JOURNAL OF VIROLOGY, Jan. 2006, p. 578–586 Vol. 80, No. 2
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.2.578–586.2006

The MC160 Protein Expressed by the Dermatotropic Poxvirus Molluscum Contagiosum Virus Prevents Tumor Necrosis Factor Alpha-Induced NF-κB Activation via Inhibition of I Kappa Kinase Complex Formation

Daniel Brian Nichols and Joanna L. Shisler*

Department of Microbiology, College of Medicine, University of Illinois, Urbana, Illinois 61801

Received 21 July 2005/Accepted 17 October 2005

The pluripotent cytokine tumor necrosis factor alpha (TNF-α) binds to its cognate TNF receptor I (TNF-RI) to stimulate inflammation via activation of the NF-κB transcription factor. To prevent the detrimental effects of TNF-α in keratinocytes infected with the molluscum contagiosum virus (MCV), this poxvirus is expected to produce proteins that block at least one step of the TNF-RI signal transduction pathway. One such product, the MC160 protein, is predicted to interfere with this cellular response because of its homology to other proteins that regulate TNF-RI-mediated signaling. We report here that expression of MC160 molecules did significantly reduce TNF-α-mediated NF-κB activation in 293T cells, as measured by gene reporter and gel mobility shift assays. Since we observed that MC160 decreased other NF-κB activation pathways, namely those activated by receptor-interacting protein, TNF receptor-associated factor 2, NF-κB-inducing kinase, or MyD88, we hypothesized that the MC160 product interfered with I kappa kinase (IKK) activation, an event common to multiple signal transduction pathways. Indeed, MC160 protein expression was associated with a reduction in in vitro IKK kinase activity and IKK subunit phosphorylation. Further, IKK1-IKK2 interactions were not detected in MC160-expressing cells, under conditions demonstrated to induce IKK complex formation, but interactions between the MC160 protein and the major IKK subunits were undetectable. Surprisingly, MC160 expression correlated with a decrease in IKK1, but not IKK2 levels, suggesting a mechanism for MC160 disruption of IKK1-IKK2 interactions. MCV has probably retained its MC160 gene to inhibit NF-κB activation by interfering with signaling via multiple biological mediators. In the context of an MCV infection in vivo, MC160 protein expression may dampen the cellular production of proinflammatory molecules and enhance persistent infections in host keratinocytes.

The NF-κB transcription factor is a powerful mediator of immune responses, controlling the transcription of cellular genes coding for proinflammatory and immune molecules (for a review, see reference 4). In unstimulated cells, NF-κB is cytoplasmically sequestered and inactive, due to an interaction with IκBα (20). For NF-κB to be released, an activated I kappa kinase (IKK) complex must first phosphorylate IκBα (11, 35), promoting IκBα ubiquitination and subsequent degradation (1, 9, 42). The freed NF-κB migrates to the nucleus where it binds to consensus DNA sequences to promote expression of specific host defense genes (4, 20).

Tumor necrosis factor alpha (TNF-α) is a pluripotent cytokine that, in binding to its cognate receptor (TNF receptor I [TNF-RI]), induces NF-κB activation or apoptosis (for a review, see reference 8). The former is a consequence of TNF-RI clustering that results in the recruitment of three signaling molecules, TNF-R-associated death domain (TRADD), receptor-interacting protein (RIP), and TNF-R-associated factor 2 (TRAF2), to form a receptor-based signalsome (8). Afterwards, an inactive IKK complex migrates to the signalsome and is phosphorylated by an IKK kinase, such as MEKK3, which is recruited to the signalsome via RIP (3). The activated IKK complex dissociates from the signalsome and, in turn, phosphorylates IκBα to initiate a cascade resulting in NF-κB activation.

To circumvent inflammation and possibly other host immune responses, some pathogens such as poxviruses express proteins designed to dampen these defense mechanisms by preventing either an activated NF-κB moiety from functioning in the nucleus or a step in the activation process (21). For instance, the leporipoxvirus myxoma virus utilizes the former strategy by expressing the M150R protein that presumably associates with NF-κB at a nuclear location (6). In contrast, the orthopoxvirus vaccinia virus expresses the N1L, A46R, and A52R proteins to inhibit Toll-like receptor-mediated NF-κB activation (5, 12). The molluscipoxvirus molluscum contagiosum virus (MCV) appears to be in the later category, in that its MC159 protein blocks death receptor-induced activation of NF-κB by acting on cytoplasmic events (7).

Since limited inflammation is associated with the persistent, benign skin neoplasms caused by MCV (17, 51), it is likely that virus-encoded proteins, in addition to the MC159 product, regulate the outcome of the host immune response. Although such responsibility has already been attributed to several proteins (36), it is probable that others remain undiscovered. In this regard, one likely candidate is the MC160 protein, which is considered a putative FLICE-inhibitory protein (FLIP) (44). Members of this family were originally defined by the presence of tandem motifs called death effector domains (DEDs) and by

* Corresponding author. Mailing address: Department of Microbiology, B103 Chemical and Life Sciences Building, 601 S. Goodwin Ave., Urbana, IL 61801. Phone: (217) 265-6450. Fax: (217) 244-6697. E-mail: jshisler@uiuc.edu.

their antiapoptotic properties (2, 16, 18, 27, 28, 47, 50). Even though the MC160 protein does not inhibit apoptosis like other FLIPs (46), it is a homolog of MCV MC159, an inhibitor of NF-κB activation (7). Thus, in this study we evaluated the effect of MC160 expression in cells stimulated to activate NF-κB either directly due to exposure to TNF-α or indirectly due to overexpression of one or more of the proteins involved in the classical NF-κB activation pathway. In this manner, we discovered that the MC160 protein was capable of inhibiting TNF-α-induced NF-κB activation, presumably by interfering with IKK1 and IKK2 complex formation and subsequent phosphorylation of IκBα.

MATERIALS AND METHODS

Cells, plasmids, and reagents. The human embryonic kidney cell line 293T (American Type Culture Collection) was maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (HyClone). This cell line was selected because it expresses large amounts of TNF-RI, is easily transfected, and has been utilized by multiple laboratories as a model system for studying the TNF-RI-mediated NF-κB activation pathway (7, 24, 26).

