

Poxviral Regulation of the Host NF- κ B Response: the Vaccinia Virus M2L Protein Inhibits Induction of NF- κ B Activation via an ERK2 Pathway in Virus-Infected Human Embryonic Kidney Cells

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Exposure of eukaryotic cells to viruses will activate the host NF- κ B transcription factor, resulting in proinflammatory and immune protein production. Vaccinia virus (VV), the prototypic orthopoxvirus, expresses products that inhibit this antiviral event. To identify novel mechanisms responsible for this effect, we made use of a VV deletion mutant (MVA) that stimulates NF- κ B activation in infected 293T cells. In this virus-host system, the extents of NF- κ B-regulated gene expression and nuclear translocation were reduced in the presence of either PD 98059 or U0126, two compounds capable of blocking ERK1 and ERK2 phosphorylation. A similar repression was also observed in cells that contained a dominant, nonactive form of ERK2 but not in cells where ERK1 phosphorylation was inhibited via overexpression of a dominant-negative mutant MEK1 protein. Presumably, proteins expressed from a wild-type VV that block ERK2 activity would also inhibit MVA-induced NF- κ B activation. Indeed, the expression of one such open reading frame, M2L, supported this prediction. First, ectopic M2L expression hampered ERK2 phosphorylation induced by exposure to phorbol myristate acetate. Second, viral M2L expression via infection of cells with a recombinant MVA construct that stably expressed M2L decreased the phosphorylation of ERK2 compared to that in cells infected with the parental MVA strain. Finally, the recombinant M2L-expressing virus restored the “wild-type” NF- κ B-inhibitory phenotype, as indicated by decreased NF- κ B migration to infected cell nuclei and interference in transcription. Thus, in 293T cells, VV apparently utilizes its M2L protein to interfere with a step(s) that would otherwise enable ERK2 phosphorylation and the consequential activation of an NF- κ B response.

The transcription factor NF- κ B regulates the expression of genes that encode proinflammatory and immune response proteins, making it an important component of the cellular defense against pathogens (reviewed in reference 5). In unstimulated cells, homo- or heterodimers of this complex reside in the cytosol due to masking of the nuclear localization signal of the p65 portion of NF- κ B via interaction with an I κ B α complex (reviewed in reference 19). However, exposure of cells to stimulants as diverse as viruses, cytokines, and lipopolysaccharides triggers a variety of distinct, classical signal transduction pathways that culminate in the activation of the I kappa kinase (IKK) complexes (13, 29). Consequently, I κ B α molecules bound to NF- κ B are phosphorylated, resulting in their subsequent polyubiquitination and degradation (3, 20, 36). The newly released NF- κ B migrates to the nucleus, where it binds to consensus nucleotide sequences in the cellular genome to facilitate transcription of specific immune defense genes (19).

Some viruses, such as human immunodeficiency virus, are thought to subvert NF- κ B for their own replicative purposes, specifically utilizing it as a viral transcription factor (reviewed in references 21 and 38), since free NF- κ B is detected in infected cultured cells and since the promoter regions in the respective viral genomes possess NF- κ B binding sites (21, 38).

Thus, for these pathogens, regulation of NF- κ B activity during infection must be delicately balanced to ensure virus gene expression without compromising virus survival (21).

In contrast to the above viruses, orthopoxviruses synthesize proteins that actually block the induction of NF- κ B activation in vitro (35, 40). Presumably, the rather unique physical nature of the poxvirus early and late promoters (32) precludes a prerequisite for NF- κ B involvement in poxvirus transcription. Thus, the members of this genus likely maintain NF- κ B in an inhibited state without negatively impacting viral gene expression. During in vivo infection, this inhibitory ability may be a defensive measure to retard NF- κ B-aided host immune responses. Indeed, this is the case for the prototypical orthopoxvirus, vaccinia virus (VV); the pathogenicity of mutant VVs incapable of expressing NF- κ B-inhibitory proteins is attenuated in infected laboratory animals relative to that observed for the wild-type virus (4, 18, 41).

Currently, the mechanisms responsible for strain MVA-induced NF- κ B activation and the respective mechanisms that prevent NF- κ B activation during infection with wild-type VVs are incompletely characterized. In this regard, VV may utilize a multifaceted approach to inhibit NF- κ B, since genetically modified viruses lacking the K1L open reading frame (ORF) still prevail in reducing NF- κ B activation (40). Hence, the initial goal of this study was to identify the signal transduction pathway(s) that is triggered by MVA infection and culminates in NF- κ B activation. The second aim was to ascertain which VV protein(s) absent in MVA interacts with the above-identified cellular components to suppress NF- κ B activity. To this end, the vulnerability of MVA-induced NF- κ B activation in

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the human kidney cell line HEK 293T was assessed by allowing virus infections to proceed in the presence of drugs that inhibited the action of proteins known or implicated to be members of different NF- κ B activation pathways. Notably, NF- κ B-regulated transcription of an introduced reporter gene was reduced when the cells were incubated in the presence of compounds specifically antagonistic for the MEK/ERK pathway or overexpressed a dominant-negative mutant ERK2 protein. Moreover, the VV M2L protein, whose corresponding gene is absent from the MVA genome, prevented phosphorylation of the ERK2 protein and subsequent NF- κ B activation. Thus, based on the results presented in this study, an additional VV protein is involved in the negative regulation of NF- κ B activity in poxvirus-infected cells.

MATERIALS AND METHODS

Cells and viruses. The human embryonic kidney cell line HEK 293T (293T) and primary chicken embryo fibroblasts (CEF) were purchased from the ATCC (Manassas, VA) and Charles River Laboratories (Wilmington, MA), respectively. All cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal calf serum (HyClone). Bernard Moss (National Institutes of Health) provided the wild-type WR strain of VV. The attenuated VV isolate MVA (27) was used to generate the MVA/5.2kb construct, whose genome contains a stably inserted 5.2-kb EcoRI fragment of DNA from the Ankara strain of VV (30). The MVA/K1L recombinant virus was created by stably inserting the entire WR K1L transcriptional unit into the deletion VI (del VI) region of the MVA genome (42).

An MVA virus containing the WR M2L transcriptional unit (MVA/M2L) was produced by homologous recombination in CEF cells. For this purpose, the M2L ORF and its linked promoter were amplified from WR virus DNA by using the primer set M2Lamp-F (5'-GAAGAAAGCGGCCCACTAATTGGTAAAAATCGATAG-3') and M2Lamp-R (5'-CCCTCTGCAGGATAGATAATACTCGGCGAC-3'). The resultant PCR product was inserted into the EagI and PstI sites of pLW44 (provided by Linda Wyatt and Bernard Moss) to create M2L/pLW44. This plasmid retained the green fluorescent protein gene linked to the VV p11 promoter for use as a stable screening marker during isolation of recombinant MVA viruses but lacked an adjacent VV H5 promoter that might otherwise have influenced the expression of the inserted M2L gene. Sequence analysis of a representative plasmid indicated that no mutations had been introduced into the amplicon. MVA/M2L progeny viruses, generated during recombination between homologous del III regions in both the MVA genome and the transfected M2L/pLW44 plasmid, were identified based on their ability to express green fluorescent protein. After four rounds of plaque purification performed in conjunction with visual screening for fluorescence, the presence of the M2L gene in the MVA del III region and the absence of contaminating parental viral DNA were verified by PCR analysis of the isolated viruses' genomes, using primers specifically binding to sites either flanking the del III region or present in the M2L gene. Stocks of pure recombinant MVA viruses were then prepared in CEF cells. M2L protein expression in MVA/M2L-infected 293T cells was verified by immunoblotting, where lysates from virus-infected cells were first electrophoretically separated and transferred to a polyvinylidene difluoride (PVDF) membrane. Subsequently, the membrane was incubated with polyclonal antisera raised against a keyhole limpet hemocyanin-conjugated peptide containing a portion of the predicted M2L protein sequence.

