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# Characterization of wild-type and mutant vaccinia virus M2L proteins' abilities to localize to the endoplasmic reticulum and to inhibit NF-κB activation during infection

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## Abstract

Proinflammatory molecules are important for attracting immune effector cells to localized areas of viral infection. One such cellular mechanism facilitating this response is the NF-κB transcription factor. While wild-type vaccinia virus expresses multiple products to inhibit NF-κB during infection, the attenuated deletion mutant MVA lacks this ability. However, introduction of the wild-type M2L ORF into the MVA genome will re-establish the parental phenotype. As the M2L protein is unique to poxviruses, we characterized it to elucidate its mechanism to quell an inflammatory response. It was discovered that the M2L protein possesses motifs characteristic of ER-localized proteins: an N-terminal signal peptide sequence, C-terminal endoplasmic reticulum (ER) retention and retrieval sequences, and N-linked glycosylation sites. Indeed, the M2L protein was demonstrated to be N-linked glycosylated and expressed early during infection. Furthermore, confocal microscopic analysis revealed that the M2L protein co-localized with cellular ER proteins. Organelle location also affects M2L protein function: the elimination of the N-terminal leader sequence from the M2L protein compromised both its ER location and its ability to inhibit virus-induced NF-κB activation. There is only partial ER localization when a second mutant M2L protein lacking potential endoplasmic reticulum retention signal is expressed. However, this C-terminal deleted mutant protein is compromised in its ability to inhibit NF-κB activation. Determination of the ER location of the M2L proteins provides important insights for its function in future investigations.

### Keywords

Vaccinia; Poxvirus; M2L; NF-κB; Endoplasmic reticulum

## Introduction

For the immune system to respond quickly to a pathogen, host cellular signal transduction events, such as those mediating NF-κB activation, must occur. In unperturbed cells, this heterodimeric transcription factor remains inactive by virtue of its interactions with IκBα molecules (Baeuerle and Baltimore, 1988). However, perturbations such as virus infection will begin a cascade of events that culminates in activation of an IKK complex (Scheidereit, 2006) that, in turn, phosphorylates its target IκBα molecules (DiDonato et al., 1997; Mercurio et al., 1997). IκBα is then poly-ubiquitinated, released from the NF-κB dimer, and degraded by the 26S proteasome (Henkel et al., 1993; Palombella et al., 1994). Freed NF-κB translocates to the nucleus, where it binds to a specific sequence within a promoters that regulate

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transcription of genes involved in anti-viral inflammatory and immune responses (reviewed in Gilmore, 2006).

NF-κB activation and the resultant immune protein expression is an important host cell defense against virus infection (reviewed in Hiscott et al., 2006; Hoffmann et al., 2006), as exemplified by the finding that laboratory mice lacking functional NF-κB dimers are more susceptible to virus infection than normal mice (Weih et al., 1997). Similarly, viral antagonism of this transcription factor is key in producing a robust disease: mutant viruses that lack genes encoding NF-κB inhibitory proteins are less virulent than their unaltered, parental viruses (Stack et al., 2005; Billings et al., 2004).

Poxviruses are complex viruses, possessing large double-stranded DNA genomes and the ability to replicate cytoplasmically in their host cell (Moss, 2007), with vaccinia virus being the best-characterized member of this family. Wild types strains of vaccinia virus code for myriad products that inhibit inflammatory and immune responses, including ones that prevent NF-κB activation (Haga and Bowie, 2005; Smith et al., 1999). In contrast, the genome of attenuated vaccinia virus strain MVA lacks many of the wild type immunomodulatory genes (Antoine et al., 1998), and infection of cells with this strain will render the activated form of this transcription factor (Oie and Pickup, 2001; Shisler and Jin, 2004). As would be expected, stable re-introduction of wild type genes whose products inhibit NF-κB activation, such as the M2L ORF, reverses this phenotype (Gedey et al., 2006).

Little is known about the M2L protein: its expression is dispensable for virus replication in cell culture (Smith et al., 1993), and no portion of the M2L protein possesses regions of homology to other proteins with known biological properties. Delineating the molecular mechanism for the M2L protein's inhibitory effect is important for understanding viral regulation of the host immune response. Further, since the M2L protein is unique to poxviruses, its mode of action may enable the identification of new anti-viral drug targets. Additionally, the M2L protein itself may be useful as a biological tool to regulate proinflammatory processes. Upon close examination of the M2L protein, several motifs indicative of endoplasmic reticulum (ER) localization were found to be present. To understand the involvement of the M2L protein, the goal of these studies was to define its intracellular location and examine the importance of this site to its function. As shown here, the M2L protein was found to be produced early during infection, a characteristic shared with other poxviral immunomodulatory gene products, and to be sequestered to the ER. Using mutational analysis of the M2L ORF, the relationship between ER location and the inhibitory effect on NF-κB activation was investigated.

## Results

### The M2L product is expressed early during vaccinia virus infection

Given the role of the M2L protein in altering host-mediated immune responses (Gedey et al., 2006), it was likely that M2L was produced early during infection, a time in which most other known poxviral immune-modulating proteins are expressed (Moss, 2007). Bolstering this hypothesis is the fact that the nucleotide sequence of the M2L ORF promoter resembles that of transcriptional regulatory elements for other confirmed early genes (Moss, 2007), and that the transcriptional termination sequence (TTTTTNT) is not embedded within the M2L ORF, a characteristic of other early genes (Moss, 2007).

To assess temporal expression during infection, M2L protein levels were examined at early and late times post-infection by probing infected-cell lysates with polyclonal anti-M2L antiserum (Fig. 1). In lanes containing WR-infected cell lysates, a 31 kDa protein was detected as early as 2 h post-infection (Fig. 1). As infections progressed over time, additional higher

molecular weight proteins of 33 kDa were recognized by the anti-M2L antiserum, with the proteins most abundant at 4 h post-infection, but still detectable at 8 and 16 h post-infection. These bands most likely represent M2L proteins since reactive proteins of identical molecular masses were not detected in mock-infected cell lysates or lysates from cells infected with a mutant WR virus lacking the M2L ORF (ΔM2L) (Fig. 1). Likewise, 31–33 kDa proteins were not detected when MVA-infected cell lysates were probed (Fig. 1), consistent with the fact that the M2L ORF is absent from the genome of this attenuated vaccinia virus (Antoine et al., 1998). In contrast, M2L protein expression in recombinant MVA/M2L-infected cells, in which the M2L ORF is present, was similar to that observed with lysates from WR-infected cells: 31–33 kDa M2L proteins were detected as early as 2 h post-infection (Fig. 1). Detected proteins of similar molecular weight also were observed in lysates from MVA//M2L-infected cells at 4 h post-infection, while an additional different-sized protein was detected in cells at 8 and 16 h post-infection. Since similar amounts of actin were present in each sample (Fig. 1), as detected by the presence of a 42 kDa band, the differences observed in M2L levels at different times were not due to varying amounts of proteins in a lane.

The presence of Ara C during virus infection did not prevent M2L protein expression in either WR- or MVA/M2L-infected cell lysates (Fig. 1), since the lower- and higher-molecular weight forms of M2L proteins were readily detected at all time points. Cytoplasmic extracts from infected cells were analyzed concomitantly for the presence of the known late vaccinia virus protein, A11R (Resch et al., 2005) to ensure that the concentration of Ara C utilized in these experiments inhibited poxviral genome replication and late gene expression. As previously reported (Resch et al., 2005), A11R proteins were detected at very low levels at 4 h post-infection, and in greater amounts at 8 and 16 h post-infection, when WR infections proceeded in regular medium (Fig. 1). Likewise, A11R proteins were detected also in ΔM2L, MVA- and MVA/M2L-infected cells at 4, 8 and 16 h post-infection, consistent with the presumed expression of the A11R ORF (Fig. 1). However, when infections proceeded in medium containing Ara C, A11R proteins were not detected at any time post-infection. This absence of A11R proteins was not due to low protein levels in cytoplasmically extracted samples since similar amounts of actin were detected in each immunoblotted sample (Fig. 1). Since Ara C, a pyrimidine nucleoside analog, inhibits vaccinia virus DNA replication (Keck et al., 1990) and also prevents poxviral intermediate and late gene expression, it appears that the M2L protein is an early protein.

## The M2L protein sequence possesses motifs associated with ER localization and retention

Previously, expression of the vaccinia M2L product was reported to greatly diminish MVA-mediated NF-κB activation in 293T cells (Gedey et al., 2006). Since no other organisms possess analogs to the M2L protein, its sequence was analyzed for other known motifs that would indicate potential function.

The predicted amino acid sequence of the M2L protein contains several motifs that infer a possible localization of the M2L protein to the endoplasmic reticulum (ER) during infection (Fig. 2). For instance, analysis of the M2L protein by using the SignalP 3.0 algorithm software predicted the presence of a signal peptide at N-terminal residues 1–19 (Fig. 2) (von Heijne, 1983). Signal peptide sequences, especially those containing a cluster of hydrophobic residues like those present in the M2L signal peptide, often direct proteins to the ER (von Heijne, 1983). Further suggestive of ER localization was the presence of four consensus sites for N-linked glycosylation (N–X–S/T, where X can be any amino acid except proline), with the motifs located at residues 49–51, 79–81, 118–120 and 154–156 (Fig. 2). Since one of the major biosynthetic functions of the ER is to covalently add sugars to proteins (reviewed by (Abeijon and Hirschberg, 1992)), the presence of these sites suggested that the M2L protein may locate to the ER for this modification to occur. While many glycosylated proteins traffic to other

cellular structures, or become secreted (Pelham, 1988;Wieland et al., 1987), the presence of two ER retention signal motifs (XDEL) at residues 199–202 and 209–212, implies that M2L remains in the ER (Munro and Pelham, 1987). Interestingly, the C-terminus of the viral product also possesses a di-lysine KXKXX motif (residues 178–182) that directs Golgi-to-ER retrieval (Jackson et al., 1993). Based on these motifs, a working hypothesis is that the M2L product is directed to the ER by its signal sequence. Next, the M2L protein is N-linked glycosylated and may briefly traffic to the Golgi apparatus before returning to the ER for the duration of infection.

## The M2L product is N-linked glycosylated

M2L proteins of various molecular weights were observed in WR- and MVA/M2L-infected cells at later times post-infection (Fig. 1). These proteins were larger than the predicted 25 kDa molecular weight for the M2L protein. Since N-linked glycosylation adds approximately 2.5 kDa of molecular mass to a protein (Kornfeld and Kornfeld, 1985), one hypothesis is that these M2L proteins with a size greater than 25 kDa represent glycosylated forms of M2L. To test this possibility, the electrophoretic mobility of M2L proteins from cells infected in the presence of glycosylation inhibitory drugs were compared to M2L proteins expressed under normal conditions. As before (Fig. 1), when either WR or MVA/M2L infections proceeded in unmodified medium, the molecular weights of the M2L proteins were between 31 and 33 kDa (Fig. 3A). In contrast, when infections proceeded in the presence of tunicamycin, a drug that prevents N-linked glycosylation (Heifetz et al., 1979), the size of detected M2L proteins decreased to 25 kDa. In contrast, when infections proceeded in the presence of monensin, a drug that prevents Golgi-mediated O-linked glycosylation (Tartakoff and Vassalli, 1979), the mobilities of the M2L proteins remained similar to that observed for untreated, WR- or MVA/M2L-infected cells (Fig. 3C). As expected, exposure to either tunicamycin or monensin did not affect the lack of M2L protein expression by the MVA or ΔM2L virus, nor did it affect the expression of cellular genes, since actin levels were similar in untreated versus treated cells.