The MC160/pCI plasmid contains the intact MC160 open reading frame (ORF) placed under the transcriptional control of a cytomegalovirus promoter (46). The MC159/pCI plasmid was similarly constructed as previously described (46). The parental pCI vector was purchased from Promega. Plasmid pHA-TRAF2, which produces a hemagglutinin (HA) epitope-tagged TRAF2 protein, was provided by Jonathan Ashwell, National Institutes of Health (30), while plasmid pRIP was a gift from Preet Chaudhary (University of Texas Southwestern Medical Center) (7). Richard Tapping (University of Illinois) provided the pFLAG-MyD88 plasmid, which produces a FLAG epitope-tagged MyD88 protein. The pFLAG-IKK1, pHA-IKK2, and pNIK plasmids were kind gifts from Ulrich Siebenlist (National Institutes of Health) (37). Plasmid pIKK2DN contains an altered IKK2 gene, which expresses a dominant negative, kinase-deficient IKK2 protein (53), was a gift from Mark Hershenson (University of Chicago). For all experiments, plasmid DNA was introduced into 293T cells using the FuGene 6 transfection reagent (Roche Diagnostics).

Polyclonal antisera specifically recognizing either the MC160 or MC159 proteins were described previously (46). Anti-HA antiserum was obtained from Sigma-Aldrich. Anti-TRAF2, anti-IKK1, anti-IKK2 (recognizing the unphosphorylated forms), anti-IKK-γ, anti-IκBα, and NF-κB p65 and p50 subunit antibodies were purchased from Santa Cruz Biotechnology. Antisera recognizing the phosphorylated forms of both IKK1 and IKK2 simultaneously were purchased from Cell Signaling Technologies. Anti-RIP antibody was acquired from BD Transduction Laboratories. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) antibodies were obtained from Pierce Biotechnology. Human recombinant TNF-α was purchased from Roche.

Luciferase reporter assays. The luciferase reporter assay employed to quantify NF-κB activation was described previously (45). In assays involving TNF-α-mediated activation, subconfluent 293T cell monolayers were cotransfected with pNF-κB*luc* (450 ng), pRL-null (50 ng), and 500 ng of either pCI or MC160/pCI, unless otherwise stated. At 24 h posttransfection, monolayers were overlaid with fresh medium lacking or containing TNF-α (10 ng/ml, unless otherwise stated). At various times afterwards, cell monolayers were lysed in passive lysis buffer (Promega), and the lysates were evaluated for sea pansy and firefly luciferase activities by using the dual luciferase reporter assay (Promega).

In other luciferase assays, NF-κB was first activated due to overexpression of either the RIP, TRAF2, MyD88, NF-κB-inducing kinase (NIK), IKK1, or IKK2 signaling proteins. In this case, subconfluent 293T cells were cotransfected with pNF-κB*luc* (450 ng), pRL-null (50 ng), either pCI (500 ng) or MC160/pCI (500 ng), and either pHA-TRAF2 (500 to 2000 ng), pHA-RIP (50 to 500 ng), pFLAG-MyD88 (250 ng), pNIK (10 to 50 ng), pFLAG-IKK1 (500 ng), or pHA-IKK2 (500 ng). When required, larger amounts of pCI were included in the transfection mixture to equalize the quantity of DNA present. At 24 h posttransfection, cell monolayers were lysed and assayed as described above.

Luciferase activity was measured as relative light units by using the Luminoskan Microplate Luminometer (Labsystems). For all assays, experiments were performed in triplicate. For each experimental point, firefly luciferase activity was divided by sea pansy luciferase activity to correct for differences in transfection efficiencies. The resultant ratios were normalized to that of cells transfected with pCI alone, whose value was taken as 1.

Some of the lysates generated during the luciferase assays were also analyzed for the presence of either MC160, RIP, TRAF2, IKK1, or IKK2 by immunoblotting. The protein concentration of each lysate was determined by a bicinchoninic acid assay (Pierce). Ten micrograms of protein was separated electrophoretically in a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Membranes were first incubated with the indicated primary antibodies and then incubated with a secondary antibody conjugated to horseradish peroxidase. Blots were developed using Pierce Super Signal West chemiluminescence reagents per the manufacturer's recommendations.

Electromobility shift assays. Subconfluent monolayers of 293T cells were transfected with 1 μg of either pCI or MC160/pCI. At 24 h posttransfection, cells were incubated with medium lacking or containing TNF-α (10 ng/ml) for the times indicated. Afterwards, cells were collected at various times and lysed in CE buffer (40) to disrupt the cytoplasmic, but not nuclear, membranes. Nuclei were 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 (40). Two micrograms of protein from each nuclear extract was incubated with 0.35 pmol of γ-^{32}P-labeled, double-stranded oligonucleotides containing binding sites for the NF-κB transcription factor (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Promega) in gel shift binding buffer (Promega) for 20 min at room temperature. Each reaction product was analyzed electrophoretically in a 6% polyacrylamide gel (Invitrogen) under nondenaturing conditions. Dried gels were exposed to phosphorimager plates (Molecular Devices), and images were developed and analyzed using the ImageGauge and ImageReader programs (Fuji). For some reactions, an excess of nonradiolabeled oligonucleotides (1.75 pmol) (Promega) possessing or lacking the NF-κB recognition site or 2 μg of monoclonal antibodies recognizing either the NF-κB p65 or p50 subunits (Santa Cruz Biotechnology) was included.

Coimmunoprecipitation assays. Coimmunoprecipitations were performed as described previously (46). Briefly, subconfluent monolayers of 293T cells were cotransfected with equal amounts of the indicated plasmids. At 24 h posttransfection, some cells were incubated in medium containing TNF-α (10 ng/ml) for 15 min. Next, cells were harvested by being scraped and pelleted by centrifugation at 10,000 × *g* for 10 s. Cells were then lysed in DED-coimmunoprecipitation lysis buffer (46) for 30 min of incubation at 4°C and then centrifuged at 10,000 × *g* and 4C for 10 min. Supernatants were removed and incubated with either anti-HA Affinity Matrix beads (Roche) or with antibodies and protein A-Sepharose beads (Amersham) for 1 h at 4°C with constant rotation. Beads and bound proteins were pelleted by a brief high-speed centrifugation (10,000 × *g* for 30 s) and washed several times in lysis buffer. Pelleted proteins were resuspended in Laemmli buffer (Pierce), boiled for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were electrophoretically transferred to a PVDF membrane, and subsequently the blots were analyzed by immunoblotting with specific primary antibodies and a horseradish-conjugated secondary antibody. Blots were developed as described above. Preimmunoprecipitated lysates were analyzed for protein expression in a similar manner.