Plasmids. Marc Hersenson (University of Chicago) kindly provided pIKK β (AA), which contains an altered IKK β gene that expresses a dominant-negative kinase-deficient IKK β protein (34). pERK2-K52R, which encodes a dominant-negative mutant ERK2 protein (23), was a gift from Melanie Cobb (University of Texas Southwestern Medical Center). pMEK1-K97M codes for a dominant-negative, kinase-deficient mutant MEK1 protein (46). Plasmids K1L/pCI, M1L/pCI, and M2L/pCI were constructed by ligating the pCI plasmid (Promega) with the K1L, M1L, and M2L ORFs, respectively, which were PCR amplified from WR DNA. Each of these plasmids uses its internal cytomegalovirus promoter to express the inserted VV K1L, M1L, or M2L ORF. Where required, plasmids were transfected into cultured cells by using the FuGene 6 transfection reagent (Roche) per the manufacturer's instructions.

Luciferase reporter assay. The luciferase reporter assay used to quantitate NF- κ B activation was described previously (40). Briefly, 50 ng of pRL-null (Promega) and 450 ng of pNF- κ B κ (Stratagene) were transfected into subconfluent 293T cell monolayers. pNF- κ B κ contains the firefly luciferase gene under

the control of a synthetic promoter containing five direct repeats of the NF- κ B binding sequence and expresses the luciferase gene in the presence of activated NF- κ B. pRL-null constitutively expresses low levels of the *Renilla reniformis* (sea pansy) luciferase, whose measurement was used to define transfection efficiencies. In some instances, 500 ng of either pIKK β (AA), pERK2-K52R, pMEK1-K97M, or a control vector devoid of the studied genes was cotransfected with the pRL-null and pNF- κ B κ constructs. At 24 h posttransfection, the 293T cells were either mock infected or infected with unmodified or recombinant MVA (multiplicity of infection [MOI] = 10). In some cases, starting at 23.5 h posttransfection, the monolayers were exposed to various concentrations of either PD 98059 (Calbiochem) or U0126 (Calbiochem) or to a single concentration of either rapamycin (Calbiochem), wortmannin (Calbiochem), Ly294002 (Calbiochem), AG1478 (Calbiochem), or SB 203580 (Calbiochem). After a 30-min incubation period, virus infection proceeded as described above, except that the respective compounds were included in the medium and remained there throughout the experiment. Regardless of the experimental conditions, all cells were harvested at 8 h postinfection by scraping, pelleted by centrifugation (30 s at 14,000 \times g), and resuspended in passive lysis buffer (Promega). The resultant lysates were incubated with reagents from a dual-luciferase reporter assay (Promega), and firefly and sea pansy luciferase activities were measured separately in relative light units by using a Luminoskan microplate luminometer (Labsystems). Each assay was performed in triplicate, and the units of firefly luciferase activity was divided by the units of sea pansy luciferase activity for each experimental point to correct for possible differences in transfection efficiencies. The resultant ratios were normalized to the value obtained using untreated, uninfected cells.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays were performed as previously described (33). In some instances, subconfluent 293T cell monolayers in 12-well plates were transfected with 500 ng of either pIKK β (AA), pMEK1-K97M, or pERK2-K52R. At 24 h posttransfection, cells were mock infected or infected with MVA (MOI = 10). In other experiments involving the use of compounds known to inhibit MEK activity, cellular monolayers were incubated in medium containing either PD 98059 or U0126 for 30 min before infection. In these cases, the respective conditions were maintained during subsequent infection of the cells with MVA (MOI = 10) and until they were harvested.

At 8 h postinfection, all monolayers were harvested by scraping, pelleted by centrifugation (30 s at 14,000 \times g), and then lysed in CE buffer (35) to disrupt the cytoplasmic, but not nuclear, membranes. Nuclei were then separated from lysates by centrifugation and washed in excess CE buffer to remove contaminating cytoplasmic proteins. Next, nuclear membranes were disrupted in the presence of NE buffer (35), and the concentrations of the extracted nuclear proteins were determined by using a bicinchoninic acid protein assay kit (Pierce). Two micrograms of each extract was incubated with 0.35 pmol of 32 P-labeled double-stranded oligonucleotides containing binding sites for the NF- κ B transcription factor (Promega) in gel shift assay system binding buffer (Promega) per the manufacturer's directions. Monoclonal anti-p65 antiserum (Santa Cruz Biotechnology) was present in some reactions, as indicated. Reactions were resolved electrophoretically in a 6% acrylamide gel (Invitrogen) under nondenaturing conditions. Afterwards, the gel was dried onto filter paper and exposed on a phosphorimager plate (Molecular Devices), and images were developed and analyzed using the ImageGauge and ImageReader programs, respectively (Fuji).

Western immunoblotting. Cytoplasmic proteins were extracted from cell lysates according to a modified version of previously described methods (35). For this purpose, previously treated cell monolayers were collected by centrifugation (30 s at 14,000 \times g), lysed in CE buffer containing protease inhibitors, and incubated for 5 min at 4°C. After a brief centrifugation step (20 s at 14,000 \times g), the resultant supernatants were collected, and the protein concentration in each preparation was determined by using a bicinchoninic acid assay (Pierce). Equivalent amounts of protein (25 μ g) from the samples were loaded into separate wells of a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore). Blots were subsequently incubated either at room temperature for 1 h or at 4°C overnight with rabbit antisera that either recognized the phosphorylated form of the tyrosines at position 204 in ERK1 and ERK2 (Santa Cruz Biotechnology) or unphosphorylated ERK2 and, to a lesser extent, ERK1 (Santa Cruz Biotechnology). After being washed to remove unbound primary antibodies, blots were incubated with a secondary antibody consisting of goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (Fisher Scientific). The immunoblots were developed by using SuperSignal West chemiluminescence reagents per the manufacturer's directions (Pierce).