## The M2L protein localizes to the ER during vaccinia virus infection

The presence of the signal peptide sequence, the N-linked glycosylation sites, and the ER retention and ER retrieval motifs in the M2L protein suggested that it would localize to, and remain in, the ER. However, the M2L product prevents NF-κB activation (Gedey et al., 2006), an intracellular signaling event, making M2L secretion unlikely. Additionally, when incubating media from virus-infected cells with anti-M2L antiserum and Protein G sepharose beads, M2L proteins were not detected in these immunoprecipitates, inferring that the M2L protein remained cell-associated (data not shown).

To directly test the hypothesis that the M2L protein localizes to the ER during infection, confocal microscopy was utilized to assess the cellular location of a C-terminally V5-epitope tagged version of the M2L protein. Since the biological function of the M2L protein had been elucidated in HEK293T cells, we chose to study M2L protein cellular location in this cell line. As shown in Fig. 4A, a low level of diffuse fluorescence was observed when either mock- or MVA-infected cells were incubated with monoclonal mouse anti-V5 IgG antibodies. In contrast, staining of fixed MVA/M2L-V5-infected cells with anti-V5 antibodies resulted in fluorescence next to and surrounding part of the nucleus, a pattern typical of ER localization. Similar results were observed when fixed MVA/M2L-V5-infected cells were instead incubated with polyclonal rabbit anti-M2L protein antiserum (Fig. 7D), inferring that the addition of a V5 epitope tag did not appear to influence the cellular location of the M2L protein.

As further evidence that M2L proteins were being retained in the ER, virus-infected cells expressing M2L protein also were co-incubated with antiserum specific for the ER luminal resident protein disulfide isomerase (PDI) (Haas and Wabl, 1983). As shown in Fig. 4A, there was a co-localization of the M2L-V5 proteins with PDI in MVA/M2L-V5-infected cells, and

this co-localization remained constant when these double-labeled, stacked images were viewed by Z-series (data not shown). Similar results also were observed when antiserum against another known cellular ER-localizing protein, BiP, was substituted for anti-PDI antiserum (data not shown). When comparing PDI location in mock- versus virus-infected cells, PDI staining patterns were similar, indicating that virus infection was not obviously affecting ER structure at early times post-infection (Fig. 4A). Similar results were observed if COS-7 cells were instead utilized as the host cell for infection (Fig. 4B). Staining of fixed MVA/M2L-V5-infected cells with anti-V5 antiserum resulted in fluorescence surrounding the nucleus, a pattern similar to that observed when the same fixed, infected cells were stained with anti-PDI.

## The signal peptide sequence at the N-terminus of M2L is responsible for ER localization of the M2L protein

Previously, the M2L protein was reported to prevent virus-induced NF-κB activation (Gedey et al., 2006). To examine if M2L ER location is important for this function, a modified M2L protein, in which the N-terminal signal sequence was removed, was created. This altered M2L protein was predicted to no longer possess a functional signal peptide motif, as predicted by the SignalP 3.0 software program, and therefore should not localize to the ER. After verifying the aberrant cellular location, this mutant M2L protein would then be assessed for its ability to inhibit virus-induced NF-κB activation to ascertain the importance of ER location for NF-κB inhibitory function. To this end, the M2L ORF was engineered such that residues 2–13 were deleted from the encoded protein, and residues encoding a V5 epitope tag were engineered onto its C-terminus.

Analysis of the molecular weight of the modified M2L protein (“M2L-V5 Δsp”) is shown in Fig. 5A. While the wild-type M2L protein is expressed as 31–33 kDa products in cells infected with vaccinia strain WR, the addition of a V5 epitope tag is estimated to increased the molecular weight of the M2L protein by approximately 1.4 kDa. In agreement with this prediction, the M2L-V5 protein expressed during infection of cells with MVA/M2L-V5 results in the expression of proteins that are 32.4–34.4 kDa (Fig. 5A). It was predicted that the removal of M2L residues 2–13 would result in a reduction of the M2L molecular mass by 1.5 kDa, resulting in 30.9–32.9 kDa M2L-V5 Δsp proteins. If, however, this mutation affected the ability of M2L proteins to localize to the ER, thereby disrupting the normally occurring post-translational modifications of the M2L protein, then the altered M2L protein’s molecular weight would be expected to be decreased further. Indeed, when cells were infected with MVA/M2L-V5 Δsp, 25 kDa M2L proteins were detected (Fig. 5A), the predicted molecular weight of an epitope-tagged mutant protein that received no post-translational modifications. The relative quantity of the modified M2L protein compared to wild type M2L protein detected in either MVA/M2L-V5-infected cells was lower, whether detection of the mutant protein was assessed at either 1, 2 or 4 h post infection (data not shown). However, unlike MVA/M2L-V5-infected cells, where the M2L protein localized to the ER, the modified M2L protein in MVA/M2L-V5 Δsp-infected cells was spread throughout the cytoplasm (Fig. 5B). Moreover, whereas the intact M2L protein co-localized with cellular PDI, there was little, if any, similar placement of PDI and the M2L-V5 Δsp protein (Fig. 5B). Further examination of M2L-V5 Δsp protein location also revealed that small populations of this protein were localizing to the nucleus. Similar results were obtained if virus-infected cells were instead incubated with anti-M2L antiserum, instead of anti-V5 antiserum to detect M2L protein location (Fig. 7).

As further proof that the M2L-V5 Δsp protein did not localize to the ER, its sensitivity or resistance to endoglycosidase H (Endo H) was assessed. Endo H cleaves mannoses and some hybrid oligosaccharides from N-linked glycoproteins. Therefore, proteins that are Endo H sensitive imply that protein localized to the ER, where it received the N-linked glycosylation. To determine the Endo H sensitivity of wild-type or mutant M2L proteins, epitope-tagged M2L

proteins from virus-infected 293T cells was immunoprecipitated with anti-V5 antiserum. Immunoprecipitates were incubated with Endo H, and reactions were analyzed for M2L protein mobility by using immunoblotting. Results are shown in Fig. 5C. While the wild-type M2L-V5 protein's mobility was approximately 35 kDa, Endo H treatment of immunoprecipitates decreased the mobility; M2L was represented as an intense 25 kDa band, and as less intense bands ranging from 25 to 35 kDa. Thus, wild-type M2L proteins were EndoH sensitive, and present in the ER. In contrast, the 25 kDa M2L-V5 Δsp protein's mobility remained the same in the presence or absence of Endo H, indicating that this mutant protein did not localize to the ER.

## The ability of the M2L protein to prevent NF-κB activation is lost when its signal peptide sequence is deleted

To assess the importance of ER location for the ability of the M2L protein to inhibit NF-κB activation, 293T cells were infected with MVA constructs expressing either wild type or mutated M2L proteins. NF-κB activation was then determined by incubating nuclear-extracted proteins from these virus-infected cells with radiolabeled oligonucleotides containing consensus NF-κB binding motifs. If NF-κB had translocated to the nucleus, an event that occurs when this host protein is activated, then it would bind to the radiolabeled oligonucleotides, and reduce their electrophoretic mobility. As previously reported (Gedey et al., 2006), MVA was apparently unable to prevent NF-κB nuclear translocation (Fig. 6, lane "MVA"). That the novel high molecular weight band did indeed represent an NF-κB-oligonucleotide complex was verified by it being shifted to a higher molecular weight due to reaction with a specific anti-NF-κB antibody (Fig. 6, lane "MVA+anti-p65"). A similar complex was also observed when a lysate from cells infected with MVA/M2L-V5 Δsp was used. In contrast, both the unmodified WR and the parental MVA/M2L-V5 exhibited the inhibitory phenotype, with no band detected in lanes containing extracts from WR-infected cells, and only a faint band observed when testing extracts from MVA/M2L-V5-infected cells. Thus, elimination of the leader sequence of the M2L protein was associated with the loss of the ability to prevent NF-κB nuclear translocation.

## A mutant M2L protein lacking the putative ER retention and retrieval motifs localizes to the ER and to the cytoplasm, and no longer prevents NF-κB nuclear translocation

To rule out the possibility that the decreased detection levels of the M2L-V5 Δsp protein as compared to wild type M2L protein was due to the anti-M2L antibodies preferentially recognizing the glycosylated forms of M2L proteins, a second mutant M2L protein expressing only residues 1–178, and thereby retaining its glycosylation sites, was created. Since this C-terminal deletion mutant protein (M2L-V5 ΔERret) lacked the regions containing the two putative ER retention motifs and the ER retrieval signal, it was predicted that the M2L-V5 ΔERret proteins, while initially localizing to the ER, would egress from the ER during infection. Similar to M2L-V5 Δsp, M2L-V5 ΔERret also possessed a C-terminal V5 epitope tag. The predicted molecular weight of this protein, if it received no N-linked glycosylation and if the signal peptide sequence was cleaved, was 19.5 kDa. As shown in Fig. 7A, infection of cells with MVA/M2L-V5 ΔERret resulted in the detection of 25 kDa products. Additionally, the relative quantity of the C-terminal deleted M2L protein was similar to the V5-epitope tagged wild-type protein (Fig. 7A).

To assess if the differences in observed versus theoretical molecular weights was due to N-linked glycosylation, infected cells were incubated in medium containing tunicamycin. Similar to before, the 34 kDa wild-type M2L-V5 protein was represented by a 25 kDa band (Fig. 7B), indicating that the wild-type protein received N-linked glycosylation, an event which occurs in the ER. When studying the mobility of M2L-V5 ΔERret, its mobility in the absence of tunicamycin is 25 kDa. However, when tunicamycin is present during infection, the mobility

of the mutant M2L protein changes to 18–19 kDa (Fig. 7B), suggesting that this mutant M2L protein migrates to the ER and receives some N-linked glycosylation modifications.

Next, the cellular location of the M2L-V5 ΔERret protein during infection was assessed by using confocal microscopy. Similar to patterns found when utilizing anti-V5 antiserum to identify M2L proteins (Fig. 4 and Fig 5), staining fixed cells with anti-V5 antiserum resulted in detection of the V5 epitope tagged form of the wild-type M2L protein in areas next to and around the nucleus, whereas the M2L-V5 Δsp protein appeared to be distributed throughout the cellular cytoplasm (Fig. 7C). As shown in Fig. 7C, M2L-V5 ΔERret also localized to areas next to and surrounding the nucleus, as detected by using anti-V5 antiserum, suggesting ER localization. However, when comparing the cellular location of the M2L-V5 ΔERret proteins with that of the PDI cellular resident ER proteins, it was observed that a population of these mutant M2L proteins no longer co-localized with PDI (Fig. 7C). Thus, it appears that that there is not complete localization of this mutant M2L protein to the ER.