In vitro kinase assays. In vitro kinase assays were performed as previously described (15). Briefly, subconfluent monolayers of 293T cells were cotransfected with pFLAG-IKK1 (250 ng), pHA-IKK2 (250 ng), and either MC160/pCI (1,500 ng) or pCI (1,500 ng). At 24 h posttransfection, cells were harvested, collected by low-speed centrifugation, lysed in kinase assay lysis buffer (15) for 10 min at 4°C, and then centrifuged at 10,000 × *g* for 10 min. Supernatants were incubated with anti-IKK1 antibody and protein A-Sepharose beads for 1 h at 4°C with continuous rocking. Beads were collected by centrifugation, washed three times with lysis buffer and once with kinase assay kinase buffer (15) and then incubated in kinase buffer containing 5 μM ATP, 5 μCi [γ-^{32}P]ATP, and 1 μg of recombinant IκBα (Cell Sciences) for 20 min at 30°C. After the addition of 5× Laemmli buffer, each sample was boiled for 5 min. Each reaction was separated by 12% SDS-PAGE, and the proteins were electrophoretically transferred to a PVDF membrane. Blots were exposed to a phosphorimaging plate (Molecular Devices). After an overnight incubation, the plates were analyzed with ImageQuant and ImageReader software (Fuji). The immunoblots were probed first with anti-IκBα antibodies and then with anti-rabbit IgG antibodies conjugated to alkaline phosphatase. Antigen-antibody complexes were detected performed by using an alkaline phosphate substrate according to the manufacturer's directions (Promega).

RESULTS

MC160 protein inhibits the expression of an NF-κB-controlled reporter gene. MC160 protein inhibition of NF-κB activation was initially assessed by comparing the TNF-α-induced

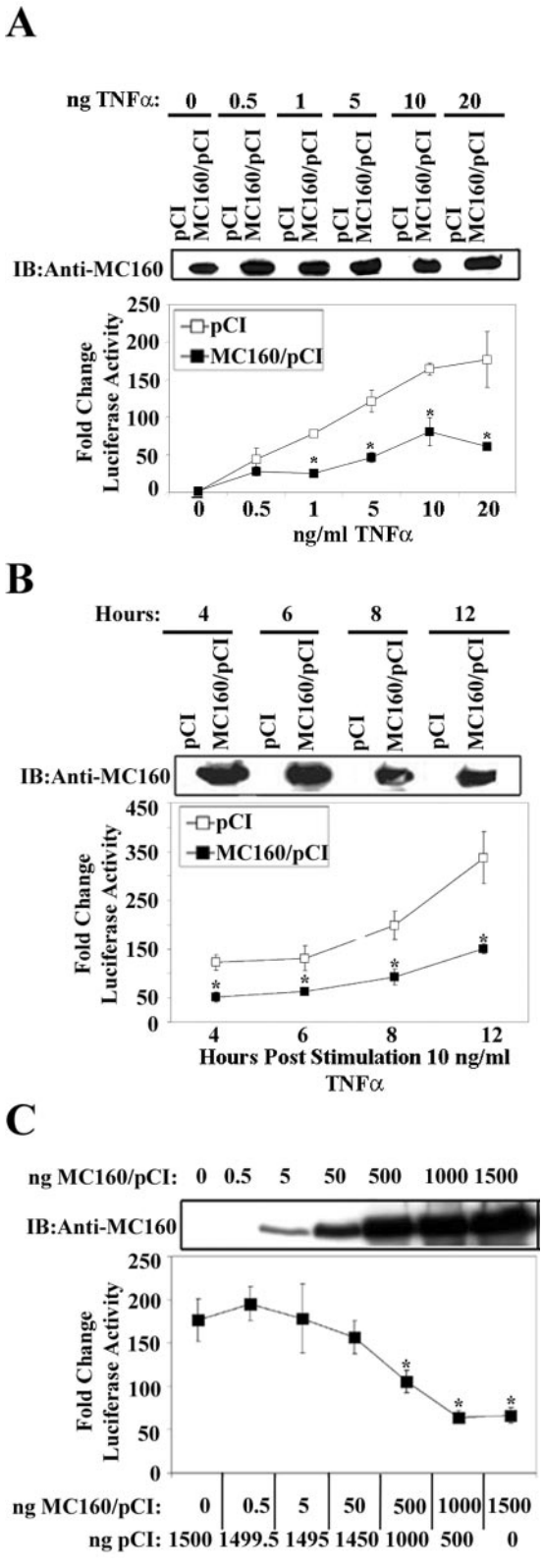

FIG. 1. The effect of MC160 expression on TNF-α-induced expression of an NF-κB transcriptionally regulated luciferase gene. (A and B) 293T cells were cotransfected with 450 ng of pNF-κB*luc*, 50 ng of pRL-null, and 500 ng of either pCI or MC160/pCI. (A) After 24 h, cells were incubated in medium lacking or containing TNF (0 to 20 ng/ml) for 4 h and then harvested. Their lysates were analyzed for luciferase activity. (B) Cells were incubated with medium lacking or containing 10 ng TNF/ml, and cells were harvested either 4, 6, 8, or 12 h later. Their lysates were then analyzed for luciferase activity. (C) Cells were transfected with reporter plasmids and the indicated amounts of either pCI or a combination of pCI and MC160/pCI. Following a 24-h incubation, cells were incubated in medium lacking or containing TNF (10 ng/ml) and were harvested 4 h later. Lysates were analyzed for luciferase activities. An asterisk (*) indicates statistically significant inhibition ($P < 0.05$). For all experiments, 10 μg of each cellular lysate was screened for MC160 expression by immunoblotting (IB) with anti-MC160 protein antisera, as illustrated by the immunoblot inset above each graph.

activities of an NF-κB-transcriptionally regulated firefly luciferase gene in MC160/pCI- and pCI-transfected cells (Fig. 1). In this experiment, TNF-α-mediated luciferase activity was reduced 1.5 to 3.1 fold in the presence of the MC160 protein (Fig. 1A). This reduction was statistically significant when determined after a 4-h incubation in the presence of either 1 ($P = 0.0001$), 5 ($P = 0.005$), 10 ($P = 0.008$), or 20 ($P = 0.032$) ng of TNF-α/ml. In comparison to exposure to 10 ng/ml TNF-α, luciferase activity was not greatly increased when pCI-transfected cells were incubated with either 20 (Fig. 1A) or 50 (data not shown) ng of TNF-α/ml. Thus, in subsequent experiments, cells were incubated with 10 ng/ml TNF-α. It should also be noted that inclusion of TNF-α in the medium did not impact MC160 expression, as similar amounts of this protein were detected in all of the MC160/pCI-transfected monolayers by immunoblotting (Fig. 1A). As vector-transfected cells were incubated for increasing times in the presence of 10 ng/ml TNF-α, luciferase activity levels increased (Fig. 1B). However, the MC160 protein significantly inhibited luciferase activity when the cells were examined at either 4 ($P = 0.005$), 6 ($P = 0.037$), 8 ($P = 0.011$), or 12 ($P = 0.023$) h (Fig. 1B). Extended incubation of cells with TNF-α did not drastically decrease MC160 expression, since similar amounts of this protein were present at all times tested (Fig. 1B).