TABLE 1. Effect of kinase phosphorylation blockers on MVA-induced activation of host NF- κ B^a

Compound	Target (reference) ^b	Concn of compound (μ M)	Inhibition of MVA-induced NF- κ B activation (%) ^c
Rapamycin	p70S6 kinase (9)	50.0	0
Wortmannin	Phosphatidylinositol 3-kinase (2)	0.1	0
U0126	MEK1 and MEK2 (16, 17)	50.0	75
LY294002	Phosphatidylinositol 3-kinase (43)	50.0	15
AG1478	EGF receptor kinase (25)	50.0	20
PD 98059	MEKs (15)	50.0	85
SB 203580	p38 MAP kinase (10)	50.0	0

^a 293T cell monolayers, previously transfected with pRL-null and pNF- κ B κ Luc, were incubated in medium containing the indicated quantities of the designated compounds for 30 min prior to, during, and 8 h after infection with MVA.

^b Principal kinase whose phosphorylation and subsequent activation are blocked by the designated compound.

^c Percent inhibition was determined by dividing the value of normalized luciferase activity measured in the lysate of MVA-infected cells exposed to the designated compound by that obtained using the lysate of cells similarly infected in the absence of any drug and multiplying the difference by 100.

RESULTS

Inhibition of MEK/ERK signal transduction pathway decreases MVA-induced NF- κ B activation. To identify the signal transduction pathway(s) utilized by 293T cells to activate NF- κ B in response to MVA infection, this host-virus system was used to screen inhibitors of the phosphorylation of various cellular kinases for the ability to inhibit virus-induced NF- κ B activity. In this assay, all of the targeted proteins were components of known NF- κ B activation pathways. The inhibition of MVA-induced NF- κ B activation was determined by a relative comparison of the extents of NF- κ B-regulated firefly luciferase expression in infected cells incubated in either the presence or absence of the compounds. As shown in Table 1, a presumed inhibition of the phosphorylation of either p70S6 (9) or p38 mitogen-activated protein (MAP) kinase (10) by rapamycin or SB 203580, respectively, had no detectable impact on virus-induced NF- κ B activity. Likewise, no noticeable effect was caused by wortmannin, whose target is phosphatidylinositol 3-kinase (2). In contrast, another preventer of phosphorylation of this kinase (43), LY294002, modestly decreased NF- κ B activity, as did AG1478, an inhibitor of epidermal growth factor receptor kinase phosphorylation (25). The greatest reductions ($\geq 75\%$) were afforded by two blockers of MEK phosphorylation, namely, PD 98059 (15) and U0126 (16, 17).

Based on the observed repression of MVA-induced NF- κ B activation by compounds that either inhibit the MEK family of kinases in general (PD 98059) (15) or inhibit primarily MEK1 and MEK2 (U0126) (16, 17), the influence of both drugs on infected 293T cell responses was studied further. First, the effects of various concentrations of each compound on virus-induced NF- κ B activation were assayed by measuring NF- κ B-regulated luciferase gene expression. Similar to previous reports (35, 40), luciferase activity was greatly increased (31-fold) in MVA-infected versus uninfected cells (Fig. 1A). In contrast, when MVA infection proceeded in medium containing 10 μ M PD 98059, a decrease in luciferase activity of approximately 20%, to about 25-fold greater than the level detected in uninfected cells, was observed. More pronounced reductions, of 68

and 77%, were observed when infections proceeded in medium containing 25 and 50 μ M PD 98059, respectively. When utilizing U0126, a 50- μ M dose resulted in the greatest inhibition of NF- κ B activation, with infected cells expressing luciferase at a level only threefold above that detected in uninfected cells (Fig. 1B). When the drug concentration was reduced to 25 μ M, the lysates possessed luciferase activities approximately twofold greater than those derived from the cells treated with 50 μ M U0126 (Fig. 1B). A further decrease of the U0126 concentration to 10 μ M apparently did not inhibit MVA-induced NF- κ B activation, since comparable 11- and 10-fold increases in luciferase activity were present in infected cells either undergoing this treatment or incubated in regular medium, respectively. Luciferase activity levels remained comparably low in uninfected cells, regardless of the presence or absence of PD 98059 or U0126. Thus, neither compound exhibited an intrinsic NF- κ B-activating ability that would have influenced the outcomes of these experiments.

Second, to validate that both drugs interfered with NF- κ B activation at a step prior to its nuclear translocation, we employed gel mobility shift assays, which detect the interaction of freed NF- κ B in protein samples extracted from nuclei with a radiolabeled oligonucleotide containing sequential, consensus

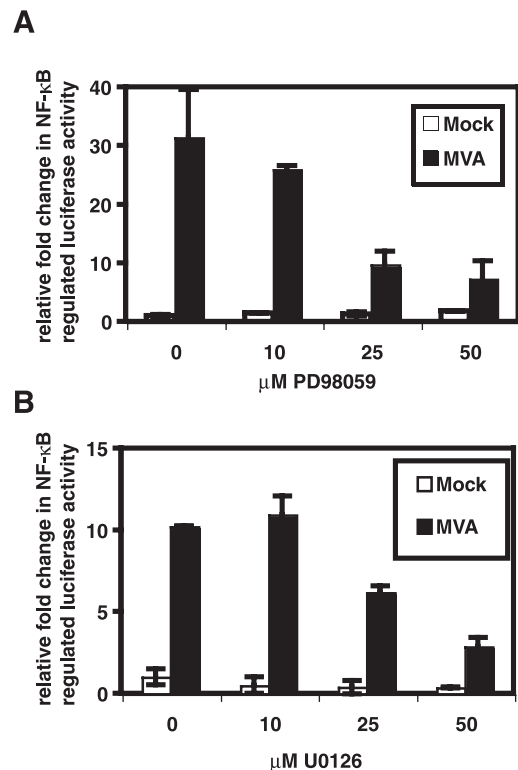


FIG. 1. Effect of either PD 98059 or U0126 on expression of an NF- κ B-controlled luciferase reporter gene in MVA-infected cells. For all experiments, subconfluent 293T cell monolayers were transfected with 450 ng of pNF- κ B κ Luc and 50 ng of pRL-null. After 24 h, the cells were incubated in medium lacking or containing the indicated concentrations of either PD 98059 (A) or U0126 (B) for 30 min prior to being mock infected or infected with MVA (MOI = 10). Where applicable, either drug was present in the medium during the adsorption stage of infection and for 8 h postinfection, at which time the cells were harvested and lysates were assayed for luciferase activity.