To assess the effect of this mutant protein on MVA-mediated NF-κB activation, 293T cells were infected with MVA constructs expressing either wild-type or mutated M2L proteins. Similar to above, NF-κB activation was determined by the migration of freed, and presumably active, transcription factor to the nucleus. When incubating nuclear-extracted proteins from uninfected cells with radiolabeled oligonucleotides containing consensus NF-κB binding motifs, there was no detectable shift in the mobility of the radiolabeled oligonucleotides, suggesting that NF-κB was absent from the nuclei of these resting cells (Fig. 8, lane “uninfected”). In contrast, MVA infection was apparently triggering NF-κB nuclear translocation (Fig. 8, lane “MVA”), as was evidenced by the appearance of a unique mobility-shifted band. The addition of antibodies specific for the p65 subunit of the NF-κB heterodimer super-shifted this unique complex (Fig. 8, lane “MVA+anti-p65”), confirming that the MVA-induced mobility-shifted band contained NF-κB. A band of similar intensity was observed when analyzing nuclear extracted proteins from cells infected with MVA/M2L-V5 ΔERret, but this band was not present when proteins from cells infected with WR were analyzed. A band of lesser intensity was present when extracts from MVA/M2L-V5-infected cells were used, an event not surprising given that this genome lacks ORFs that code for NF-κB inhibitory function, namely K1L (Shisler and Jin, 2004). These trends are noted when cells were harvested at either 1 or 2 h post-infection. Thus, the removal of the ER retention and retrieval sequences from the M2L protein yielded the same NF-κB activation phenotype as when the N-terminal signal sequence of the M2L protein was absent, even though some of these mutant M2L proteins still localized to the ER.

## Discussion

The vaccinia M2L protein was characterized as a means of understanding how it functions molecularly to inhibit virus-induced NF-κB activation. Here, we demonstrate that the M2L protein is expressed early during virus infection, a trait shared with other poxviral immunomodulatory agents (Moss, 2007). Also, the M2L protein remains intracellular and is N-linked glycosylated, indicative of its localization to the ER. Moreover, microscopic analysis confirmed that M2L proteins traffic to the ER, with this organelle location dependent on its N-terminal signal peptide sequence and, to a lesser extent, on its C-terminal retention motifs. Finally, either leaderless M2L proteins or M2L proteins lacking their putative ER retention and retrieval motifs lose their ability to deter NF-κB activation at early times post-infection.

The M2L protein is expressed as a 31–33 kDa product during the course of infection. There are two scenarios that would explain the detection of this doublet: either the M2L protein is cleaved during infection or the protein is post-translationally modified. Since expression of the M2L protein in the presence of tunicamycin, a drug that inhibits N-linked glycosylation,

renders the production of a single 25-kDa M2L protein, it appears that the later possibility is most likely, with the 31 kDa and 33 kDa proteins representing high- versus low-glycosylation forms. It was unlikely that the wild-type M2L proteins localized to the Golgi apparatus, a hypothesis that was supported with the observation that M2L protein mobility was unaltered when infected cells were treated cells with monensin, a drug that inhibits Golgi apparatus-mediated O-linked glycosylation (Tartakoff and Vassalli, 1979). Additionally, M2L proteins did not co-localize with other known Golgi apparatus-associated proteins as determined by using confocal microscopy (data not shown).

Motifs present in the M2L protein suggested that it entered the ER. Indeed, analysis of a mutant M2L-V5 protein missing the putative N-terminal signal peptide sequence (M2L-V5 Δsp) revealed that the modified protein no longer localized to the ER. Coincident with this data was the observation that the M2L-V5 Δsp protein was of a molecular weight that indicated it had not received N-linked glycosylation modifications, biochemical events that occur in the ER.

It was observed that the mutant M2L-V5 Δsp protein was detected at lower levels than the wild-type M2L-V5 protein when using immunoblotting (Fig. 6). One possibility for this difference in detection levels may be that the avidity of the anti-M2L antiserum for the unglycosylated form of M2L is lower than for the glycosylated form. Thus, a construct expressing mutant M2L proteins that still allow for glycosylation, while losing predicted ER location signals, M2L-V5 ΔER ret, was created and tested for its ability to inhibit NF-κB activation. Unlike M2L-V5 Δsp proteins, M2L-V5 ΔERret protein detection was similar to that of wild-type M2L proteins.

The C-terminal region of the M2L protein contains 2 ER retention motifs (XDEL) and an ER retrieval motif (KXKXX), implying that M2L proteins, after post-translational modifications, would remain in the ER for the duration of infection. Removal of these motifs, then, was expected to result in M2L-V5 ΔERret proteins that would initially enter the ER, but ultimately migrate out of the ER. Since the mobility of this mutant protein decreased when cells were infected in medium containing tunicamycin, it was likely that M2L-V5 ΔERret proteins were indeed N-linked glycosylated, and therefore entered the ER. Notably, while some M2L-V5 ΔERret proteins still co-localized with PDI, a portion of the ΔERret proteins no longer co-localized, suggesting that only some of the mutant M2L proteins lost their ER association phenotype. It is unknown why some of the mutant M2L proteins still remain in the ER. Images of ΔERret proteins were obtained up to 4 h post-infection. Whether the entire population may eventually migrate out of the ER at later times post-infection remains to be tested. Nevertheless, even with this partial phenotype, the M2L-V5 ΔERret protein is compromised in its ability to inhibit NF-κB activity at early times post-infection.

The ER comprises the large reticular network that not only acts as a storage site for calcium, but also regulates protein synthesis, protein modification, and the proper folding and transport of proteins (Gorlach et al., 2006). Events germaine to viral replication, namely high level production of viral proteins, will often cause ER stress, leading to at least two chemically distinct signal transduction pathways, termed the ER overload response (EOR) and the unfolded protein response (UPR) (Kaufman, 1999). Not surprisingly, multiple viruses express proteins to regulate ER homeostasis (He, 2006; Mulvey et al., 2007; Pahl and Baeuerle, 1995b; Pahl et al., 1996; Waris et al., 2002), thereby maintaining a productive cellular environment for viral replication.

It is thought that EOR is an immediate cellular response to viral infection, presumably activating cellular genes whose products would limit virus infection. During EOR, calcium is released from the ER into the cytoplasm, proceeded by an increase in intracellular reactive oxygen species (ROS) (Pahl and Baeuerle, 1996). Through undefined signal transduction

mechanisms, this release of calcium and ROS results in NF-κB activation (Pahl and Baeuerle, 1995a, 1996). Given the requirement of ER localization for the M2L protein's NF-κB inhibitory function, one speculation is that this viral protein blocks an early event in the EOR pathway, namely calcium release from the ER. At least one cellular protein, RTN3HAP, is involved in triggering calcium release during EOR (Kuang et al., 2005). Thus, M2L interaction with this or other similar ER-based calcium release channels, such as the ryanodine receptors and the INSP3 receptors (Gorlach et al., 2006) may exert this predicted effect. It was reported previously that the M2L product also prevents ERK2 phosphorylation, an event necessary for MVA-induced NF-κB activation of 293T cells (Gedey et al., 2006). Although the M2L and ERK2 proteins are not likely to be present in common cellular compartments (Pearson et al., 2001), there is a body of evidence showing a causal relationship between cytoplasmic calcium increases (perhaps those triggered via the EOR) and MEK/ERK activation (Kim et al., 2005; Mogensen et al., 2003), with calcium release and ERK activation intimately associated with NF-κB activation. Thus, the M2L protein's ability to prevent ERK2 phosphorylation may be linked with its ability to inhibit calcium release from the ER.

In contrast to the EOR, the UPR occurs when there is an accumulation of improperly folded proteins in the ER. The UPR is characterized by the activation of cellular kinases such as ATF6 and IRE1, to stimulate the production of proteins that enhance the folding capacity of the ER, and PERK, which phosphorylates eIF2α to induce a translational arrest (Harding et al., 2002). Historically, only the EOR was thought to trigger activation of NF-κB via the accumulation of proteins in the ER (Kaufman, 1999). More recent publications, however, find that UPR and NF-κB activation occur concomitantly, with the inhibition of the UPR also preventing NF-κB activation (Deng et al., 2004; Misra et al., 2006). Thus, assessing the role of M2L on the UPR as its mechanism for inhibiting NF-κB activation is warranted.

Yet another important innate immune defense is Toll-like receptor (TLR) recognition of viral genomes, subsequently resulting in NF-κB activation. For virus-induced TLR signaling, TLRs 3, 7 and 9 are probably most important because they recognize viral components such as double-stranded RNA, single stranded RNA and unmethylated DNA, respectively (O'Neill and Bowie, 2007). Recently, these viral sensing TLR proteins were shown to interact with the cellular UNC93B ER membrane protein (Brinkmann et al., 2007), with a point mutation in UNC93B abolishing this interaction. In laboratory mice expressing mutant UNC93B proteins, the TNF cytokine, whose expression is dependent upon the activated form of NF-κB, is no longer produced in response to the natural ligands of TLRs 7 or 9 (Brinkmann et al., 2007; Tabeta et al., 2006). These studies infer that the ER localization of these TLRs, and their interaction with UNC93B, may be important for NF-κB activation. Whether ER-localized vaccinia M2L proteins may be preventing such interactions is the subject of future studies. If future data support this hypothesis, then the M2L protein would utilize a different mechanism for inhibiting TLR-mediated NF-κB activation than those deployed by the previously characterized vaccinia A52R, A46R and N1L proteins (Bowie et al., 2000; DiPerna et al., 2004).

The M2L protein is highly conserved amongst members of the *Orthopoxvirus* genus (www.poxvirus.org), suggesting that its function may be important for pathogenesis *in vivo*. In addition, predicted proteins with approximately 50% similarity are also present in myxoma virus (gp120-like ORF) and rabbit fibroma virus (gp154L) (www.poxvirus.org). Whether these viral products also localize to the ER and inhibit NF-κB activation remains to be determined. Interestingly, there are two other poxviral proteins (vaccinia B7R and myxoma M-T4) that reside in the ER and are virulence factors (Price et al., 2000; Barry et al., 1997). For instance, deletion of the B7R ORF from the vaccinia virus genome results in a genetically altered virus that is attenuated when administered intradermally to mice (Price et al., 2000). Similarly, removal of the myxoma M-T4 ORF results in a deletion mutant virus that produces an

attenuated myxamatosis in rabbits (Barry et al., 1997). Additionally, unlike the parental virus, this altered virus activates apoptosis in cultured and primary lymphocytes (Barry et al., 1997). Whether either protein affects ER stress as its mechanism for virulence remains to be tested. Multiple viruses express mechanisms to regulate ER stress. Given the importance of ER location to the M2L ability to inhibit NF-κB activation, it is interesting to speculate that poxviruses, like other viruses, may also express functionally similar proteins to purposefully regulate ER-mediated signaling pathways, thereby ensuring a productive infection *in vivo*.

## Materials and methods

### Cells and viruses

The human HEK293T (293T) and monkey BS-C-1 kidney fibroblast cell lines were obtained from the American Type Culture Collection, while chicken embryo fibroblast (CEF) cells were purchased from Charles Rivers Laboratories. All cells were cultivated in EMEM supplemented with 10% fetal calf serum (FCS; Hyclone). The wild type vaccinia virus strain Western Reserve (WR) and the attenuated strain modified vaccinia Ankara (MVA)(Mayr et al., 1975) were used in these studies. MVA/M2L is a recombinant virus in which the WR M2L transcriptional unit was stably inserted into the MVA del III region (Gedey et al., 2006). BS-C-1 and CEF cells were utilized to amplify the WR- and MVA-based vaccinia viruses, respectively.

