler JL. Immunology 101 at poxvirus U: immune evasion genes. Semin Immunol 2001;13 (1):59–66. [PubMed: 11289800]

Moss BL, Gross S, Gammon ST, Vinjamoori A, Piwnica-Worms D. Identification of a ligand-induced transient refractory period in nuclear factor-kappaB signaling. J Biol Chem 2008;283 (13):8687–98. [PubMed: 18203717]

Oie KL, Pickup DJ. Cowpox virus and other members of the orthopoxvirus genus interfere with the regulation of NF-kappaB activation. Virology 2001;288 (1):175–87. [PubMed: 11543670]

Panus JF, Smith CA, Ray CA, Smith TD, Patel DD, Pickup DJ. Cowpox virus encodes a fifth member of the tumor necrosis factor receptor family: a soluble, secreted CD30 homologue. Proc Natl Acad Sci U S A 2002;99 (12):8348–53. [PubMed: 12034885]

Perkins ND, Gilmore TD. Good cop, bad cop: the different faces of NF-kappaB. Cell death and differentiation 2006;13 (5):759–72. [PubMed: 16410803]

Perkus ME, Goebel SJ, Davis SW, Johnson GP, Limbach K, Norton EK, Paoletti E. Vaccinia virus host range genes. Virology 1990;179 (1):276–86. [PubMed: 2171207]

Perkus ME, Limbach K, Paoletti E. Cloning and expression of foreign genes in vaccinia virus, using a host range selection system. J Virol 1989;63 (9):3829–36. [PubMed: 2547999]

Pickup DJ. Understanding orthopoxvirus interference with host immune responses to inform novel vaccine design. Expert Rev Vaccines 2007;6 (1):87–95. [PubMed: 17280481]

Ramsey-Ewing AL, Moss B. Complementation of a vaccinia virus host-range K1L gene deletion by the nonhomologous CP77 gene. Virology 1996;222 (1):75–86. [PubMed: 8806489]

Sancho MC, Schleich S, Griffiths G, Krijnse-Locker J. The block in assembly of modified vaccinia virus Ankara in HeLa cells reveals new insights into vaccinia virus morphogenesis. J Virol 2002;76 (16): 8318–34. [PubMed: 12134037]

Saraiva M, Alcami A. CrmE, a novel soluble tumor necrosis factor receptor encoded by poxviruses. J Virol 2001;75 (1):226–33. [PubMed: 11119592]

Scheidereit C. IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. Oncogene 2006;25 (51):6685–705. [PubMed: 17072322]

Seet BT, Johnston JB, Brunetti CR, Barrett JW, Everett H, Cameron C, Sypula J, Nazarian SH, Lucas A, McFadden G. Poxviruses and immune evasion. Annu Rev Immunol 2003;21:377–423. [PubMed: 12543935]

Shisler JL, Jin XL. The vaccinia virus K1L gene product inhibits host NF-kappaB activation by preventing IkappaBalpha degradation. J Virol 2004;78 (7):3553–60. [PubMed: 15016878]

Smith CA, Hu FQ, Smith TD, Richards CL, Smolak P, Goodwin RG, Pickup DJ. Cowpox virus genome encodes a second soluble homologue of cellular TNF receptors, distinct from CrmB, that binds TNF but not LT alpha. Virology 1996;223 (1):132–47. [PubMed: 8806547]

Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of molecular biology 1975;98 (3):503–17. [PubMed: 1195397]

Spehner D, Gillard S, Drillien R, Kirn A. A cowpox virus gene required for multiplication in Chinese hamster ovary cells. J Virol 1988;62 (4):1297–304. [PubMed: 2831390]

Staib C, Drexler I, Sutter G. Construction and isolation of recombinant MVA. Methods Mol Biol 2004;269:77–100. [PubMed: 15114009]

Sutter G, Moss B. Nonreplicating vaccinia vector efficiently expresses recombinant genes. Proc Natl Acad Sci U S A 1992;89 (22):10847–51. [PubMed: 1438287]