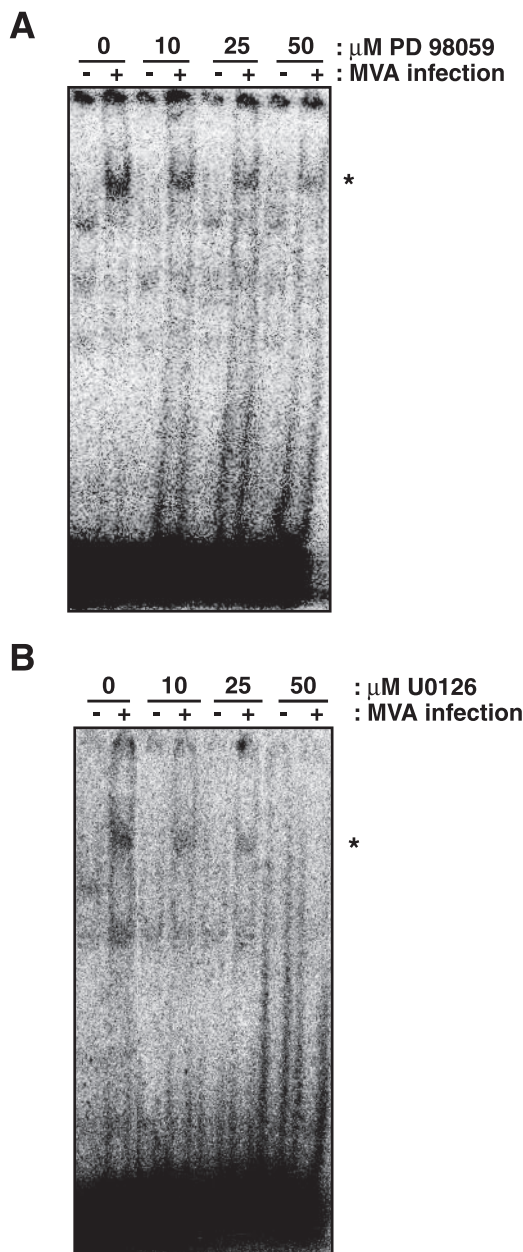


FIG. 2. Effect of either PD 98059 or U0126 on NF-κB nuclear translocation in MVA-infected cells. In all experiments, 293T cells were incubated in medium lacking or containing the indicated concentrations of either PD 98059 (A) or U0126 (B) for 30 min prior to being mock infected or infected with MVA (MOI = 10). Where applicable, either drug was present in the medium during the adsorption stage of infection and for 8 h postinfection, at which time the cells were harvested. Two micrograms of protein extracted from nuclei prepared from each sample was incubated with ³²P-radiolabeled oligonucleotides containing consensus NF-κB binding sites and analyzed via an electrophoretic mobility shift assay. The location of shifted bands containing NF-κB is indicated by an asterisk.

NF-κB binding sequences. As expected, nuclear extracts from monolayers that were uninfected or unexposed to either drug failed to alter oligonucleotide mobility (Fig. 2A and B). In contrast, a unique shifted band (indicative of bound NF-κB) was observed when the oligonucleotides were incubated with

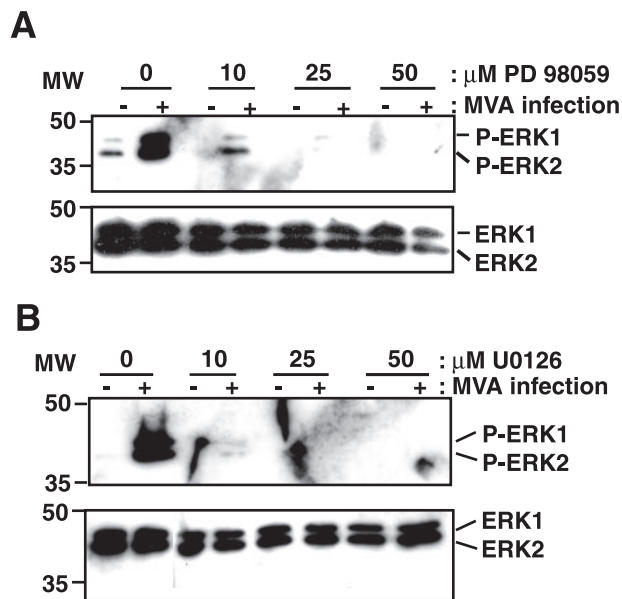


FIG. 3. Effects of PD 98059 and U0126 on ERK1/2 phosphorylation in MVA-infected cells. Subconfluent 293T cell monolayers were incubated in medium lacking or containing the indicated concentrations of either PD 98059 (A) or U0126 (B) for 30 min prior to being mock infected or infected with MVA (MOI = 10). Where applicable, either compound was present in the medium during the adsorption stage of infection and for 8 h postinfection, at which time the cells were lysed in CE buffer. Equal amounts (25 μg) of prepared cytoplasmic extracts were electrophoretically separated in 12% SDS-polyacrylamide gels and transferred to PVDF membranes. Samples were analyzed by probing separate immunoblots with antisera recognizing the phosphorylated (top panels) (P-ERK1 and P-ERK2) and unphosphorylated (bottom panels) (ERK1 and ERK2) forms of ERK1 and ERK2.

extracts prepared from MVA-infected cells. The relative amount of this band, as indicated by the recorded intensity of its signal, greatly decreased when utilizing extracts of cells exposed to an amount of PD 98059 (25 or 50 μM) or U0126 (25 or 50 μM) previously shown to inhibit MVA-induced NF-κB activation (Fig. 1). As confirmation that the shifted band contained the NF-κB complex, we observed the presence of a supershifted band when antisera recognizing NF-κB subunits were included in reactions containing extracts from MVA-infected cells (data not shown). Thus, it seemed that both compounds acted prior to the entrance of NF-κB into the nucleus.

Third, to establish that, as reported previously (15–17), PD 98059 and U0126 did indeed inhibit MEK1 and MEK2 activity, we utilized immunoblotting, detecting the phosphorylated forms of the respective targets (ERK1 and ERK2), to examine the status of these proteins in cells exposed to either of the two drugs (Fig. 3). While enhanced phosphorylation of both ERKs was associated with MVA infection of 293T cells, this cellular response was decreased when the infected cells were incubated in the presence of the lowest tested amount (10 μM) of either drug. Increasing the concentration of either compound 2.5- to 5-fold resulted in an inability to detect phosphorylated ERK1 (P-ERK1) or ERK2 (P-ERK2). These reductions were not attributed to ERK1 or ERK2 degradation since the relative quantities of each remained fairly constant, as measured by