An MVA virus containing a DNA sequence encoding a V5 epitope tag fused to the C-terminus of the WR M2L transcriptional unit (MVA/M2L-V5) was produced by homologous recombination in CEF cells. To accomplish this, the M2L ORF and its linked promoter were PCR amplified from WR viral DNA, using two sequential PCR reactions to add DNA coding for a V5 epitope tag to the C-terminus of the M2L ORF. To this end, oligonucleotide primers M2LFOR (5′-TGCGCATGCACGAGCTGTACAAGTAAGCGGCCGCAACTAATT-3′) and M2LREV1 (5′-CAGCAGGGGGTTGGGGATGGGCTTGCCCTCTCTATAACA-3′) were utilized. DNA coding for the V5 epitope product is underlined. This PCR product was gel purified to remove excess, unincorporated oligonucleotide primers. Next, DNA encoding the remainder of the V5 epitope was engineered onto the C-terminus of the aforementioned M2L amplicon in a second, sequential PCR amplification reaction that utilized primers M2LFOR and M2LREV2 (5′-GTACTGCAGTTAGGTGCTGTCCAGGCCCAGCAGGGGGTT-3′). The DNA sequence coding for the V5 epitope in M2LREV2 is underlined. The resultant M2L-V5 amplicon was inserted into the *Eag*I and *Pst*I sites of a modified pLW44 (provided by Drs. Linda Wyatt and Bernard Moss) to create plasmid M2L-V5/pLW44. This modified plasmid no longer possesses an H5 promoter directly upstream of the inserted M2L-V5 amplicon, thereby allowing M2L-V5 to be expressed only during the early times post-infection, under the control of the naturally occurring M2L promoter. M2L-V5/pLW44 also retains the green fluorescent protein (GFP) gene linked to the vaccinia p11 promoter, allowing for GFP fluorescence to be utilized as a stable screening marker during isolation of recombinant MVA viruses (described below). Sequence analysis of a representative plasmid demonstrated the presence of the C-terminal V5 epitope tag and the absence of mutations in the wild type M2L promoter and ORF.

To create a recombinant MVA virus expressing M2L-V5, CEFs were infected with MVA and transfected subsequently with plasmid M2L-V5/pLW44. At 48 h post-infection, cells were harvested and lysed. Virus-containing lysates were incubated with fresh CEF monolayers, and cells infected with recombinant MVA/M2L-V5 viruses were identified based on their ability to express GFP. GFP-expressing foci were plaque purified at least four times and pure stocks were amplified in CEF cells. The presence of the M2L-V5 ORF in the MVA del III region, as well as the absence of contaminating parental viral DNA, was verified by PCR analysis of the isolated viruses' genomes, using primers recognizing sequences that either flanked the MVA del III region or were present within the M2L ORF. Expression of the M2L-V5 protein in virus-

infected cells was confirmed via probing immunoblotted lysates of infected cells with polyclonal rabbit anti-M2L antibodies raised against M2L residues 130–145 (Gedey et al., 2006) (diluted 1:1000 in TBS-T), followed by incubation of membranes with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Fisher Scientific), which was diluted 1:20,000 in TBS-T. Immunoblots were then washed to remove excess antibodies and developed by using the SuperSignal West chemilluminescence reagents, as per manufacturer's directions (Pierce).

Similar to above, an MVA construct expressing a mutant M2L protein lacking residues 2–13 (MVA/M2L-V5 Δsp), thereby removing most of the predicted N-terminal peptide sequence, was produced by using homologous recombination in CEF cells. For this purpose, the mutated M2L amplicon was PCR amplified from plasmid M2L-V5/pLW44 using primers ΔSPFOR (5′-TCGGCATGCACGAGCTGT-3′) and ΔSPREV (5′-CTAATTTGTACCCGAGCATCTTAC-3′). An *Ava*I restriction enzyme site was present in primer ΔSPFOR (underlined), allowing for the introduction of a unique *Ava*I site into the mutant M2L amplicon. Next, the gel-purified PCR product was ligated into the *Ava*I and *Eag*I sites of the modified pLW44. Stable introduction of this mutant ORF into the del III region of the MVA genome, as well as the selection, purification and amplification of recombinant viruses, and the detection of the inserted modified M2L DNA, were performed as described above. Expression of M2L-V5 Δsp mutant proteins during infection was assessed by immunoblotting, where infected-cell lysates were electrophoretically separated, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) and incubated first with polyclonal rabbit anti-M2L antiserum (Gedey et al., 2006), and then with HRP-conjugated goat anti-rabbit IgG (Fisher Scientific), using the dilutions mentioned above. Immunoblots were then washed to remove excess antibodies and developed by using the SuperSignal West chemilluminescence reagents (Pierce).

A recombinant MVA construct expressing a mutant M2L product deleted of residues 179–220 (MVA/M2L-V5 ΔERret) was created by homologous recombination in CEF cells, similar to the methods utilized above. To begin, the C-terminal truncated M2L amplicon and its natural promoter was PCR amplified from plasmid M2L-V5/pLW44. Two sequential PCR reactions were performed to allow for the addition of DNA coding for a V5 epitope tag onto the C-terminus of the truncated M2L ORF. The first PCR reaction utilized oligonucleotides ERFOR (5′-TCT CGGCATGCACGAGCTGTACAAGTAAGCGGCCGCAACTAA TT-3′) and ERREV1 (5′-GGGGATGGGCTTGCCGGGGTGTAGGTAATGGGG-3′), while the subsequent PCR reaction utilized primers ERFOR and ERREV2 (5′-CTTCTGCAGTTAGGTGCTGTCCAGGCCCAGCAGGGGGTT GGGGATGGG-3′). DNA sequences coding for the V5 epitope are underlined in oligonucleotides ERREV1 and ERREV2. The purified amplicon, which has *Eag*I and *Pst*I sites engineered into the N- and C-termini, respectively, was inserted into the same sites of a modified pLW44 vector, creating plasmid M2L-V5 ΔERret/pLW44. The stable introduction of this mutant ORF into the MVA genome, the identification of recombinant viruses expressing GFP, the purification of recombinant viruses away from parental MVA viruses, and the verification of the presence of the modified M2L ORF in the del III region of the MVA genome, was performed as described above. Expression of M2L-V5 ΔERret mutant proteins during infection was assessed by immunoblotting as described above, using polyclonal rabbit anti-M2L antiserum (Gedey et al., 2006) and HRP-conjugated goat anti-rabbit IgG (Fisher Scientific). Dilutions of antiserum used for immunoblotting are mentioned above.

A WR construct deleted for the M2L ORF (ΔM2L) was produced by using homologous recombination in BS-C-1 cells. For this purpose, a 4.2 kb DNA segment containing the M2L ORF and flanking sequences was PCR amplified from the WR genome, using oligonucleotides M2A (5′-GACTTCATCATGAATTCCCG- 3′) and M2B (5′-

CAATAATTCGGATCCCTTCATTC-3′). The resultant PCR amplicon possessed *Eco*RI and *Bam*HI restriction enzyme sites at the 5′ and 3′ ends, respectively, since these DNA sequences were present in primers M2A and M2B, as denoted by the underlined regions in the above oligonucleotides. The purified PCR products were then ligated into pNEB193 (New England Biolabs) at the *Eco*RI and *Bam*HI sites. The resultant construct was named pNEB/M2L, and was sequenced to verify that no mutations had been introduced into the viral DNA. The pZIPPY-NEO/GUS plasmid (Dvoracek and Shors, 2003) was digested with the *Bgl*II and *Hin*dIII restriction enzymes, and the neo-gus cassette was isolated from this plasmid by using agarose gel electrophoresis. This DNA segment contains the *Escherichia coli* neomycin resistance gene (*neo*) under the control of the vaccinia p7.5 promoter, and the *E. coli gusA* gene under the control of a modified vaccinia H5 promoter. Thus, when this cassette is stably inserted into the WR genome, recombinant viruses are identified based on their ability to form plaques in the presence of geneticin (G418) and to produce β-glucuronidase, an event measurable by the production of a blue color when virus-infected cells are incubated with the X-glucuronidase (X-glu) reagent (Clontech Laboratories). Next, the neo-gus cassette was blunt-end ligated into the *Sty*I and *Age*I restriction enzyme sites in pNEB/M2L, resulting in plasmid pNEB/ΔM2L/ neo-gus. Using this process, most of the M2L ORF was removed from pNEB193, and replaced with the neo-gus cassette. However, enough viral DNA flanking the M2L ORF was retained in the plasmid to allow for successful homologous recombination.

BS-C-1 cell monolayers were transfected with pNEB/ΔM2L/ neo-gus using the FuGene6 transfection reagent (Roche) and then infected with WR. At 24 h post-infection, cells were harvested by scraping, lysed by freeze-thawing three times, and sonicated. Virus-containing cellular lysates were incubated with fresh BS-C-1 cellular monolayers that had been pre-incubated in medium containing 1 mg/ml G418 (Invitrogen Corporation) for 24 h. After a 1-h adsorption phase, virus-containing medium was removed, and cellular monolayers were incubated in medium containing 1.0% low-melting point agarose and 1 mg/ml G418. At 48 h later, a second layer of medium containing 1.0% low-melting point agarose and 0.2 mg/ml X-glu (Clontech Laboratories) was added. Recombinant viruses that produced blue-colored plaques were identified and collected. These plaques were further purified to remove contaminating parental virus using the same techniques, for a total of four rounds of plaque purification. The absence of the M2L ORF in the recombinant WR-based viruses was verified by PCR analysis of the genomes of plaque-purified viruses, and by immunoblotting lysates from ΔM2L-infected cells. For immunoblotting, infected-cell lysates were electrophoretically separated, transferred to a PVDF membrane (Millipore) and membraneswere incubated with polyclonal rabbit anti-M2L antiserum (Gedey et al., 2006) in TBS-T, followed by incubation with HRP-conjugated goat anti-rabbit IgG (Fisher Scientific), using dilutions of antibodies mentioned above. Immunoblots were developed as discussed above.