Sutter G, Ramsey-Ewing A, Rosales R, Moss B. Stable expression of the vaccinia virus K1L gene in rabbit cells complements the host range defect of a vaccinia virus mutant. J Virol 1994;68 (7):4109–16. [PubMed: 8207789]

Tsung K, Yim JH, Marti W, Buller RM, Norton JA. Gene expression and cytopathic effect of vaccinia virus inactivated by psoralen and long-wave UV light. J Virol 1996;70 (1):165–71. [PubMed: 8523521]

Vijaysri S, Jentarra G, Heck MC, Mercer AA, McInnes CJ, Jacobs BL. Vaccinia viruses with mutations in the E3L gene as potential replication-competent, attenuated vaccines: intra-nasal vaccination. Vaccine 2008;26 (5):664–76. [PubMed: 18096276]

Yang YL, Reis LF, Pavlovic J, Aguzzi A, Schafer R, Kumar A, Williams BR, Aguet M, Weissmann C. Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. EMBO J 1995;14 (24):6095–106. [PubMed: 8557029]

Zhang P, Samuel CE. Induction of protein kinase PKR-dependent activation of interferon regulatory factor 3 by vaccinia virus occurs through adapter IPS-1 signaling. J Biol Chem 2008;283(50):34580–34587. [PubMed: 18927075]

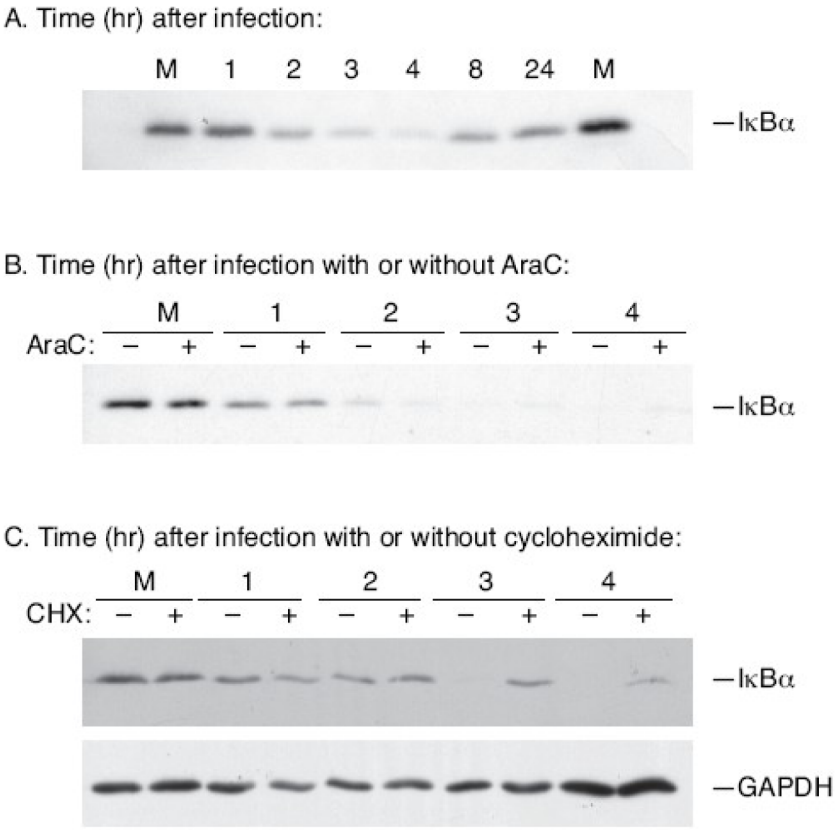

Fig. 1.