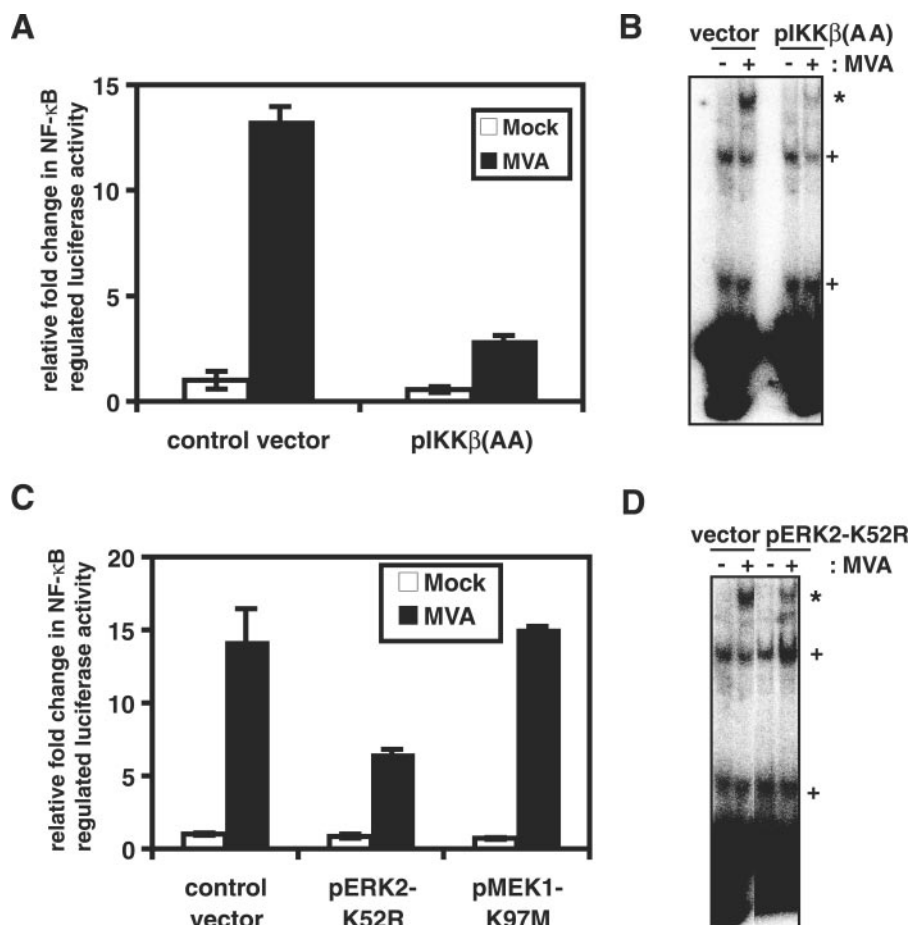


FIG. 4. Effect of expression of dominant-negative, kinase-deficient forms of IKK β , MEK1, and ERK2 on NF- κ B activation in MVA-infected cells. (A and C) 293T cell monolayers were cotransfected with 450 ng of pNF- κ B Luc , 50 ng of pRL-null, and 500 ng of either empty vector, pIKK β (AA) (A), pMEK1-K97M (C), or pERK2-K52R (C). At 24 h posttransfection, the cells were mock infected or infected with MVA (MOI = 10). After 8 h, the cells were harvested and lysates were assayed for luciferase activity. (B and D) Subconfluent 293T cell monolayers were transfected with 500 ng of either vector control plasmid, pIKK β (AA) (B), or pERK2-K52R (D). After 24 h, the cells were mock infected or infected with MVA (MOI = 10). At 8 h postinfection, the monolayers were harvested, and 2 μ g of protein extracted from nuclei prepared from each sample was incubated with 32 P-radiolabeled oligonucleotides containing consensus NF- κ B binding sites and analyzed via an electrophoretic mobility shift assay. The locations of shifted bands containing NF- κ B and nonspecific bands are indicated by an asterisk and plus signs, respectively.

immunoblotting with antibodies recognizing only the native (unphosphorylated) forms of the kinases. Thus, either drug could block the induction of ERK1 and ERK2 phosphorylation by MVA infection without physical elimination of either kinase.

Activation of NF- κ B in response to MVA infection is reliant on ERK2 phosphorylation. Based on the above results, MVA infection of 293T cells appeared to initiate a cascade that resulted in the activation of the MEK1 and MEK2 proteins, followed by their phosphorylation of ERK1 and ERK2 and the subsequent nuclear translocation of NF- κ B. To confirm a requirement for these kinases, we evaluated the effect of expression of deficient kinases during MVA-induced NF- κ B activation. As a source of deficient kinases, vectors designed to overexpress either a dominant-negative mutant MEK1 or ERK2 protein were transfected into 293T cells prior to MVA infection. However, a potential drawback to utilizing this approach is that a low transfection efficiency would limit the production of the mutant protein and could accordingly yield a false-negative result. Thus, we assessed the practicality of this

approach by measuring the ability of plasmid pIKK β (AA), capable of producing a dominant-negative, kinase-defective mutant IKK β protein (34), to repress MVA-induced NF- κ B activation. Since the IKK β protein is usually necessary for the release of NF- κ B from a complex with I κ B molecules (29), competitive inhibition of its activity should inhibit NF- κ B. Since the level of luciferase activity was reduced approximately 75% (from 13-fold to 3.5-fold) when MVA infections proceeded in pIKK β (AA)-transfected cells versus vector control-transfected cells (Fig. 4A), we concluded that an adequate transfection efficiency was achieved with this system. The decrease in luciferase activity accurately reflected an inhibition of NF- κ B nuclear migration, as measured by electrophoretic mobility shift assays. As expected, the nuclear proteins from uninfected, control vector-transfected cells failed to alter oligonucleotide mobility (Fig. 4B), indicating a lack of NF- κ B nuclear translocation. In contrast, a shifted band was detected when assaying nuclear proteins from control vector-transfected 293T cell monolayers that were subsequently infected with

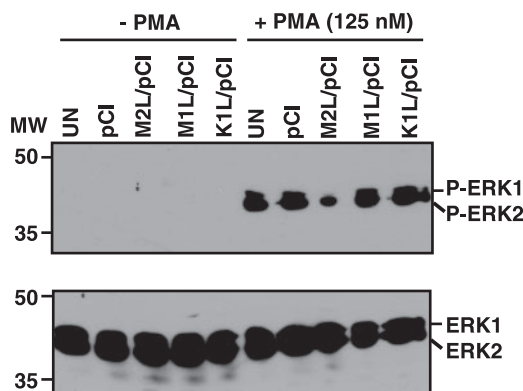


FIG. 5. Effect of autonomous expression of VV K1L, M1L, or M2L on ERK2 phosphorylation in PMA-induced cells. Subconfluent 293T cell monolayers were either mock transfected (UN) or transfected with 1 μ g of either pCI, M2L/pCI, M1L/pCI, or K1L/pCI. After 24 h, the cells were incubated in medium lacking or containing 125 nM PMA for 2 h, at which time they were harvested and lysed in CE buffer. Equal amounts (25 μ g) of prepared cytoplasmic extracts were electrophoretically separated in 12% SDS-polyacrylamide gels and transferred to PVDF membranes. Samples were analyzed by probing separate immunoblots with antisera recognizing the phosphorylated (top panel) and unphosphorylated (bottom panel) forms of ERK1 and ERK2.

MVA. This specific band was supershifted to a higher-molecular-weight complex when reactions included antisera against the NF- κ B p65 subunit (data not shown), confirming that NF- κ B was responsible for inducing the original shifted band. The signal intensity of this novel band, but not those of the nonspecific bands, was greatly reduced when the oligonucleotides were incubated with nuclear extracts from pIKK β (AA)-transfected, MVA-infected cells (Fig. 4B). Thus, transfected plasmids could be used to regulate MVA-induced activation of NF- κ B by interfering with a pathway(s) enabling the nuclear translocation of this transcription factor.