## Western immunoblotting for M2L protein expression

Cytoplasmic proteins were extracted from infected-cell lysates, according to a modified version of methods described previously (Gedey et al., 2006; Oie and Pickup, 2001). Briefly, 293T cellular monolayers were infected with indicated viruses at an MOI of 10. In some experiments, 40 μg/ml cytosine arabinoside (Ara C) (Sigma-Aldrich) was present in the medium during the absorption phase of infection and post-infection. At either 2, 4, 8 or 16 h post-infection, cell monolayers were scraped from the cell culture plates and pelleted during centrifugation for 1 min at 14,000×*g* at 4 °C. Alternatively, cellular monolayers were incubated in medium containing either tunicamycin (10 μg/ml; Sigma-Aldrich) or monensin (1 μM; Sigma-Aldrich) 30 min prior to infection, and were continuously incubated in supplemented medium during and after the absorption phase of virus infection. For these experiments, infected cell monolayers were harvested from cell culture substrates by scraping at 20 h post-infection. As above, cells were collected and pelleted by centrifugation (1 min at 14,000×*g*). For both protocols,

supernatants were removed, and cellular pellets were resuspended and lysed in CE buffer (Oie and Pickup, 2001) containing HALT protease inhibitors (Pierce) for 5 min at 4 °C. After a brief centrifugation (1 min at 14,000×*g*), the resultant supernatants were collected, and the protein concentration in each preparation was determined by using the BCA Protein Assay kit (Pierce). An equivalent amount of protein (25 μg) from each sample was mixed in a 1:1 ratio with 2× Laemmli buffer (Fisher) containing 10% 2-mercaptoethanol, boiled for 5 min, and loaded into separate wells of a SDS-12% polyacrylamide gel. Proteins were separated electrophoretically and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Membranes were incubated in blocking buffer (TBS containing 5% powdered milk and 0.1% Tween-20) for at least 1 h. Next, membranes were incubated at 4 °C overnight with the primary antibodies in TBS-T (TBS containing 0.1% Tween-20 and 0.5% milk). For detection of M2L proteins, blots were incubated with polyclonal rabbit anti-M2L antiserum (Gedey et al., 2006) diluted 1:1000 dilutions in TBS-T. After washing the membranes in TBS-T to remove unbound primary antibodies, blots were incubated with TBS-T containing a 1:20,000 dilution of HRP-conjugated goat anti-rabbit IgG (Fisher Scientific). Immunoblots were then washed to remove excess antibodies and developed by using the SuperSignal West chemilluminescence reagents, as per manufacturer's directions (Pierce). In some cases, the same immunoblots or identical, duplicate immunoblots were probed with either polyclonal rabbit anti-actin antiserum (Sigma-Aldrich) or polyclonal rabbit anti-A11R antiserum (Resch, Weisberg, and Moss, 2005) diluted 1:1000 in TBS-T, followed by incubation with HRP-conjugated goat anti-rabbit IgG (Fisher Scientific) diluted 1:10,000 in TBS-T before being developed using the SuperSignal West chemilluminescence reagents (Pierce).

## Confocal microscopy

293T cells were seeded onto glass cover slips (Fisher Scientific) and incubated overnight at 37 °C. Cellular mono-layers were infected with the indicated vaccinia viruses (MOI=10). At 1 to 2 h post-infection, infected-cell monolayers were washed with PBS, incubated in a 4% paraformaldehyde solution (Fisher Scientific) for 25 min, washed with PBS, and then incubated in a 0.1% Triton X-100 solution for 10 min (Bio-Rad) to allow for permeabilization. Next, fixed cells were washed with PBS and incubated in confocal blocking buffer (PBS containing 3% bovine serum albumin; BSA) (Fisher Scientific) for 1 h. Fixed monolayers were then incubated for at least 1 h with either monoclonal rabbit IgG (Jackson ImmunoResearch), monoclonal mouse anti-V5 IgG (Invitrogen), rabbit polyclonal anti-BiP IgG (Abcam), rabbit polyclonal anti-M2L IgG (Gedey et al., 2006), or rabbit polyclonal anti-PDI IgG (Stressgen). These primary antibodies were diluted at 1:250 or 1:500 in blocking buffer. Next, cellular monolayers were washed with excess volumes of confocal blocking buffer, and subsequently incubated with either Alexa Fluor 488 dye-conjugated donkey anti-mouse IgG or Alexa Fluor 647 dye-conjugated goat anti-rabbit IgG antibodies (Molecular Probes) for 30–45 min (1:1000–1:2000 dilutions), washed with confocal blocking buffer to remove unbound antibody and then mounted on microscope slides. All of these incubations proceeded at room temperature. Images were acquired by using a Leica SP2 laser scanning confocal microscope, and processed using the Leica SP2 software (Leica).

## Gel electromobility shift assays

Electromobility shift assays were performed as previously described (Gedey et al., 2006). Briefly, subconfluent 293T cellular monolayers in 6-well plates were infected with the indicated viruses (MOI=10). At 1 h post-infection, cells were harvested by scraping, and pelleted by centrifugation (1 min at 14,000×*g*) at 4 °C. Pellets were lysed in CE buffer (Oie and Pickup, 2001) containing HALT protease inhibitors (Pierce) to disrupt the cytoplasmic, but not the nuclear membranes. Nuclei were then separated from the lysates by centrifugation (5 min at 14,000×*g* at 4 °C) and washed in excess CE buffer to remove contaminating cytoplasmic proteins. Nuclei were then resuspended in NE buffer (Oie and Pickup, 2001) to

disrupt their nuclear membranes. Samples were centrifuged to separate soluble and insoluble proteins. Soluble proteins were collected to new tubes and their concentrations were determined by using the BCA Protein Assay Kit (Pierce). 5 μg of each extract was incubated with 0.35 pmol of $^{32}$P-labeled double-stranded oligonucleotides containing binding sites for the NF-κB transcription factor (Promega) in Gel Shift Assay System Binding buffer (Promega) as per manufacturer's directions. In some reactions, 1 μg of monoclonal mouse anti-p65 IgG (Santa Cruz Biotechnology), and antibody recognizing the p65 subunit of the p65:p50 NF-κB heterodimer, was present. Following incubation at room temperature, reactions were resolved electrophoretically in a 6% acrylamide gel (Invitrogen) under non-denaturing conditions. Afterwards, the gel was dried onto filter paper, exposed to a phosphorimager plate (Molecular Devices), and images were developed and analyzed using the ImageGauge and ImageReader programs, respectively (Fuji).

### Immunoprecipitation and EndoH treatments

293T cellular monolayers were infected with MVA-based viruses at an MOI of 10. At 2 h post-infection, cells were harvested by scraping, and collected by centrifugation. Cellular pellets were re-suspended in IP lysis buffer (140 mM NaCl, 10 mM Tris (pH 7.2), 2 mM EDTA (pH 8.0), 1% NP40) containing HALT protease inhibitors (Pierce). Clarified lysates were incubated with Protein G-Sepharose 4B beads (Invitrogen) previously conjugated to monoclonal anti-V5 antibodies (Invitrogen). The reaction was incubated at 4 °C with constant rotation for at least 2 h. Next, immunoprecipitates were collected by centrifugation, and beads were washed with large volumes of IP lysis buffer three times. Next, collected beads were subjected to EndoH digestion, as per the manufacturer's directions. Briefly, 3 μl of the EndoH 10X denaturing solution (New England Biolabs) was added to each tube containing beads, and tubes were heated to 100 °C for 10 min, and then incubated on ice for 2 min. Samples were centrifuged at 13,000×*g* for 5 min at room temperature. Supernatants were collected, and incubated with 1000 U EndoH in G5 buffer (New England Biolabs) for 2 h at 37 °C. EndoH digestion was halted when samples then received 2× loading buffer and 2-mercaptoethanol. Next, samples were incubated at 100 °C for 5 min, cooled on ice, and samples analyzed by SDS-12% PAGE. Immunoblotting for M2L protein expression was performed as described above.

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## References

Abeijon C, Hirschberg CB. Topography of glycosylation reactions in the endoplasmic reticulum. Trends Biochem. Sci 1992;17(1):32–36. [PubMed: 1533966]

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):365–396. [PubMed: 9601507]

Baeuerle PA, Baltimore D. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. Science 1988;242(4878):540–546. [PubMed: 3140380]

Barry M, Hnatiuk S, Mossman K, Lee SF, Boshkov L, McFadden G. The myxoma virus M-T4 gene encodes a novel RDEL-containing protein that is retained within the endoplasmic reticulum and is important for the productive infection of lymphocytes. Virology 1997;239(2):360–377. [PubMed: 9434727]

Billings B, Smith SA, Zhang Z, Lahiri DK, Kotwal GJ. Lack of N1L gene expression results in a significant decrease of vaccinia virus replication in mouse brain. Ann. N.Y. Acad. Sci 2004;1030:297–302. [PubMed: 15659810]

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):10162–10167. [PubMed: 10920188]

Brinkmann MM, Spooner E, Hoebe K, Beutler B, Ploegh HL, Kim YM. The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling. J. Cell Biol 2007;177 (2):265–275. [PubMed: 17452530]

Deng J, Lu PD, Zhang Y, Scheuner D, Kaufman RJ, Sonenberg N, Harding HP, Ron D. Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2. Mol. Cell. Biol 2004;24(23):10161–10168. [PubMed: 15542827]

DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature 1997;388(6642):548–554. [PubMed: 9252186]

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):36570–36578. [PubMed: 15215253]

Dvoracek B, Shors T. Construction of a novel set of transfer vectors to study vaccinia virus replication and foreign gene expression. Plasmid 2003;49(1):9–17. [PubMed: 12583996]

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. J. Virol 2006;80(17):8676–8685. [PubMed: 16912315]

Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. Oncogene 2006;25(51):6680–6684. [PubMed: 17072321]

Gorlach A, Klappa P, Kietzmann T. The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. Antioxid. Redox Signal 2006;8(9–10):1391–1418. [PubMed: 16986999]

Haas IG, Wabl M. Immunoglobulin heavy chain binding protein. Nature 1983;306(5941):387–389. [PubMed: 6417546]

Haga IR, Bowie AG. Evasion of innate immunity by vaccinia virus. Parasitology 2005;(130 Suppl):S11–S25. [PubMed: 16281988]

Harding HP, Calfon M, Urano F, Novoa I, Ron D. Transcriptional and translational control in the Mammalian unfolded protein response. Annu. Rev. Cell Dev. Biol 2002;18:575–599. [PubMed: 12142265]

He B. Viruses, endoplasmic reticulum stress, and interferon responses. Cell Death Differ 2006;13(3): 393–403. [PubMed: 16397582]

Heifetz A, Keenan RW, Elbein AD. Mechanism of action of tunicamycin on the UDP-GlcNAc:dolichyl-phosphate Glc-NAc-1-phosphate transferase. Biochemistry 1979;18(11):2186–2192. [PubMed: 444447]

Henkel T, Machleidt T, Alkalay I, Kronke M, Ben-Neriah Y, Baeuerle PA. Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. Nature 1993;365(6442):182–185. [PubMed: 8371761]

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):6844–6867. [PubMed: 17072332]

Hoffmann A, Natoli G, Ghosh G. Transcriptional regulation via the NF-kappaB signaling module. Oncogene 2006;25(51):6706–6716. [PubMed: 17072323]

Jackson MR, Nilsson T, Peterson PA. Retrieval of transmembrane proteins to the endoplasmic reticulum. J. Cell Biol 1993;121(2):317–333. [PubMed: 8468349]

Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev 1999;13(10):1211–1233. [PubMed: 10346810]

Keck JG, Baldick CJ Jr, Moss B. Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late trans-activator genes. Cell 1990;61(5):801–809. [PubMed: 2344616]

Kim MS, Lim WK, Park RK, Shin T, Yoo YH, Hong SH, An NH, Kim HM. Involvement of mitogen-activated protein kinase and NF-kappaB activation in Ca2+-induced IL-8 production in human mast cells. Cytokine 2005;32(5):226–233. [PubMed: 16343928]

Kornfeld R, Kornfeld S. Assembly of asparagine-linked oligosaccharides. Ann. Rev. Biochem 1985;54:631–664. [PubMed: 3896128]

Kuang E, Wan Q, Li X, Xu H, Liu Q, Qi Y. ERCa2+ depletion triggers apoptotic signals for endoplasmic reticulum (ER) overload response induced by overexpressed reticulon 3 (RTN3/HAP). J. Cell. Physiol 2005;204(2):549–559. [PubMed: 15799019]

Mayr A, Hochstein-Mintzel V, Stickl H. Creation of an attenuated strain of Ankara, MVA. Infection 1975;3:6–14.

Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, Rao A. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. Science 1997;278(5339):860–866. [PubMed: 9346484]

Misra UK, Deedwania R, Pizzo SV. Activation and cross-talk between Akt, NF-kappaB, and unfolded protein response signaling in 1-LN prostate cancer cells consequent to ligation of cell surface-associated GRP78. J. Biol. Chem 2006;281(19):13694–13707. [PubMed: 16543232]

Mogensen TH, Melchjorsen J, Hollsberg P, Paludan SR. Activation of NF-kappa B in virus-infected macrophages is dependent on mitochondrial oxidative stress and intracellular calcium: downstream involvement of the kinases TGF-beta-activated kinase 1, mitogen-activated kinase/extracellular signal-regulated kinase kinase 1, and I kappa B kinase. J. Immunol 2003;170(12):6224–6233. [PubMed: 12794154]

Moss, B. Poxviridae: The Viruses and Their Replication. In: Knipe, D.; Howley, P., editors. Fields Virology. Vol. Fifth edition. Vol. vol. 2. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 2905-2946.2 vols

Mulvey M, Arias C, Mohr I. Maintenance of endoplasmic reticulum (ER) homeostasis in herpes simplex virus type 1-infected cells through the association of a viral glycoprotein with PERK, a cellular ER stress sensor. J. Virol 2007;81(7):3377–3390. [PubMed: 17229688]

Munro S, Pelham HR. A C-terminal signal prevents secretion of luminal ER proteins. Cell 1987;48(5): 899–907. [PubMed: 3545499]

O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat. Rev., Immunol 2007;7(5):353–364. [PubMed: 17457343]

Oie KL, Pickup DJ. Cowpox virus and other members of the ortho-poxvirus genus interfere with the regulation of NF-kappaB activation. Virology 2001;288(1):175–187. [PubMed: 11543670]

Pahl HL, Baeuerle PA. A novel signal transduction pathway from the endoplasmic reticulum to the nucleus is mediated by transcription factor NF-kappa B. EMBO J 1995a;14(11):2580–2588. [PubMed: 7781611]

Pahl HL, Baeuerle PA. Expression of influenza virus hemagglutinin activates transcription factor NF-kappa B. J. Virol 1995b;69(3):1480–1484. [PubMed: 7853480]

Pahl HL, Baeuerle PA. Activation of NF-kappa B by ER stress requires both Ca2+ and reactive oxygen intermediates as messengers. FEBS Lett 1996;392(2):129–136. [PubMed: 8772190]

Pahl HL, Sester M, Burgert HG, Baeuerle PA. Activation of transcription factor NF-kappaB by the adenovirus E3/19K protein requires its ER retention. J. Cell Biol 1996;132(4):511–522. [PubMed: 8647884]

Palombella VJ, Rando OJ, Goldberg AL, Maniatis T. The ubiquitin–proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell 1994;78(5): 773–785. [PubMed: 8087845]

Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr. Rev 2001;22(2): 153–183. [PubMed: 11294822]

Pelham HR. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. EMBO J 1988;7(4):913–918. [PubMed: 3402439]

Price N, Tscharke DC, Hollinshead M, Smith GL. Vaccinia virus gene B7R encodes an 18-kDa protein that is resident in the endoplasmic reticulum and affects virus virulence. Virology 2000;267(1):65–79. [PubMed: 10648184]

Resch W, Weisberg AS, Moss B. Vaccinia virus nonstructural protein encoded by the A11R gene is required for formation of the virion membrane. J. Virol 2005;79(11):6598–6609. [PubMed: 15890898]

Scheidereit C. IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. Oncogene 2006;25(51):6685–6705. [PubMed: 17072322]

Shisler JL, Jin XL. The vaccinia virus K1L gene product inhibits host NF-kappaB activation by preventing IkappaBalpha degradation. J. Virol 2004;78(7):3553–3560. [PubMed: 15016878]

Smith GL, Symons JA, Alcami A. Immune modulation by proteins secreted from cells infected by vaccinia virus. Arch. Virol 1999:111–129.

Smith KA, Stallard V, Roos JM, Hart C, Cormier N, Cohen LK, Roberts BE, Payne LG. Host range selection of vaccinia recombinants containing insertions of foreign genes into non-coding sequences. Vaccine 1993;11(1):43–53. [PubMed: 8427036]

Stack J, Haga IR, Schroder M, Bartlett NW, Maloney G, Reading PC, Fitzgerald KA, Smith GL, Bowie AG. Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. J. Exp. Med 2005;201(6):1007–1018. [PubMed: 15767367]

Tabeta K, Hoebe K, Janssen EM, Du X, Georgel P, Crozat K, Mudd S, Mann N, Sovath S, Goode J, Shamel L, Herskovits AA, Portnoy DA, Cooke M, Tarantino LM, Wiltshire T, Steinberg BE, Grinstein S, Beutler B. The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. Nat. Immunol 2006;7(2):156–164. [PubMed: 16415873]

Tartakoff A, Vassalli P. Plasma cell immunoglobulin M molecules. Their biosynthesis, assembly, and intracellular transport. J. Cell Biol 1979;83(2 Pt 1):284–299. [PubMed: 115892]

von Heijne G. Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem 1983;133(1): 17–21. [PubMed: 6852022]

Waris G, Tardif KD, Siddiqui A. Endoplasmic reticulum (ER) stress: hepatitis C virus induces an ER-nucleus signal transduction pathway and activates NF-kappaB and STAT-3. Biochem. Pharmacol 2002;64(10):1425–1430. [PubMed: 12417255]

Weih F, Warr G, Yang H, Bravo R. Multifocal defects in immune responses in RelB-deficient mice. J. Immunol 1997;158(11):5211–5218. [PubMed: 9164938]

Wieland FT, Gleason ML, Serafini TA, Rothman JE. The rate of bulk flow from the endoplasmic reticulum to the cell surface. Cell 1987;50(2):289–300. [PubMed: 3594573]

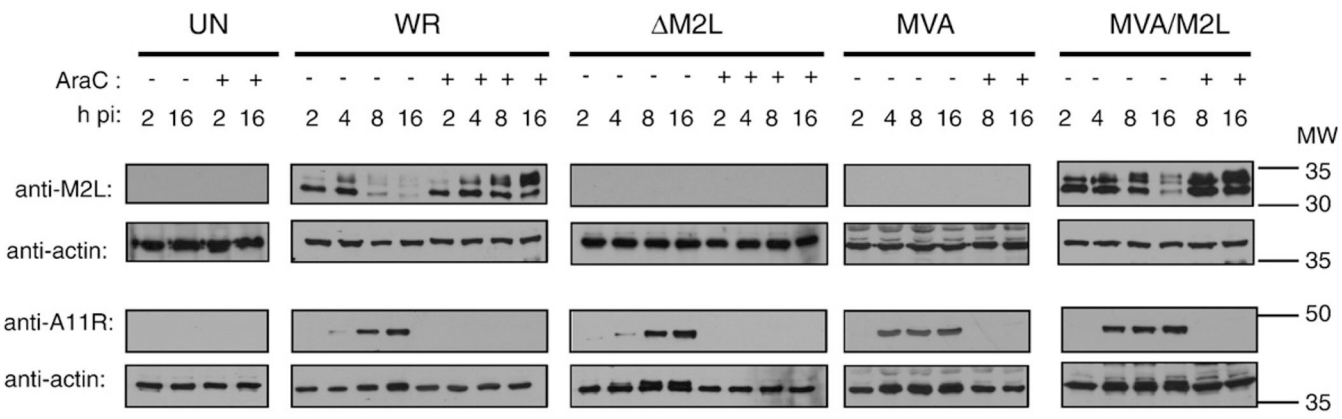


**Fig. 1.**

Temporal M2L protein expression in virus-infected cells. 293T cellular monolayers were mock-infected (UN) or infected with either WR, ΔM2L, MVA or MVA/M2L (MOI=10). For some samples, absorption of virus and virus infection proceeded in medium containing Ara C (40 μg/ml medium). Cells were harvested and lysed in CE buffer at the indicated hours post-infection. 25 μg of cytoplasmic proteins from each sample were analyzed by SDS-12% PAGE and transferred to a PVDF membrane. Membranes were probed with anti-M2L antiserum. After development based on chemilluminescence, blots were re-incubated with anti-actin antiserum and subsequently developed to detect actin proteins. Identical blots were probed with anti-A11R antibodies, developed, and then re-probed for actin protein presence by using anti-actin antibodies. The relative positions of the molecular weight markers (MW) are indicated on the right hand side of the figure.


**Fig. 2.**

Representation of the vaccinia virus M2L protein. The predicted amino acid sequence of the vaccinia M2L protein is shown. The putative signal peptide sequence (residues 1–19) is boxed, with the predicted cleavage site between residues 17 and 18 denoted by a carat (“^”). The four putative N-linked glycosylation sites are underlined. C-terminal residues specific for ER retention are highlighted in black boxes, while those specific for an ER retrieval sequence are in a gray box. Motifs were identified by using the SignalP 3.0 algorithm software.

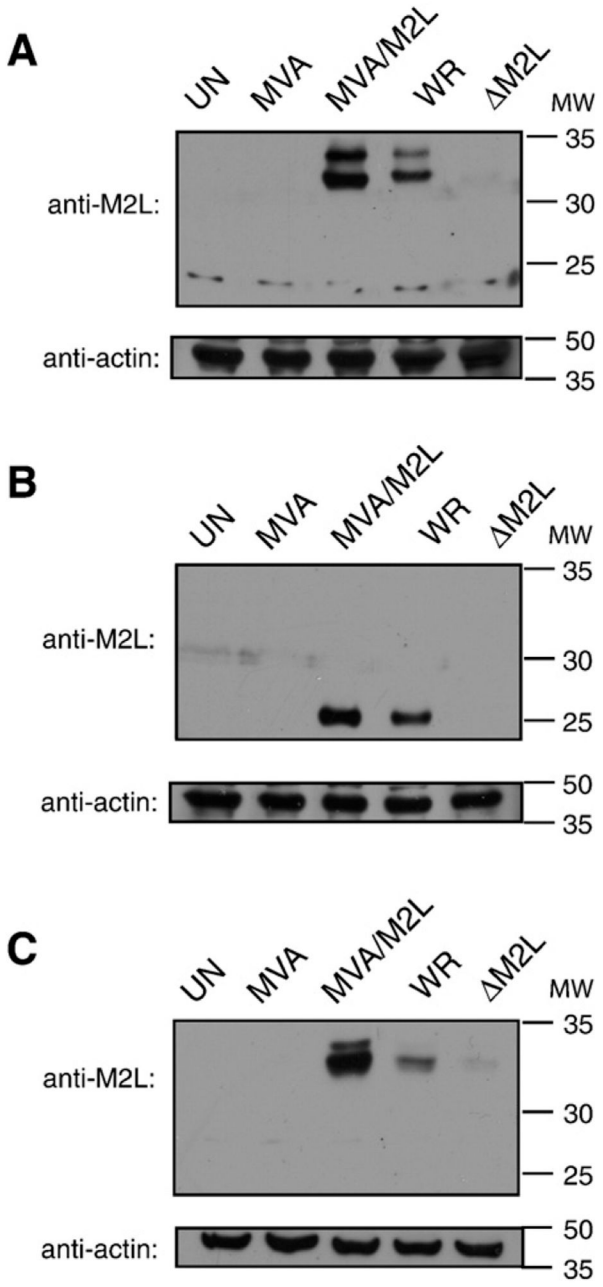


**Fig. 3.**

The effect of glycosylation inhibitors on M2L protein mobility. 293T cell monolayers were mock-infected (UN) or infected with either MVA, MVA/M2L, WR or ΔM2L (MOI=10) in medium that lacked any drug supplements (A), or contained 10 μg/ml tunicamycin (B), or 1 μM monensin (C). At 20 h post-infection, cells were harvested, lysed, and 25 μg of each lysate was electrophoretically separated in a SDS-12% PAG. Proteins were transferred to PVDF membranes, and the membranes were probed with anti-M2L antiserum. Reactive proteins were detected by chemilluminescence. Blots were re-incubated with anti-actin antibodies and subsequent signals for the detection of actin proteins were developed. The relative positions of the molecular weight markers (MW) are indicated on the right hand side of the figure.