MC160-mediated inhibition of TNF-α-induced luciferase activity also correlated with the intracellular amount of this viral protein (Fig. 1C), further confirming the inhibitory function of MC160. Statistically significant reductions in luciferase activity were observed only when cells were transfected with 500 to 1,500 ng MC160/pCI ($P < 0.02$), conditions where MC160 expression was the greatest (Fig. 1C). In all experiments, MC160 expression did not induce luciferase activity in untreated cells, implying that MC160 did not activate NF-κB.

NF-κB transcriptional activation is reduced in MC160-expressing cells. NF-κB activation by TNF-α was also examined by using electromobility shift assays to measure binding of nucleus-located NF-κB to a radiolabeled oligonucleotide containing an NF-κB consensus sequence. As expected, nuclear extracts from unstimulated cell populations failed to alter oligonucleotide mobility, indicating that NF-κB was inactive (Fig. 2). In contrast, a unique mobility-shifted band (indicative of activated NF-κB) of similar intensities was observed when nuclear extracts from pCI- and MC160/pCI-transfected cells stimulated with TNF-α for 15, 30, or 45 min (Fig. 2) were used. However, the amount of the NF-κB-containing band was greatly reduced when extracts from MC160-expressing cells exposed to TNF-α for 60 to 120 min were used instead of ones

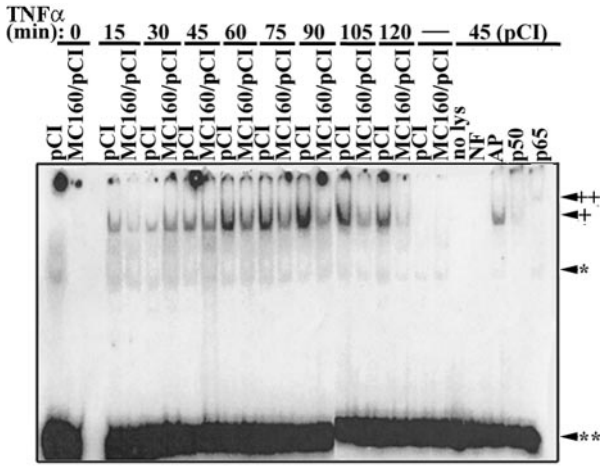

FIG. 2. The effect of MC160 expression on TNF-α-induced NF-κB nuclear translocation. 293T cells were transfected with 1 μg of either pCI or MC160/pCI. After 24 h, cells were incubated in medium lacking (—) or containing TNF (10 ng/ml) for the times indicated. Cells were harvested and lysed, and nuclei were separated from cell lysates. Two micrograms of nuclear protein from each sample was incubated with radiolabeled oligonucleotides containing consensus NF-κB binding sites. Nuclear extracts from pCI-transfected cells treated with TNF for 45 min were also incubated with either nonradiolabeled oligonucleotides (NF), oligonucleotides devoid of an NF-κB consensus sequence (AP), or anti-p50 (p50) or anti-p65 (p65) antisera. The lane labeled "no lys" indicates that no nucleus-extracted proteins were present in the reaction mixture. Reactions were resolved by 6% PAGE under nondenaturing conditions. A mobility-retarded complex containing NF-κB is indicated by a plus symbol (+), while supershifted NF-κB-containing complexes are indicated by a double plus symbol (++). A nonspecific band and the band composed of unbound oligonucleotides are shown by a single (*) or double (**) asterisk, respectively.

from vector-transfected cells treated under the same conditions. Likewise, the addition of antibodies specific for either the NF-κB p50 or p65 subunit to extracts from TNF-α-treated, pCI-transfected cells decreased the signal intensity of the NF-κB-containing band (Fig. 2, lanes p50 and p65) in comparison to samples lacking the antibodies. In general, the disappearance of the mobility-shifted band is evidence (albeit indirect) that anti-NF-κB antibodies specifically interacted with NF-κB. In a separate set of reactions, the addition of nonradiolabeled oligonucleotides containing NF-κB binding sites eliminated the NF-κB-containing band (Fig. 2, lane NF), while a similar quantity of nonspecific oligonucleotides had no effect (Fig. 2, lane AP). This specific competition demonstrated that NF-κB was indeed binding to NF-κB consensus sequences present in the radiolabeled oligonucleotides.

The MC160 protein inhibits either RIP-, TRAF2-, NIK-, or MyD88-induced NF-κB activation. After observing that NF-κB activation was inhibited in the presence of the MC160 protein, the next goal was to determine the step(s) of the TNF-RI-mediated NF-κB activation pathway that this viral protein blocked. In this regard, we studied the effect of MC160 expression on luciferase activity mediated by overexpression of either the RIP or TRAF2 accessory molecules. As shown in Fig. 3A, luciferase activity levels increased in cells transfected with 100 to 500 ng pRIP (26). In contrast, luciferase activity was two- to threefold lower in cells coexpressing MC160 (Fig. 3A), and the decrease was statistically significant in cells transfected with MC160/pCI and either 100, 250, or 500 ng of pRIP ($P < 0.05$). Increased RIP protein expression neither degraded MC160 nor prevented MC160 expression, since the levels of this viral protein were similar in the appropriate samples (data not shown). TRAF2 overexpression also increased intracellular luciferase activity (Fig. 3B), although not to the same extent as RIP overexpression, as previously reported (25). Similar to the results above, MC160 expression reduced TRAF2-mediated luciferase activity, in this case four- to eightfold (Fig. 3B), with statistically significant inhibition occurring when cells were transfected with 1,000 ($P = 0.0001$) or 2,000 ($P = 0.006$) ng of pHA-TRAF2. Immunoblotting of the respective lysates indicated the presence of the MC160 protein and TRAF2 in transfected cell lysates, demonstrating that the production of either protein was not impacted by transfection with large amounts of expression vectors (data not shown).