Next, the effects of plasmids carrying genes encoding dominant-negative, kinase-deficient forms of MEK1 (pMEK1-K97M) or ERK2 (pERK2-K52R) on MVA-induced NF- κ B activation in 293T cells were assessed (Fig. 4C). In contrast to the 14- and 15-fold increases in luciferase activity found in MVA-infected cells transfected with either the control vector or pMEK1-K97M compared to that in similarly treated, uninfected cells, only a comparable 7-fold increase was detected in infected cells transfected with pERK2-K52R. The extent of this reduction (approximately 50%) was comparable to the 75% value obtained with pIKK β (AA) and thus indicated the involvement of ERK2, but not of MEK1, and presumably its substrate, ERK1, in virus-induced activation of NF- κ B. This was further supported by the ability of transfected pERK2-K52R to partially inhibit NF- κ B nuclear translocation in MVA-infected cells, presumably via interference with phosphorylation of the natural ERK2 protein (Fig. 4D). As described above, the addition of anti-p65 antisera to reactions containing nuclear extracts from vector-transfected, MVA-infected cells resulted in the disappearance of the shifted band and the appearance of a supershifted band, thus confirming that the shifted band contained NF- κ B (data not shown).

The VV M2L protein prevents phosphorylation of ERK2 and inhibits MVA-induced NF- κ B activation. It was reported

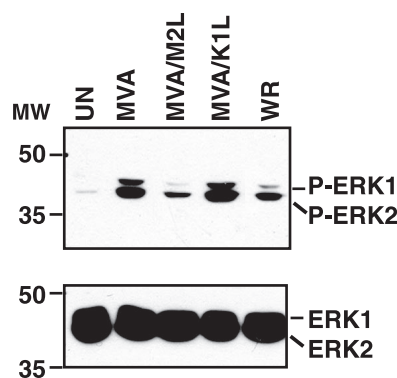


FIG. 6. Effect of M2L gene expression on ERK phosphorylation in MVA-infected cells. 293T cell monolayers were mock infected (UN) or infected at an MOI of 10 with either MVA, MVA/M2L, MVA/K1L, or WR. At 2 h postinfection, cells were collected and lysed in CE buffer. Equal amounts of prepared cytoplasmic extracts (25 μ g) were electrophoretically separated in 12% SDS-polyacrylamide gels and transferred to PVDF membranes. Samples were analyzed by probing separate immunoblots with antisera recognizing the phosphorylated (top panel) and unphosphorylated (bottom panel) forms of ERK1 and ERK2.

previously that the stable insertion of a 5.2-kb region of parental Ankara DNA into the MVA genome produced a recombinant virus that no longer fully activated NF- κ B in 293T cells (40). Since this fragment contains three intact ORFs (M2L, M1L, and K1L) that are either absent or fragmented in the MVA genome (30), one hypothesis is that one or more of the encoded products inhibits NF- κ B activation by interfering with at least one step in the pathway requiring ERK2 phosphorylation. We tested this hypothesis by examining the effect of expression of the three individual ORFs on phosphorylation of ERK2 in stimulated 293T cells. To this end, the status of ERK2 in stimulated 293T cells that had been transfected previously with expression vectors containing the VV K1L gene (K1L/pCI), M1L gene (M1L/pCI), or M2L gene (M2L/pCI) was examined.

Rather than use MVA for induction, the phorbol myristate acetate (PMA) compound, which activates the MEK/ERK pathway and triggers the formation of P-ERK2 in 293T cells (22), was included in the medium. As shown in Fig. 5, a 2-h exposure to PMA was sufficient to induce ERK2 phosphorylation, an event not detected in any of the untreated transfected cells. No decrease of ERK2 phosphorylation was noticed in the PMA-treated cells transfected with K1L/pCI, M1L/pCI, or the unmodified parental pCI vector. In contrast, ectopic expression of the M2L protein resulted in a discernible decrease in the presence of P-ERK1 and P-ERK2 in PMA-exposed cells. This loss was not due to ERK protein degradation in M2L/pCI-transfected cells since ERK1 and ERK2 quantities were similar in all lysates, regardless of whether the cells had been transfected or activated with PMA.

To test if the presence of the M2L gene had a similar effect on MVA-induced activation of MEK/ERK interactions, we examined the effect of insertion of the VV M2L transcriptional unit into the MVA genome on the resultant recombinant's ability to trigger ERK2 phosphorylation. Similar to the results of an earlier experiment (Fig. 3), both P-ERK1 and P-ERK2 were present in greater amounts in lysates from MVA-infected

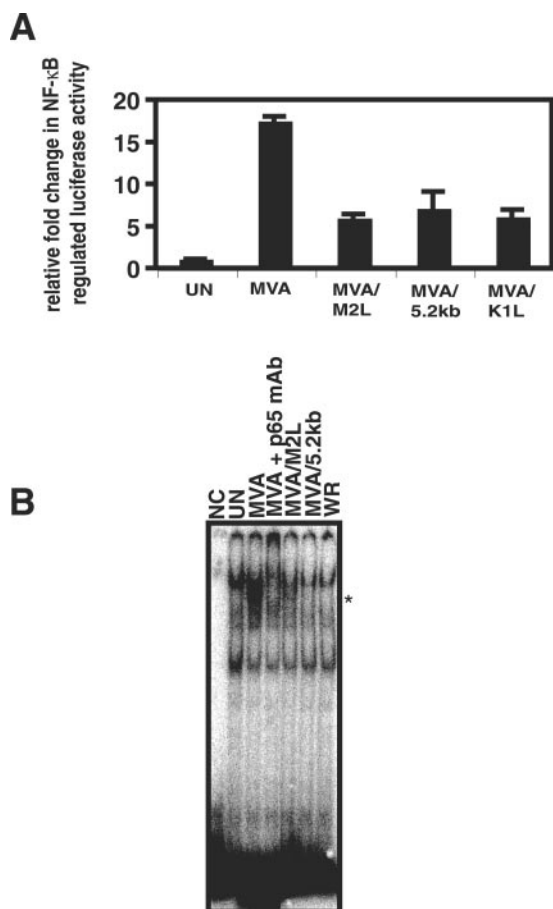


FIG. 7. Effect of M2L protein expression on NF- κ B activation in MVA-infected cells. (A) Subconfluent 293T cell monolayers were transfected with 450 ng of pNF- κ B λ and 50 ng of pRL-null. At 24 h posttransfection, cells were either mock infected (UN) or infected at an MOI of 10 with MVA, MVA/M2L, MVA/5.2kb, or MVA/K1L. At 8 h postinfection, cells were harvested and lysates were assayed for luciferase activity. (B) 293T cell monolayers were either mock infected (UN) or infected at an MOI of 10 with MVA, MVA/M2L, MVA/5.2kb, or WR. At 20 h postinfection, the cells were harvested, and 2 μ g of protein extracted from nuclei prepared from each sample was incubated with 32 P-radiolabeled oligonucleotides containing consensus NF- κ B binding sites and analyzed via an electrophoretic mobility shift assay. An additional reaction mix containing nuclear extracts from MVA-infected cells and a monoclonal anti-p65 antiserum was also analyzed. The position of the shifted band containing NF- κ B is indicated by an asterisk.

cells than in those from mock-infected cells (Fig. 6). For comparison, the levels of both phosphorylated proteins were dramatically reduced in lysates from cells infected with MVA/M2L or the M2L ORF-possessing WR strain of VV. This inhibition was specific for the M2L protein, as ERK2 phosphorylation was not diminished in cells infected with a different MVA recombinant whose DNA contained a novel VV K1L transcriptional unit insert (MVA/K1L). Moreover, the M2L protein did not cause ERK2 degradation, since the relative amount of ERK2 remained constant, regardless of whether the cells were mock infected or infected with any of the four viruses.