(A) MVA-induced IκBα depletion is transient and begins during the early phase of viral replication. HEK 293T cells were mock infected (M) with PBS or infected with MVA (20 pfu/cell). At the indicated times post-infection, cells were harvested and cytoplasmic extracts were prepared. Proteins in the extracts were resolved by PAGE, and transferred to nitrocellulose membranes. IκBα was detected with rabbit antibody against human IκBα and visualized by ECL. Equal protein loading and the efficient transfer of proteins to the membrane were verified using the fluorescent SYPRO Total Protein Stain. (B) Inhibition of DNA synthesis does not affect MVA-induced IκBα depletion. At the start of the infection, HEK 293T cells were either treated with 40 ng/mL of cytosine arabinoside (AraC), or left untreated. Cells were mock infected (M) with PBS, or infected with MVA (20 pfu/cell). At the indicated times post-infection, cells were harvested, and the depletion of IκBα was monitored as described in Fig. 1A. (C) Virus-induced IκBα depletion is reduced in MVA-infected cells when protein synthesis is inhibited with cycloheximide. At the start of infection, HEK 293T cells were either treated with 100 μg/ml cycloheximide (CHX), or left untreated. Cells were mock infected (M) with PBS, or infected with MVA (20 pfu/cell). At the indicated times post-infection, cells were harvested, and the depletion of IκBα was monitored as described in Fig. 1A. The membrane was subsequently processed for the detection of GAPDH as an additional control for protein loading per lane.

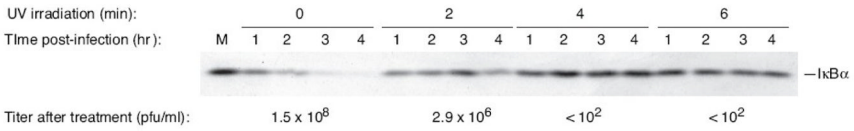

Fig. 2.

IκBα depletion is not induced by infection with UV-inactivated MVA. HEK 293T cells were mock-infected (M) with PBS, or infected with MVA incubated with psoralen cross-linker followed by 0, 2, 4, or 6 min. of UV irradiation. The quantity of virus used in each inoculum was equivalent to that of 20 pfu/cell before UV irradiation. At 1, 2, 3, and 4 hours post-infection, cells were harvested and cytoplasmic extracts were prepared. Proteins in the extracts were resolved by PAGE, and transferred to nitrocellulose membranes. IκBα was detected with rabbit antibody against human IκBα and visualized by ECL. Equal protein loading and efficient transfer were verified using the fluorescent SYPRO Total Protein Stain. The infectious titers (pfu/ml) of each inoculum after UV-irradiation were determined by plaque assay in BHK21 cells.

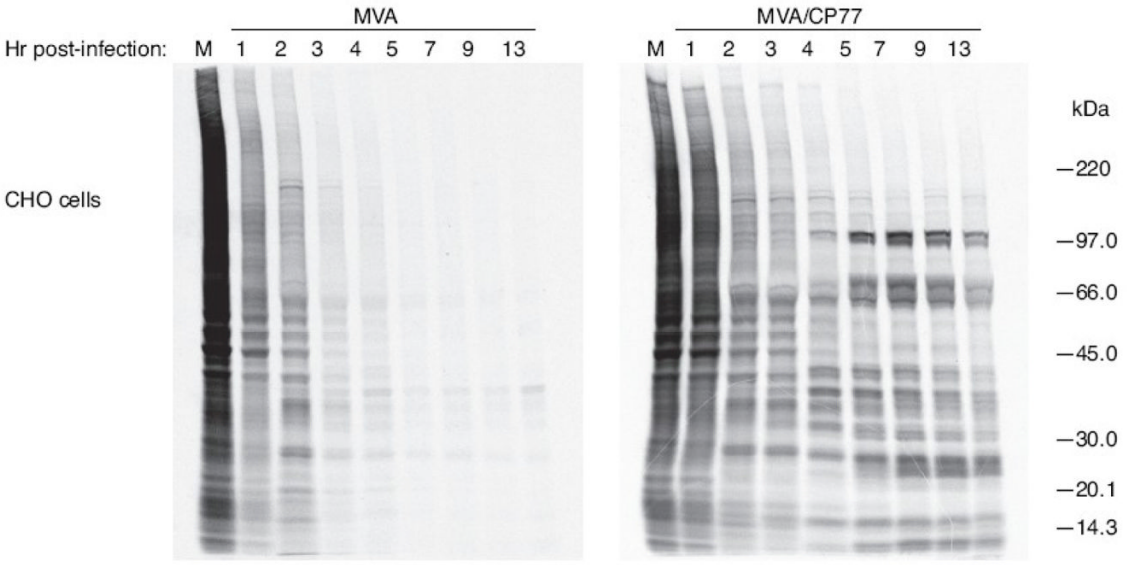

Fig. 3.