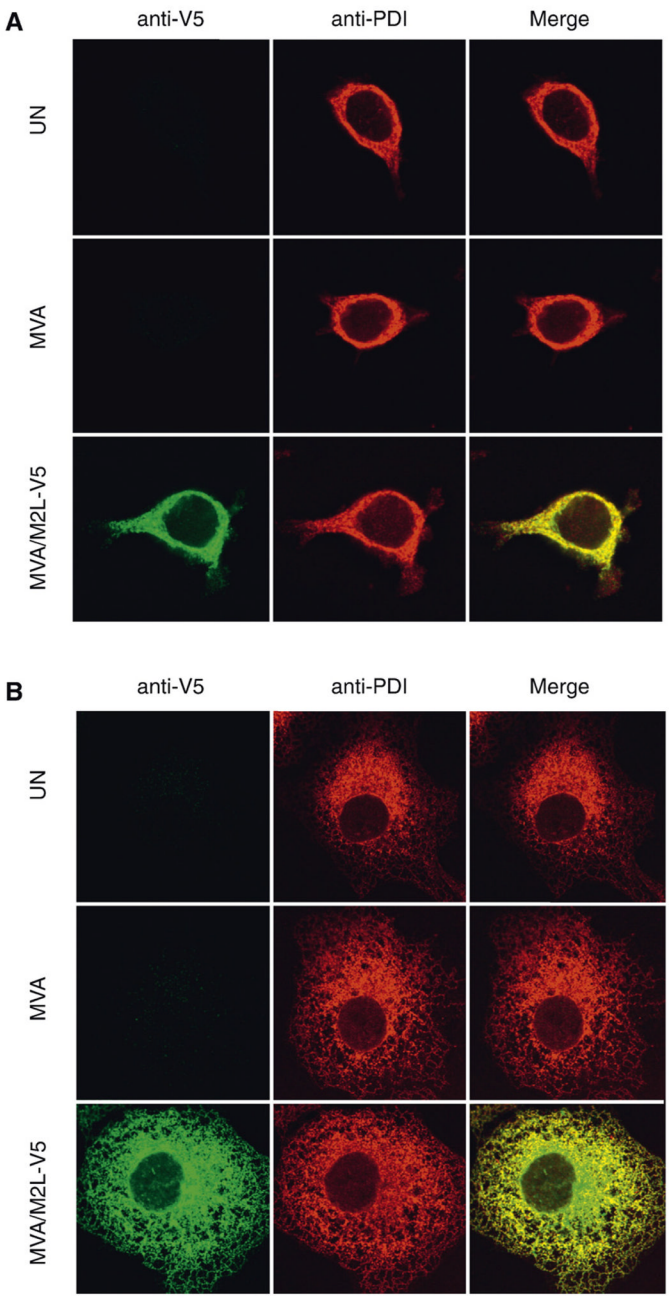


**Fig. 4.**

Co-localization of the M2L and ER proteins during virus infection. 293T (A) or COS-7 (B) cell monolayers were mock-infected (UN) or infected with either MVA orMVA/M2L-V5 (MOI=10). At 2 h post-infection, cell monolayers were fixed, permeabilized, and incubated with a mixture of mouse monoclonal anti-V5 and rabbit polyclonal anti-PDI antisera. After washing, samples were subsequently incubated with Alexa Fluor 488 dye-conjugated donkey anti-mouse or Alexa Fluor 647 dye-conjugated goat anti-rabbit secondary antibodies. Images were acquired using the Leica SP2 Laser Scanning confocal microscope.

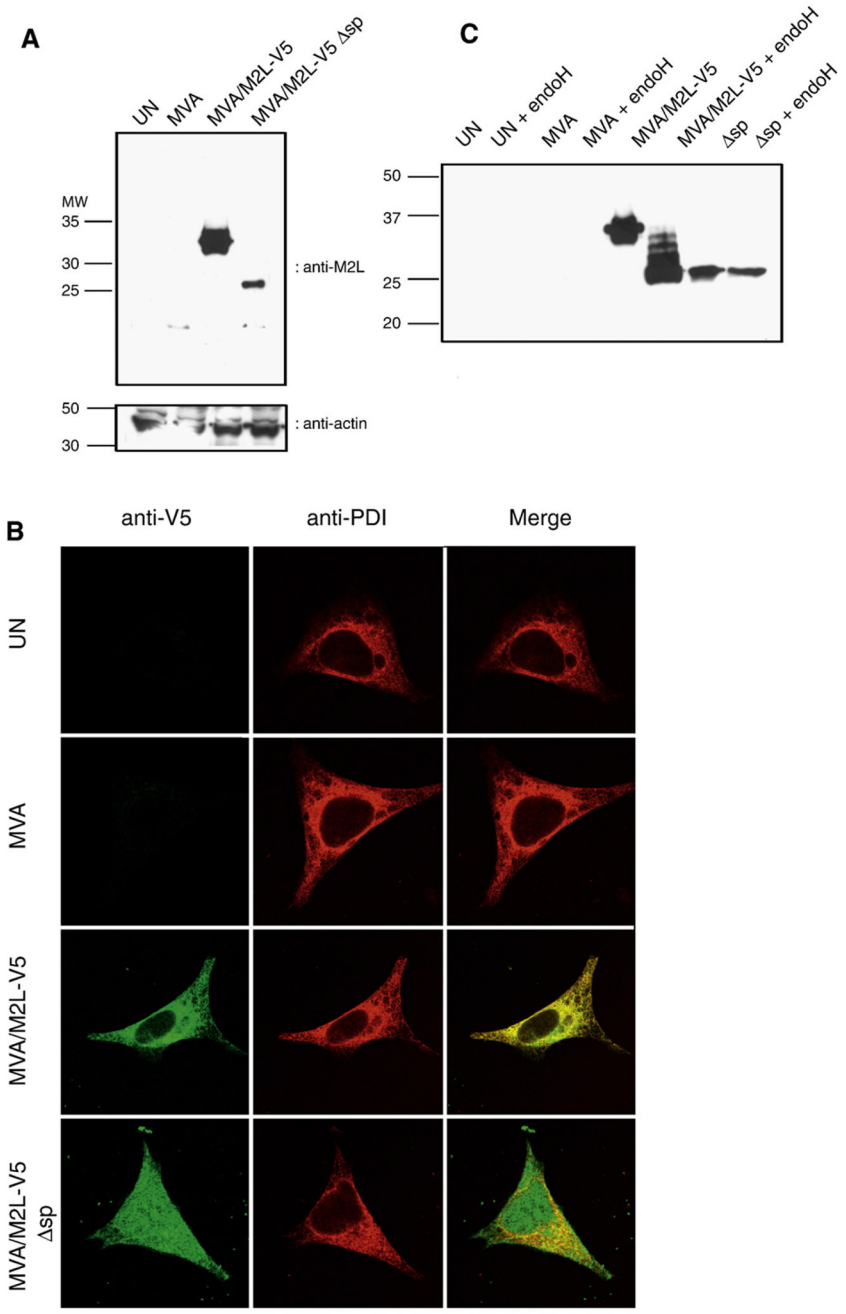


**Fig. 5.**

Cellular localization of an altered M2L protein lacking the N-terminal signal peptide sequence. 293T cellular monolayers were infected with either MVA, MVA/M2L-V5, or MVA/M2L-V5Δsp (MOI=10) or mock-infected (UN). (A) At 4 h post-infection cell lysates were prepared and analyzed electrophoretically on SDS-12% PAG. Proteins were transferred to PVDF membranes and the membranes were incubated with anti-M2L protein antiserum. Reactive proteins were detected by using chemilluminescence. (B) At 2 h post-infection cell monolayers were fixed, permeabilized and incubated with a mixture of mouse monoclonal anti-V5 and rabbit polyclonal anti-PDI antisera. After washing, samples were subsequently incubated with either Alexa Fluor 488 dye-conjugated donkey anti-mouse or Alexa Fluor 647 dye-conjugated

goat anti-rabbit secondary antibodies. Images were acquired using the Leica SP2 Laser Scanning confocal microscope. (C) At 2 h post-infection, cells were lysed, and clarified lysates were incubated with anti-V5 antibodies that had been previously conjugated to Protein G-Sepharose beads. Immunoprecipitated samples were either untreated or treated with EndoH, and then analyzed electrophoretically by 12% SDS-PAGE. Proteins were subsequently transferred to PVDF membranes. Membranes were incubated with anti-V5 antiserum and reactive proteins were detected by chemilluminescence.

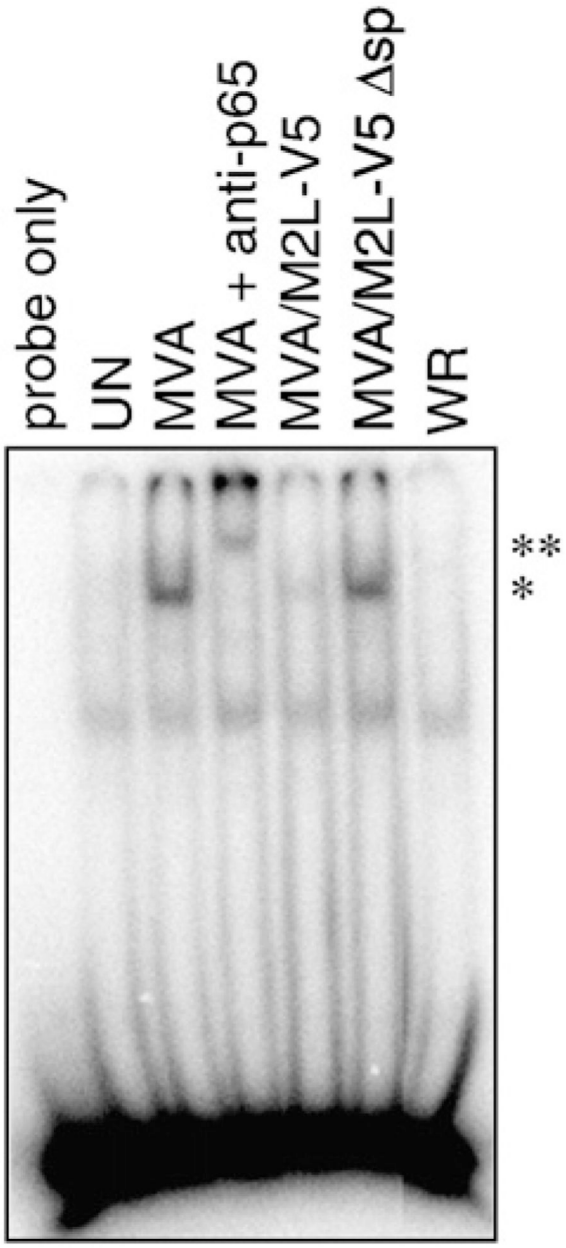


**Fig. 6.**

The effect of deleting the signal peptide signal on M2L protein-mediated inhibition of MVA-induced NF-κB activation. 293T cellular monolayers were infected with the indicated viruses (MOI=10) or mock-infected (UN). At 1 h post-infection, nuclei were isolated from the cells, lysed and clarified by centrifugation. The soluble fractions were incubated with $^{32}$P-labeled double-stranded oligonucleotides containing binding sites for the NF-κB transcription factor. In one case (MVA+anti-p65), 1 μg of monoclonal anti-p65 antiserum was included. Following incubation at room temperature, reactions were resolved electrophoretically in a 6% acrylamide gel (Invitrogen) under non-denaturing conditions. One reaction, indicated as “probe only” contained no cellular proteins. Afterwards, the gel was dried onto filter paper, exposed to a

phosphorimager plate (Molecular Devices) and images were developed. An asterisk (*) indicates a specific NF-κB-containing band, while a double asterisk (**) indicates a super-shifted NF-κB complex.