To ask if the MC160 protein could affect other NF-κB activation pathways, an assessment of whether this viral protein inhibited either NIK- or MyD88-induced NF-κB activation was made. When cells were transfected with a plasmid capable of expressing NIK, luciferase activity was enhanced compared to that in the nontransfected cells (Fig. 3C). This increase was presumably due to the ability of NIK to phosphorylate IKK1 (31), thus indirectly activating NF-κB. However, NIK-induced luciferase activity was reduced substantially when the MC160 and NIK proteins were coexpressed (Fig. 3C), indicating that MC160 partially prevented NIK-mediated NF-κB activation. The reduction in luciferase activity when cells were cotransfected with MC160/pCI and either 10 or 50 ng pNIK was statistically significant ($P = 0.008$ and $P = 0.007$, respectively). Likewise, overexpression of MyD88, a mediator of Toll-like receptor-induced NF-κB activation (34), resulted in a strong induction of luciferase activity (Fig. 3D). However, coexpression of MC160 decreased this artificially induced enhancement by 4.4 fold (Fig. 3D). In this case, the amount of MC160/pCI necessary to cause inhibition of MyD88-induced NF-κB activation did not appear to be abnormally great, since transfection of cells with a similar quantity of pIKK2DN, which should presumably result in the production of a comparable protein, in this case a dominant defective IKK2, squelched luciferase activity to a similar degree (Fig. 3D).

The MC160 protein inhibits IKK kinase activity. Since the MC160 protein reduced signaling via molecules involved in TNF-α (namely, RIP and TRAF2) and NIK- and MyD88-mediated activation of NF-κB, we predicted that this viral protein was inhibiting phosphorylation of cellular proteins by IKK, an event common to each of these pathways (20). In support of this hypothesis, we observed that MC160 expression was associated with statistically significant 35-fold reduction and 8-fold reduction in IKK1- and IKK2-mediated luciferase activity, respectively (Fig. 4A).

Next, we detected IKK activity in MC160-expressing cells via an in vitro kinase assay, in which a reduction in IKK complex formation by the MC160 protein would be measured by the specific associated decrease in phosphorylation of IκBα (Fig. 4B) (15). When the source was lysates from cells overexpressing the IKK1 and IKK2 subunits in the absence of the MC160 protein, a phosphorylated form of recombinant IκBα was easily detected. However, when either MC160 expression also occurred in the cells or the cells were only transfected with a

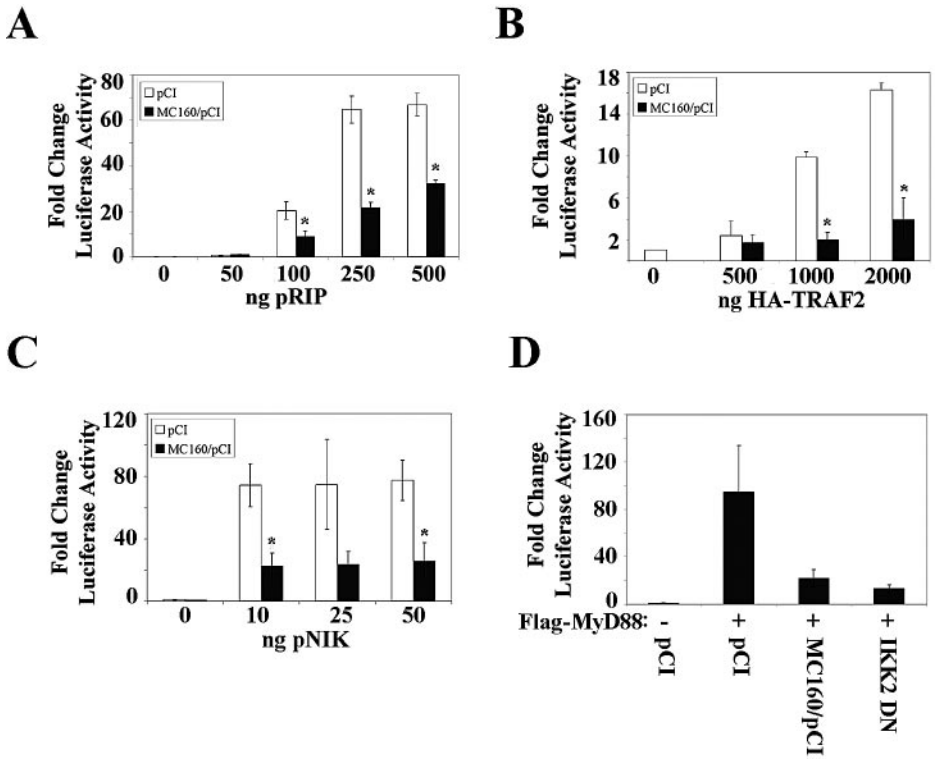

FIG. 3. The effect of MC160 expression on either RIP-, TRAF2-, NIK-, or MyD88-induced NF-κB activation. 293T cells were cotransfected with pNF-κB*luc* (450 ng), pRL-null (50 ng), either pCI (500 ng) or MC160/pCI (500 ng), and either pHA-RIP (50 to 500 ng) (A), pHA-TRAF2 (500 to 2,000 ng) (B), pNIK (10 to 50 ng) (C), or pFlagMyD88 (250 ng) (D). A separate set of cells were transfected with 500-ng MC160/pCI and 500-ng pIKK2DN (Fig. 3D only). At 24 h posttransfection, cells were harvested and lysates were analyzed for luciferase activity. An asterisk (*) indicates statistically significant inhibition ($P < 0.05$).

neutral plasmid, a greatly reduced amount of radioactively labeled IκBα was observed. Since an external provision of IκBα was made to the reactions, the diminution could only be attributed to an inadequate quantity of active IKK complexes due either to intervention by the MC160 protein or to a lack of IKK1 and IKK2 available for complex formation, respectively.