Expression of the cowpox virus *CP77* gene enables MVA protein synthesis in CHO cells. CHO cells were mock-infected (M) with PBS, infected with MVA, or infected with MVA/CP77, which expresses the *CP77* gene (each infection at 20 pfu/cell). At the indicated times post-infection, after a 30-minute incubation in methionine-free and cysteine-free media, cells were incubated in media containing [^{35}S]-labeled methionine and cysteine. After 30 minutes of incubation, cells were harvested, and whole-cell lysates were prepared. Proteins were resolved by SDS-PAGE through an 8–16% Tris-glycine gradient gel (Invitrogen). Equal protein loading was verified by Coomassie blue staining (data not shown). The gels were then dried, and radiolabeled proteins were visualized by autoradiography. The mobilities of molecular mass standards are indicated.

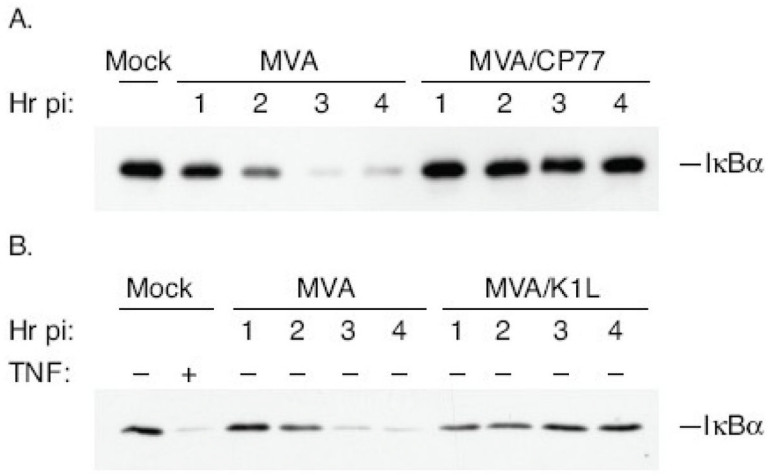

Fig. 4.

MVA-induced IκBα degradation is inhibited by the expression of either the cowpox virus *CP77* gene or the vaccinia virus *K1L* gene. (A) HEK 293T cells were mock infected with PBS, or infected with MVA, or with MVA/CP77 (20 pfu/cell). At the indicated times post-infection, cells were harvested, and the depletion of IκBα was monitored as described in Fig. 1A. (B) HEK 293T cells were mock infected with PBS, or infected with MVA, or with MVA/K1L (20 pfu/cell). For a positive control, TNF-treated cells were treated with 10ng/ml of human TNF for 20 minutes before harvest. At the indicated times post-infection, cells were harvested, and the depletion of IκBα was monitored as described in Fig. 1A.