To test that the introduction of the M2L ORF into the MVA

genome altered the virus's ability to activate NF- κ B, transcriptional regulation by and nuclear translocation of this transcription factor was assessed in MVA/M2L-infected cells. Although MVA/M2L infection enhanced luciferase activity approximately 6-fold compared to that in uninfected cells, this increase actually represented a >50% inhibition of the 16-fold luciferase increase in MVA-infected cells (Fig. 7A). As previously reported (40), a similar decrease in NF- κ B-controlled luciferase gene expression was observed when MVA/5.2kb was utilized. Since MVA/5.2kb infection did not additionally impair luciferase activity, it appeared that only the M2L protein, not the K1L product, was required for partial repression of the host's NF- κ B response to MVA infection. Coincident with the results from the reporter assay, we observed a reduction in NF- κ B nuclear translocation in cells infected with either MVA/M2L, MVA/5.2kb, or WR compared to that in MVA-infected cells (Fig. 7B). The shifted band was no longer detectable in a separate reaction containing extracts from MVA-infected cells and antibody recognizing the p65 subunit of NF- κ B. This disappearance is evidence, albeit indirect, that the antibody interacted with NF- κ B. Therefore, MVA's inability to prevent NF- κ B activation and nuclear translocation is partially mitigated by the introduction of the M2L ORF.

DISCUSSION

Viral regulation of NF- κ B has the potential to influence inflammation and the severity of viral disease. Thus, defining viral mechanisms and products that either activate or inhibit this transcription factor will give insights into mechanisms that control the pathogenesis of poxvirus-induced disease. In the present study involving characterization of the cellular signal transduction pathway activated in response to a VV deletion mutant which lacks the prerequisite genetic information to alter this reaction (35, 40), we observed that a necessary, but not all-inclusive, step towards NF- κ B activation involved ERK2 phosphorylation. Interestingly, MVA-induced NF- κ B activation could be partially counteracted by stably introducing and expressing the M2L gene product. Moreover, the M2L action seemed independent of virus infection, since ectopic M2L expression inhibited PMA-induced ERK2 phosphorylation.

ERK2 (extracellular signal-regulated kinase) belongs to a larger family of MAP kinases, which include the c-Jun N-terminal kinase/stress-activated protein kinase, p38 kinase, and the highly similar ERK1 protein (39). ERK proteins are phosphorylated and activated by their respective, upstream MAP kinase kinases, i.e., MEK1 and MEK2 activate ERK1 and ERK2, respectively (39). Previous reports have identified ERK2, in cooperation with ERK1, as contributing to NF- κ B activation (8, 12, 24, 26). According to one report, ERK2-mediated NF- κ B activity is partially due to NIK-regulated activation of the MEK/ERK pathway (12). Our observation that NF- κ B activity is decreased in MVA-infected cells overexpressing dominant-negative mutant NIK proteins compared to that in cells expressing only wild-type NIK proteins (data not shown) suggests that NIK may be partially or fully responsible for ERK2 activation in this system as well. Yet other publications reported that ERK2-mediated NF- κ B activation occurs via a Ras/Raf-1 pathway (8, 26). Since the overexpression of

dominant-negative mutant Ras or Raf-1 proteins does not impede MVA's signaling ability (data not shown), it is unlikely that this pathway is engaged during MVA infection in 293T cells.

It was observed that either exposure to nonlethal quantities of PD 98059 or U0126 or the overexpression of a dominant-negative, kinase-deficient form of ERK2 did not completely abolish MVA-induced NF- κ B activation, as measured by luciferase reporter and electrophoretic mobility shift assays. One potential explanation for this observation is that ERK2 functions cooperatively with another protein to eventually activate NF- κ B and that both participants must be inactivated to completely repress NF- κ B activation. Two such candidate proteins are ERK1 and ERK5, since complexes of either with ERK2 can activate NF- κ B (8, 12, 24, 26, 37, 47). In support of the possibility that the ERK2-ERK1 complex is responsible, the extent of ERK1 phosphorylation in MVA- or PMA-stimulated cells was reduced in the presence of the M2L protein (Fig. 6). However, the noninvolvement of ERK1 was indicated by the failure of cells transfected with a plasmid expressing a dominant-negative mutant MEK1 protein, whose overexpression would presumably enable it to interfere with ERK1 phosphorylation, to inhibit the degree of MVA-induced NF- κ B activation. In considering a role for ERK5, it should be noted that this kinase's activity is also inhibited by PD 98059 and U0126, albeit at concentrations greater than those required for repression of ERK2 phosphorylation (37). Moreover, unlike the case for ERK1, transient expression of a kinase-defective mutant form of ERK5 actually diminished MVA-induced, NF- κ B-regulated luciferase activity (data not shown). However, our efforts involving the use of immunoblotting and antibodies that specifically recognize phosphorylated ERK5 to detect the modified form of this protein in the lysates of MVA-infected cells have been unsuccessful to date (data not shown).

Alternatively, an MVA infection eliciting a second, ERK2-independent NF- κ B activation pathway would explain the partial inhibition observed in dominant-negative ERK2 mutant-expressing cells (Fig. 8). Interestingly, we demonstrated that another VV protein, K1L, hampers NF- κ B-controlled cellular and marker (luciferase) gene transcription in 293T cells (40). However, unlike M2L, K1L did not impede ERK2 phosphorylation in cells stimulated with either MVA or PMA. Since infection with either MVA/M2L or MVA/K1L inhibited NF- κ B-regulated luciferase activity similarly and to the same level as that during MVA/5.2kb infection (Fig. 7), one possibility is that the K1L product functions downstream of ERK2 activation to prevent NF- κ B activation. It is unlikely that K1L and M2L interact directly to inhibit ERK2, since no association between these two proteins was detected in a yeast two-hybrid system (28). Yet another conclusion that can be drawn from the observed independence of K1L and M2L is that they each inhibit distinct signal transduction pathways to repress NF- κ B activation. If VV infection does indeed antagonize a cell to employ more than one transduction pathway for NF- κ B activation, then it would not be surprising that separate interactions between different host and virus proteins are necessary for complete inhibition of the NF- κ B response. Whether this phenomenon occurs in 293T cells is currently unknown, but in establishing a molecular mechanism for K1L in these cells, we would rule out the involvement of p70S6 kinase, phosphatidyl-