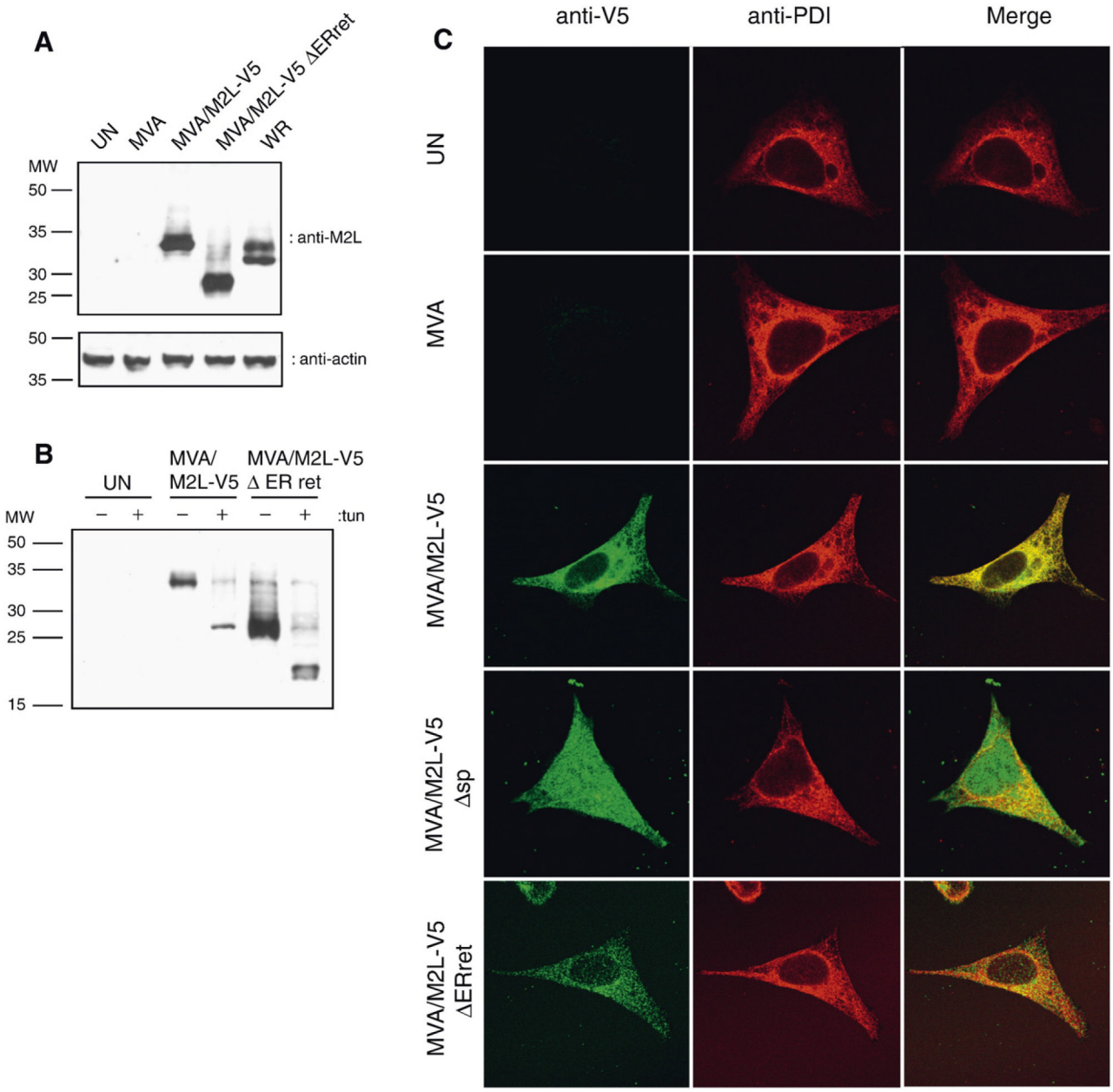


**Fig. 7.**

Cellular localization of an altered M2L protein lacking the C-terminal region containing ER retention and ER retrieval motifs. 293T cell monolayers were either mock-infected (UN) or infected with either MVA, MVA/M2L-V5, MVA/M2L-V5 ΔER ret or WR (MOI=10). At 2 h post-infection, (A) cells were harvested and lysed, and lysates were separated by using SDS-12% PAGE. After electrophoretic transfer of the proteins to a PVDF membrane, the membrane was probed with anti-M2L antiserum, followed by incubation with secondary HRP-conjugated goat anti-rabbit IgG. Blots were developed by chemilluminescence, re-probed with anti-actin antiserum and developed. (B) Mock-infected cells or cells infected with either MVA/M2L-V5 or MVA/M2L-V5 ΔERret (MOI=10) were incubated in regular medium or in medium containing 10 μg/ml tunicamycin. At 22 h post-infection, cells were harvested, lysed, and 30

μg of each lysate was electrophoretically separated in a SDS-12% PAG. Proteins were transferred to PVDF membranes, and the membranes were probed with anti-M2L antiserum. Reactive proteins were detected by chemilluminescence. (C) At 2 h post-infection, cell monolayers were fixed, permeabilized and incubated with a mixture of mouse monoclonal anti-V5 and rabbit polyclonal anti-PDI antisera. Next, samples were washed and incubated with either Alexa Fluor 647 dye-conjugated goat anti-rabbit or Alexa Fluor 488 dye-conjugated donkey anti-mouse secondary antibodies. Images were acquired using the Leica SP2 Laser Scanning confocal microscope.

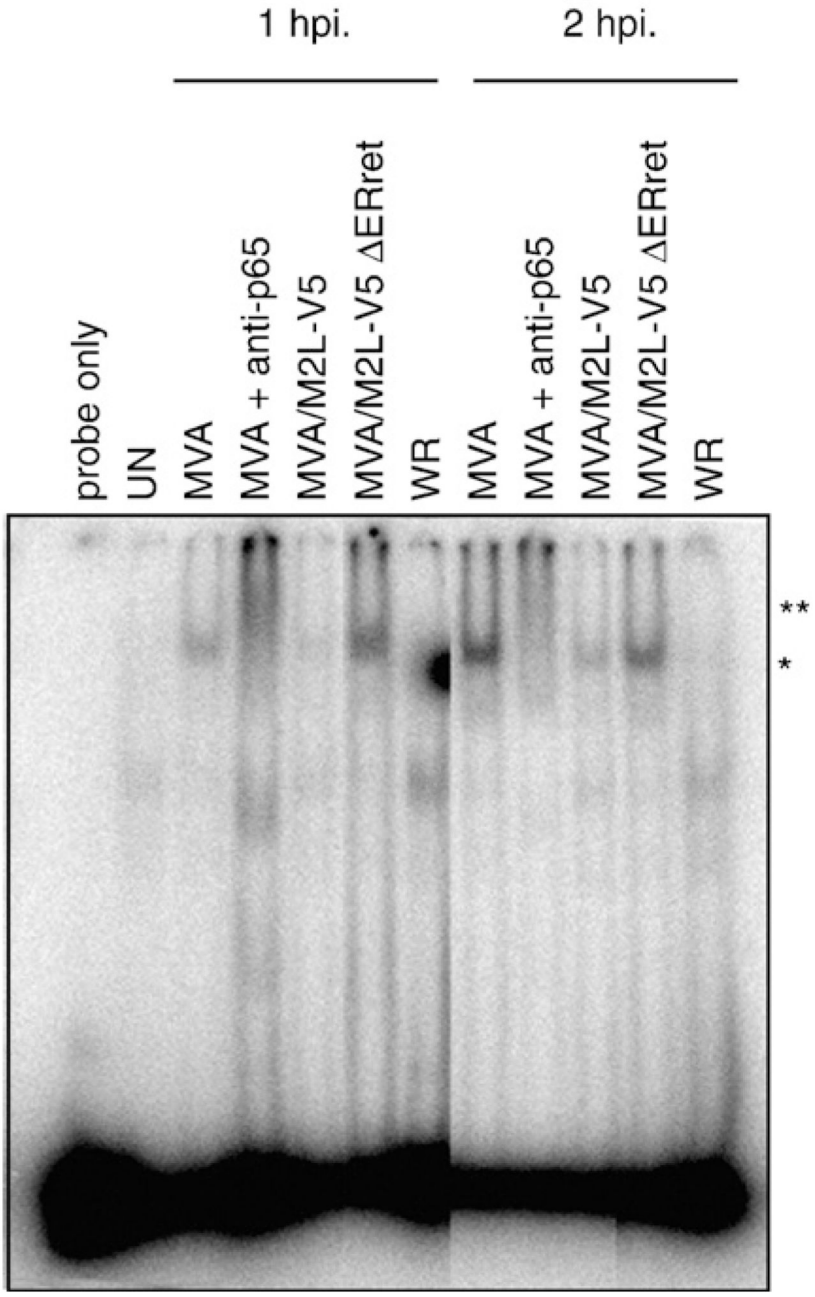


**Fig. 8.**

The effect of the M2L-V5 ΔER ret protein on MVA-induced NF-κB activation. 293T cellular monolayers were infected with either MVA, MVA/M2L-V5, MVA/M2L-V5 ΔER ret, or WR (MOI=10) or mock-infected (UN). At either 1 or 2 h post-infection, nuclei were isolated from the cells, and soluble nuclear proteins were extracted, and then incubated with $^{32}$P-labeled double-stranded oligonucleotides containing binding sites for the NF-κB transcription factor. In one case (MVA+anti-p65), 1 μg of monoclonal anti-p65 antiserum was included in the reactions. Yet another sample (probe only) lacked nuclear-extracted proteins. Following incubation at room temperature, reactions were resolved electrophoretically in a 6% acrylamide gel (Invitrogen) under non-denaturing conditions. The gel was dried onto filter paper, exposed

to a phosphorimager plate (Molecular Devices) and images were developed. An asterisk (*) indicates a specific NF-κB-containing band, while a double asterisk (**) indicates a super-shifted NF-κB complex.