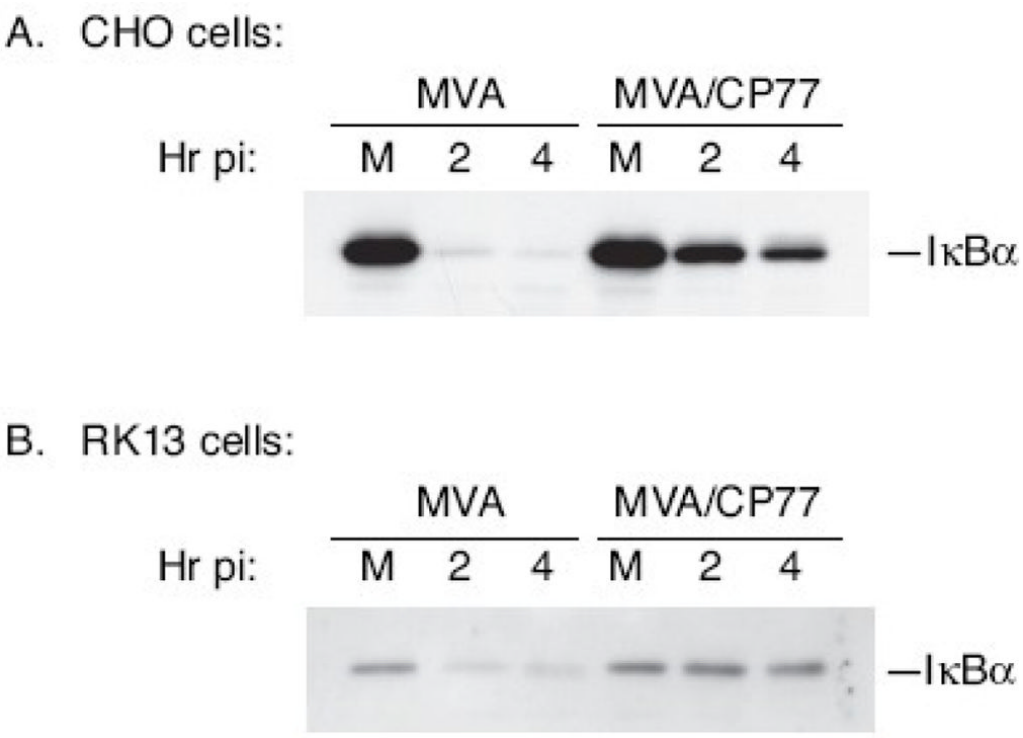

Fig. 5.

MVA-induced IκBα degradation is impaired in CHO cells and RK13 cells by the expression of the *CP77* gene. CHO or RK13 cells were mock infected (M), or infected with MVA or MVA/CP77 (20 pfu/cell). At the indicated times post-infection, cells were harvested, and the depletion of IκBα was determined as described in Fig. 1A.

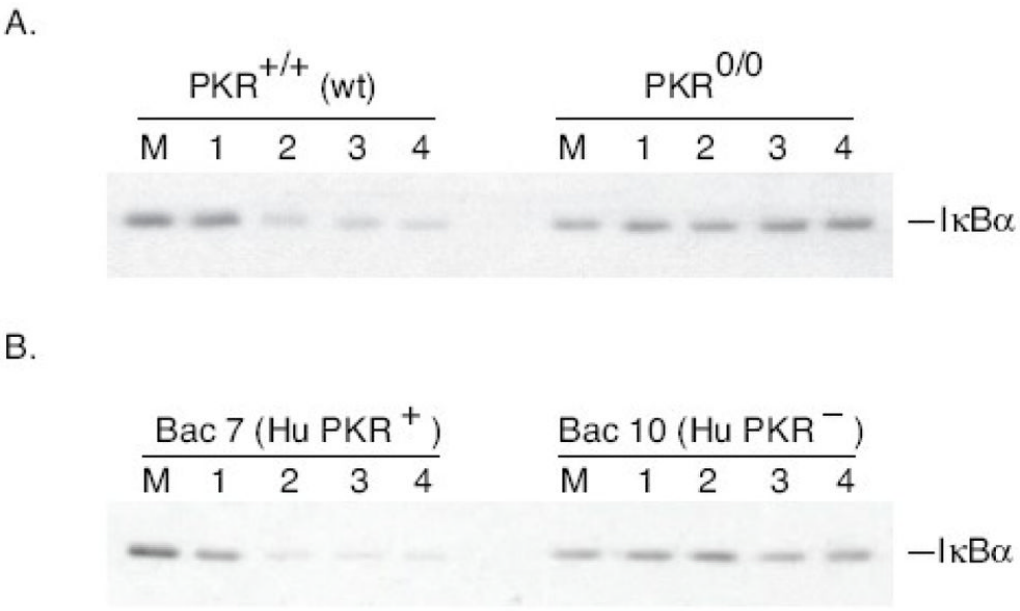

Fig. 6.