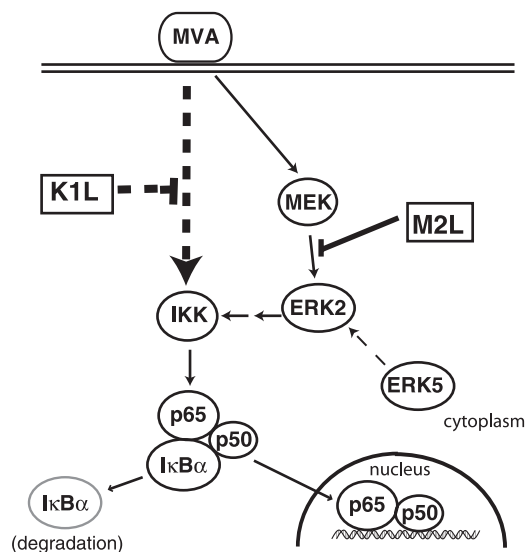


FIG. 8. Molecular model depicting the induction and suppression of NF- κ B activation during VV infection of 293T cells. Our current hypothesis is that MVA infection of 293T cells triggers the phosphorylation of ERK2 via MEK proteins. Next, the IKK complex is targeted by P-ERK2, possibly in conjunction with ERK5, and the activated IKK group in turn phosphorylates I κ B α . Consequently, the modified I κ B α is degraded, and the released NF- κ B is able to migrate but temporarily remains in the nucleus to coordinate the transcription of a variety of host immune response genes. When available, M2L will interfere with MEK-directed phosphorylation of ERK2 and thereby partially inhibit VV-induced NF- κ B activity. The remainder of the suppression is likely provided by K1L, which negatively interacts with the component(s) of an ERK2-independent pathway that promotes NF- κ B activation.

inositol 3-kinase, and p38 MAP kinase, since compounds that inhibit their activities did not reduce MVA-induced NF- κ B activation (Table 1). Future experiments that are designed to examine the effects of M2L and K1L gene expression on events after ERK phosphorylation and on other signal transduction pathways in a variety of cell lines will be necessary to establish the correct model.

Interestingly, when A31 cells are used in lieu of 293T cells as the host, ERK1/2 activation may be required for VV to establish a highly productive infection (1, 11). In these studies, not only did exposure to the WR strain promote phosphorylation of the two kinases, but inclusion of the ERK1/2-inhibitory agent PD 98059 resulted in a decreased yield of infectious progeny. Several lines of evidence suggest that the utilization of ERK1/ERK2 for WR virus replication in A31 cells is independent of these kinases' function in activating NF- κ B in MVA-infected 293T cells. First, ERK phosphorylation in virus-infected A31 cells is somewhat reliant on the presence of VV growth factor (1). However, inhibition of epidermal growth factor receptor signaling by AG1478 did not substantially alter MVA-induced NF- κ B activity in 293T cells (Table 1). Thus, it would seem that the ERK activation response is mediated through different mechanisms in the two cell lines. Second, while UV-irradiated WR virus retained the ability to elicit the formation of P-ERK1 and P-ERK2 in A31 cells (1), similarly inactivated MVA was no longer capable of inducing NF- κ B-mediated luciferase gene expression in 293T cells (data not

shown), inferring that the expression of an early gene product controls the activity of the MEK/ERK pathway in 293T cells.

Attempts to unravel the complexity of ERK involvement in both the host response to poxvirus infection and replication of this pathogen are further aggravated by the report that active ERK1/2 inhibits myxomavirus replication in mouse embryo fibroblasts (44). Moreover, suppression of ERK1/ERK2 phosphorylation reversed this restriction, thereby enabling the infectious cycle to proceed in this otherwise nonpermissive cell line (44). A similar, though less extreme, host restriction was noted with regard to MVA replication in human cells, in that the virus yield and plaque size were smaller than those observed when cells of other mammalian origins served as the host (7). Our observation that PD 98059 or U0126 treatment of MVA-infected 293T cells or two other human carcinoma cell lines did not increase the size of virus-induced foci suggests that it is unlikely that activated NF- κ B or ERK2 was responsible for limiting MVA replication in these cells (data not shown). Nevertheless, further understanding of the interrelationships of ERK and NF- κ B with regard to the regulation of poxvirus infection may provide the means to ultimately control virus pathogenesis and replication.

One expectation of a viral suppressor of the host immune response is that the gene product either be present at the time of infection or be produced immediately thereafter. Although the M2L protein does not appear to be a structural component of the virus, its rapid appearance during infection is predicted based upon the physical similarity of its gene promoter to other poxvirus early transcriptional regulatory elements and the early transcription termination signal, TTTTNT (31), embedded within its coding sequence. Indeed, transcripts from this region have been found in wild-type VV strain WR-infected cells at early times postinfection (45), and the protein itself has been detected during immunoblotting of WR-infected 293T cell lysates, prepared as early as 2 h postinfection, with anti-M2L antiserum (data not shown). Furthermore, this protein may be representative of a defense mechanism shared by many poxviruses, since M2L gene homologs have been identified in the genomes of other *Orthopoxvirus* members, such as *Variola virus*, and in the DNAs of members of the *Leporipoxvirus* and *Yatapoxvirus* genera. M2L shares no significant amino acid homology with any cellular protein described so far and also lacks any identifiable motifs, except for a putative, N-terminal signal sequence that could be used to attribute functionality. The importance of this region with regard to M2L cellular localization and ERK2-inhibitory activity will be the focus of future studies.

In addition to the M2L product, which acts on the MEK/ERK pathway, VV expresses four other known NF- κ B-inhibitory products. The K1L protein also prevents MVA-mediated NF- κ B activation (40), via undefined mechanisms. The A52R and A46R products each inhibit the NF- κ B activation pathway (6, 14) by impeding signalosome complexes necessary for IKK activation: while A52R associates with interleukin 1 receptor-associated kinase 2 and tumor necrosis factor receptor-associated factor 6 (18), the A46R protein targets Toll-like interleukin 1 resistance domain-containing proteins, such as MyD88 (41). The MVA genome possesses the MVA159R and MVA028R ORFs, which are 99% and 50% similar to the WR A46R and A52R ORFs, respectively. Since MVA infection activates

NF- κ B in 293T cells and would presumably produce biologically active A52R and A46R products, one possibility is that signaling pathways inhibited by these proteins are not activated in the 293T system. In support of this hypothesis, we found that the overexpression of a dominant-negative MyD88 mutant protein did not inhibit MVA-induced NF- κ B activation, as measured by a luciferase reporter assay (data not shown). Since Toll-like receptor 3 signaling is MyD88 independent, the role of this pathway during MVA infection cannot be ruled out. Alternatively, if NF- κ B activity was greatly increased in cells infected with a mutant MVA virus deleted for either or both A46R and A52R ORFs compared to that in cells infected with the parental MVA virus, then we would conclude that both Toll-like receptor and MEK/ERK signaling is responsible for NF- κ B activation in this cell line. The VV N1L product, which shares similarity to the A52R product, appears to act at a later point than the VV proteins discussed above, interacting with the TANK-binding kinase 1 subunit of the IKK complex to prevent IKK activation (14). Extracellular or intracellular stimuli utilize unique signal transduction pathways to converge upon the IKK complex, thereby triggering phosphorylation and subsequent degradation of the I κ B substrate and release of the NF- κ B transcription factor from its interaction with I κ B (19). The observation that VV expresses multiple proteins to neutralize individual pathways is reflective of an in vivo infection that elicits the expression of myriad cellular proteins known to activate this transcription factor.

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