IKK1-IKK2 subunit interactions are undetectable in the presence of the MC160 protein, but MC160 interaction with either protein was undetectable by coimmunoprecipitation. While the IKK heteromeric complex consisted of multiple proteins, interactions between the IKK1, IKK2, and IKK-γ proteins were sufficient for IKK activity. If the MC160 protein were to bind to one of these three subunits, then the IKK complex would not be activated. Accordingly, we attempted to detect the association of epitope-tagged IKK1 (FLAG-tagged) and IKK2 (HA-tagged) molecules in the presence or absence of MC160 by using coimmunoprecipitation assays (Fig. 5). It appeared that MC160 did not directly interact with the IKK2 protein, since MC160 did not coimmunoprecipitate with the HA-tagged IKK2 protein (Fig. 5A). Since similar quantities of HA-tagged IKK2 protein were detected in all immunoprecipitated samples (Fig. 5A), MC160 or IKK1 expression did not appear to impact the plasmid-directed generation of IKK2. Conversely, as expected from the preserved inhibitory ability of the MC160 protein, the increased presence of IKK1 and IKK2 did not prevent production of this viral protein (Fig. 5A). In a similar manner, MC160-IKK1 interactions were undetectable by coimmunoprecipitation methods (Fig. 5B), although an increased amount of IKK1 was noticed in the immunoprecipitates from samples not expressing the MC160 protein. Interestingly, we observed that IKK1-IKK2 interactions were undetectable when MC160 proteins were expressed (Fig. 5A). This is in contrast to vector-transfected cells, where FLAG-IKK1 proteins were readily found in anti-HA immunoprecipitated samples, demonstrating IKK1-IKK2 interactions (Fig. 5A). The IKK-γ subunit acts as a regulatory protein, stabilizing interactions between IKK1 and IKK2. Since MC160–IKK-γ interactions were not detected via coimmunoprecipitation (Fig. 5C), it seemed unlikely that MC160 directly interacted with this regulatory subunit to prevent IKK1-IKK2 interactions.

To more closely mimic the physiological signals that an MCV-infected host cell may encounter, we repeated the coimmunoprecipitation experiments, assaying for IKK1-IKK2 interactions in cells incubated with medium containing TNF-α (Fig. 6). Similar to results with cells that were incubated in regular medium, decreased levels of IKK1 proteins were present in IKK2-immunoprecipitated samples when MC160 proteins were present (Fig. 6). None of the viral or cellular proteins we were studying nonspecifically bound to protein A-Sepharose beads, since neither MC160, IKK1, nor IKK2 was detected in samples that had been immunoprecipitated with a nonspecific antibody. Also, MC160 protein levels were equal in lysates from cells that were incubated in medium absent for or con-

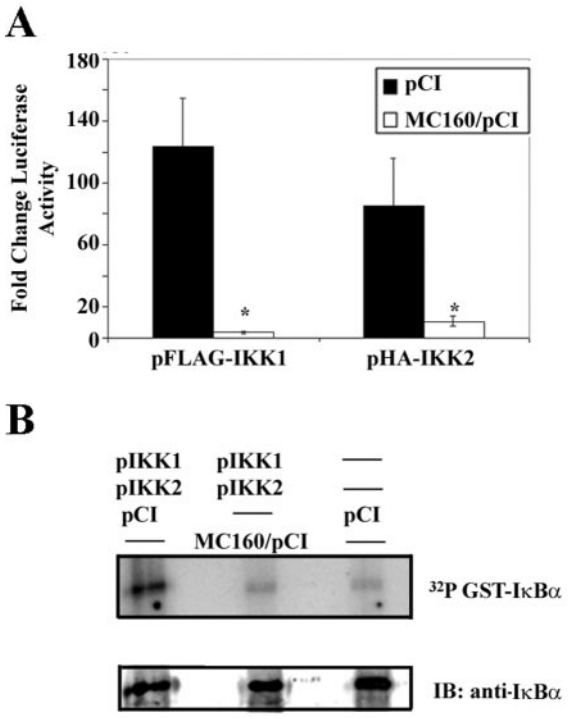

FIG. 4. The effect of MC160 expression on IKK activation. (A) 293T cells were cotransfected with 450 ng pNF-κB*luc*, 50 ng pRL-null, 500 ng of either pCI or MC160/pCI, and 500 ng of either pFLAG-IKK1 or pHA-IKK2. After 24 h, cells were harvested and lysates were assayed for luciferase activities. An asterisk (*) indicates statistically significant inhibition ($P < 0.05$). (B) 293T cells were cotransfected with 250 ng pFLAG-IKK1, 250 ng pHA-IKK2, and 1.5 μg of either pCI or MC160/pCI. After 24 h, cells were harvested, lysed, and incubated with anti-IKK1 antibodies and protein A-Sepharose beads. Immunoprecipitated samples were incubated with recombinant IκBα and [γ-^{32}P]ATP in a kinase reaction buffer. Proteins were separated by 12% SDS-PAGE and transferred to PVDF membranes. The membranes were first exposed to phosphorimaging plates (top) and then subsequently probed for IκBα by immunoblotting (bottom) with an alkaline phosphatase-conjugated secondary antibody.

taining TNF-α, indicating that the presence of TNF-α did not affect MC160 expression.

MC160 expression coincides with a decrease in IKK1 expression levels. While IKK1 and IKK2 subunits are normally inactive in resting cells, ectopic overexpression of these proteins results in their phosphorylation and subsequent activation, an event detectable by probing immunoblotted lysates from pCI-transfected cells with antisera that simultaneously recognized phosphorylated IKK1 and IKK2 (Fig. 7). In contrast, there was a decrease in phospho-IKK levels in MC160-expressing cells. This decrease was not due to overexpression of protein, since expression of the MCV MC159 product did not significantly alter IKK phosphorylation levels when IKK1 and IKK2 were coexpressed. Further analysis of these lysates revealed that IKK1 levels, but not IKK2 or actin levels, were reduced in MC160-expressing cells compared to either MC159/pCI- or pCI-transfected cells. Therefore, the possibilities that the transfection process or MC160 expression globally decreased protein expression were ruled out. Similar results were observed when only IKK1 was overexpressed: phospho-IKK1 and total IKK1 levels were decreased in MC160-expressing cells compared to cells transfected with either pCI or MC159/pCI. Phosphorylation of IKK2 was also decreased in cells cotransfected with MC160/pCI and pHA-IKK2, indicating that MC160 inhibited activation of both kinase subunits. Yet, total IKK2 levels were similar in pCI- versus MC160/pCI-transfected cells, indicating that MC160 did not affect IKK2 protein levels. In MC159/pCI-transfected cells, IKK2 was present at levels similar to that seen with pCI-transfected cells, but phospho-IKK2 levels were decreased. Similar results were observed if cells were incubated in medium containing TNF-α, a cytokine known to induce IKK1 and IKK2 phosphorylation (data not shown).

DISCUSSION

TNF-α is produced by keratinocytes, the natural host cells for MCV (14), in response to stresses such as UV radiation and endotoxins (29, 32). This powerful proinflammatory cytokine up-regulates the expression of immune molecules via NF-κB activation (52). Here, we present data that the MC160 protein prevents TNF-α-induced NF-κB activation in 293T cells as a potential mechanism for MCV to inhibit the production of antiviral immune response molecules. Kidney epithelium-derived 293T cells, while not the natural host cell type for MCV, are a common and well-defined model system for studying TNF-RI-mediated signal transduction, making their use important in characterizing this original function for the MC160 protein. One prediction, which we are currently testing in our laboratory, is that the MC160 protein will function similarly in human keratinocytes, cells that are normally infected by MCV.