MVA-induced IκBα degradation is impaired in MEFs lacking PKR. $PKR^{0/0}$ and $PKR^{+/+}$ MEFs were mock infected (M) with PBS, or infected with MVA (20 pfu/cell). At the indicated times post-infection, cells were harvested, and the degradation of IκBα was monitored as described in Fig. 1A. (B) MVA-induced IκBα degradation in $PKR^{0/0}$ MEFs is restored by expression of human PKR. BAC 7 cells, which are $PKR^{0/0}$ MEFs that express the human PKR gene, and BAC 10 cells, which are $PKR^{0/0}$ MEFs that contain an unexpressed human PKR gene, were mock infected (M) with PBS, or infected with MVA (20 pfu/cell). At the indicated times post-infection, cells were harvested, and the depletion of IκBα was determined as described in Fig. 1A.

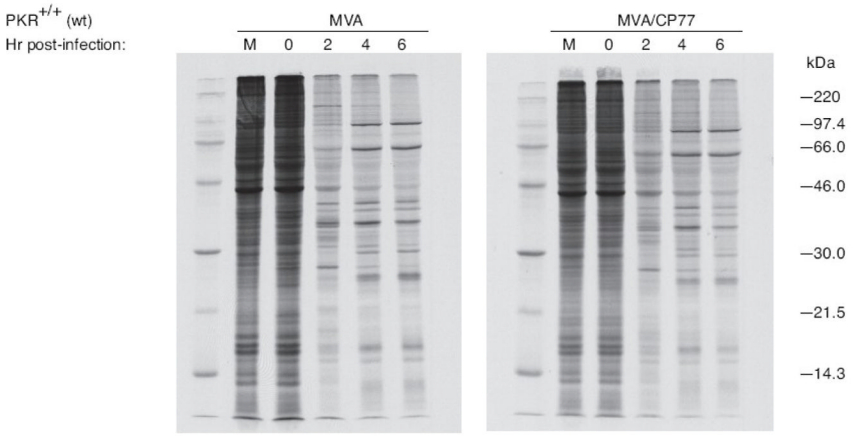

Fig. 7.

Host cell and viral protein synthesis is similar in $PKR^{+/+}$ MEFs infected with either MVA or MVA/CP77. The $PKR^{+/+}$ cells were infected (20 pfu/cell) with MVA, or with MVA/CP77, which expresses the *CP77* gene (a t 20 pfu/cell). At the indicated times post-infection, after a 30-minute incubation in methionine-free and cysteine-free media, cells were incubated in media containing [^{35}S]-labeled methionine and cysteine. After 30 minutes of incubation, cells were harvested, and whole-cell lysates were prepared. Proteins were resolved by SDS-PAGE through a 12.5 % acrylamide gel. Equal protein loading was verified by Pierce Gelcode blue staining (data not shown). The gels were then dried, and radiolabeled proteins were visualized by autoradiography. The mobilities of molecular mass standards are indicated.

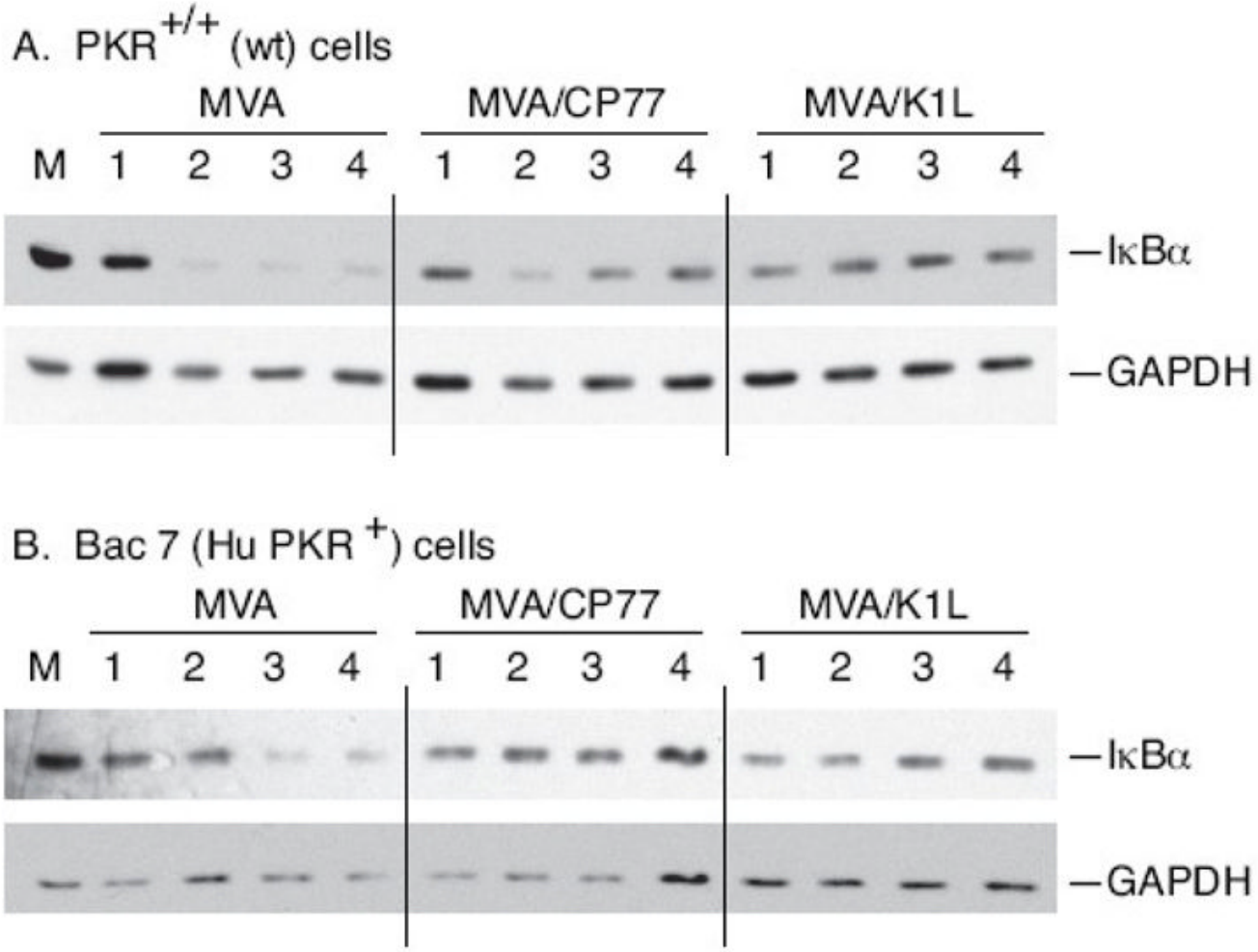

Fig. 8.

MVA-induced IκBα depletion in $PKR^{+/+}$ MEFs is impaired by the expression of either the cowpox virus *CP77* gene or the vaccinia virus *K1L* gene. (A) $PKR^{+/+}$ cells were infected with MVA, MVA/CP77, or MVA/K1L (20 pfu/cell). At the indicated times post-infection, cells were harvested, and the depletion of IκBα was monitored as described in Fig. 1A. (B) BAC 7 cells, which are $PKR^{0/0}$ MEFs that express the human PKR gene, were infected with MVA, MVA/CP77, or MVA/K1L (20 pfu/cell). At the indicated times post-infection, cells were harvested, and the depletion of IκBα was monitored as described in Fig. 1A. The membranes were subsequently processed for the detection of GAPDH as controls for protein loading per lane.