The TNF-RI mediates the classical NF-κB activation pathway by virtue of TNF-R-associated death domain, RIP, and TRAF2 forming a signalsome, that in turn allows IKK to dock to it and become activated (8, 10). We originally hypothesized that MC160 bound to RIP or TRAF2 to prevent IKK from associating with the signalsome and subsequently being phosphorylated. However, observations demonstrating no detectable interactions between the MC160 protein and either RIP or TRAF2 (data not shown) ruled out this possibility. Since expression of the MC160 protein also inhibited NIK- and MyD88-induced NF-κB activation, a more likely scenario was that MC160 prevented an event common to all of the above pathways, namely, IKK activation. This prediction was borne out to be true, and data demonstrated that cells expressing the MC160 protein did not possess active, phosphorylated IKK or IKK1-IKK2 complexes necessary for IKK activity.

We did not detect a MC160 protein interaction with IKK1 or IKK2 by coimmunoprecipitation, diminishing the likelihood that MC160 prevented formation of the IKK complex by binding to either kinase subunit. IKK1-IKK2 interactions, which are necessary for IKK autoactivation, occur via association of both proteins to IKK-γ (20). Since IKK1-IKK2 interactions in MC160-expressing cells were undetectable, another possible mechanism for MC160 function is that the MC160 protein prevents the formation of an IKK complex by binding to the IKK-γ subunit. In this regard, one other viral FLIP that also contains two tandem DEDs, the human herpesvirus 8 ORF-K13 protein, has previously been shown to interact with IKK-γ, albeit to activate NF-κB (33). However, MC160 interactions

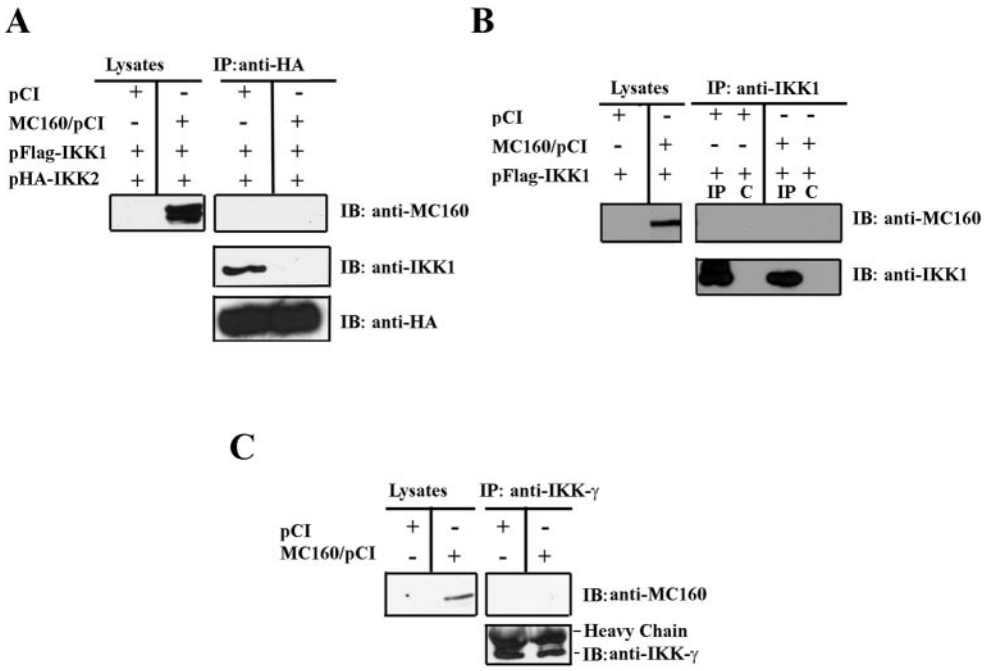

FIG. 5. The effect of MC160 expression on IKK subunit interactions. (A) 293T cells were cotransfected with 500 μg pFLAG-IKK1, 500 μg pHA-IKK2, and 1 μg of either pCI or MC160/pCI. At 24 h posttransfection, cells were lysed and incubated with anti-HA antibody conjugated to protein A-Sepharose beads. Immunoprecipitated samples (IP) were separated by 12% SDS-PAGE, and proteins were transferred to a PVDF membrane. Immunoblots (IB) were probed with the indicated antisera. (B) 293 T cells were transfected with 1 μg of either pFlag-IKK1 or MC160/pCI. At 24 h posttransfection, cells were lysed and incubated with either anti-IKK1 antibody (IP) or with antisera nonspecific for IKK1 (C) conjugated to protein A-Sepharose beads. Immunoprecipitated samples (IP) and control samples (C) were separated by 12% SDS-PAGE, and proteins were transferred to a PVDF membrane. Immunoblots (IB) were probed with the indicated antisera. (C) 293 T cells were transfected with 1 μg of either pCI or MC160/pCI. At 24 h posttransfection, cells were lysed and incubated with anti-IKK-γ conjugated to protein A-Sepharose beads. Immunoprecipitated samples were separated by 12% SDS-PAGE, and proteins were transferred to a PVDF membrane. Immunoblots (IB) were probed with the indicated antisera. For all experiments, preimmunoprecipitated lysates were also analyzed by immunoblotting for MC160 protein expression.

with endogenous IKK-γ were undetectable in our assays, ruling out this putative MC160 mechanism.

We found that phosphorylated IKK1 and IKK2 were dramatically decreased in MC160-expressing cells. Further analysis revealed that total IKK1 levels, but not IKK2 levels, were decreased. Several reports show that the phosphorylation of the IKK2 subunit is essential for TNF-α-induced NF-κB activation, while deletion of the IKK1 subunit does not alter this signal transduction pathway. Thus, our current hypothesis is that MC160-mediated prevention of IKK2 phosphorylation is the more important mechanism for MC160 function. Other cellular proteins, such as the TANK binding kinase 1 (TBK1)

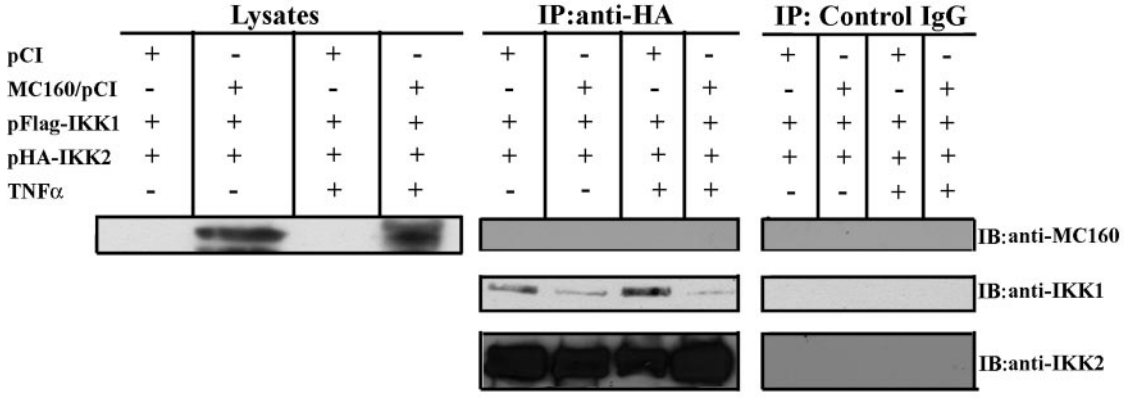

FIG. 6. The effect of MC160 expression on IKK1-IKK2 interactions in the presence or absence of TNF-α. Subconfluent 293T cells were cotransfected with 500 μg pFLAG-IKK1, 500 μg pHA-IKK2, and 1 μg of either pCI or MC160/pCI. At 24 h posttransfection, fresh medium was added either lacking (−) or containing (+) 10 ng/ml TNF-α. Following a 15-min incubation, cells were lysed, and lysates were incubated with protein A-Sepharose beads and either anti-HA or mouse IgG antibody. Immunoprecipitated samples (IP) were separated by 12% SDS-PAGE, and proteins were transferred to a PVDF membrane. Immunoblots (IB) were probed with the indicated antisera. As a control, preimmunoprecipitated lysates were also analyzed by immunoblotting for MC160 protein expression.

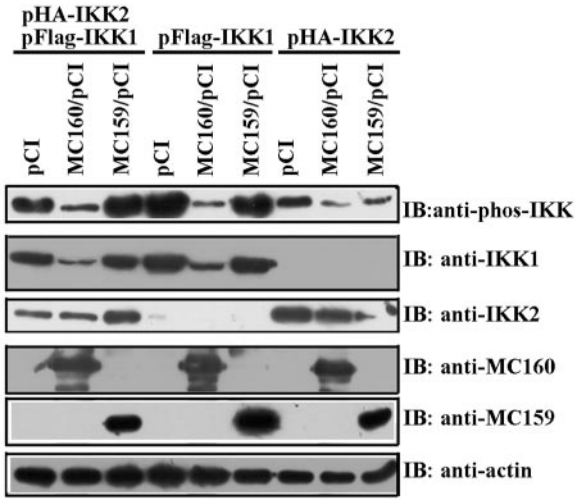

FIG. 7. Levels of phosphorylated and unphosphorylated IKK1 and IKK2 proteins in the presence of MC160. Subconfluent 293T cells were transfected with either 250 ng of pFlag-IKK1 and 250 ng of pHA-IKK2, or 500 ng of pFlag-IKK1, or 500 ng of pHA-IKK2. In addition, cells were simultaneously cotransfected with 500 ng of either pCI, MC160/pCI, or MC159/pCI. At 24 h posttransfection, cells were lysed and 30 μg of cytoplasmic extracts was separated by 12% SDS-PAGE. Proteins were electrophoretically transferred to PVDF membrane, and immunoblots (IB) were probed with the indicated antisera.

(41) and ELKS (13) have been shown to interact with and activate IKK subunits as a necessary event during TNF-RI-mediated NF-κB activation. Whether these cellular proteins bind to IKK subunits to enhance IKK stability is unknown. Thus, one possibility is that the MC160 protein may exert its effects indirectly by binding to one of these cellular proteins, thereby compromising the stability of the IKK1 protein in activated cells or preventing phosphorylation of either or both subunits. At least one other poxvirus protein, the vaccinia virus N1L protein, interacts with TBK1 and prevents TNF-α-induced NF-κB activation (12). Whether IKK1 stability is compromised in these cells remains to be tested. Despite no amino acid similarity between the MC160 and N1L protein, whether the former does associate with TBK1 or other IKK binding proteins to prevent IKK stability and/or activity will be the focus of future studies.

Recently, an alternative NF-κB activation pathway, in which IKK1 subunits form a homodimer that phosphorylates IκB proteins to enable NF-κB activation, was characterized (4). In this nonclassical cascade, the IKK1 homodimer is phosphorylated by a cellular kinase, such as NIK (39). Since we demonstrate here that the MC160 protein inhibited both NIK- and IKK1-induced luciferase activities and decreased IKK1 levels, it is possible that MC160 negatively impacts the alternative pathways.

It should be noted that, like the MC160 protein, the MCV MC159 protein contains two tandem DEDs (44). Interestingly, this virus protein also inhibits TNF-α- and MyD88-induced NF-κB activation (38). However, despite the DED I and DED II regions being 45% and 35% identical at the amino acid level, respectively, between the two virus proteins (44), it appears that they prevent TNF-α-induced NF-κB activation via different mechanisms. For instance, as shown here, MC160 expression decreased IKK1 levels whereas MC159 did not. Also, the MC160 protein inhibited RIP-induced NF-κB activation (Fig. 3), while the MC159 protein does not (38), suggesting that only the former affects MEKK-3-induced NF-κB activation (an event mediated by RIP). Moreover, the latter binds to RIP and TRAF2 (7), while such interactions involving the MC160 protein were not detected, indicating that the two proteins target different cellular proteins in vivo. In this regard, the MC160 protein possesses a unique C-terminal region; we are currently investigating a potential role for this amino acid structure regarding inhibition of NF-κB activation and/or host protein binding.

Recent reports have shown that TNF-α induces a biphasic NF-κB activation response, with the transient phase dependent upon IκBα degradation and the persistent phase reliant on IκBβ degradation (23, 43, 49). Not surprisingly, each type of NF-κB activation results in the expression of different chemokines and proinflammatory proteins in cultured cells at different times after exposure to TNF-α (22, 23, 43, 49). Since the MC159 protein inhibits IκBβ, but not IκBα degradation, it is likely that this viral protein blocks the persistent phase of activation (38). Thus, it is tempting to speculate that MCV expresses the MC160 protein to inhibit the transient NF-κB activation phase and that both viral proteins are required to completely prevent TNF-α-mediated signaling that host cells may encounter during infection. While we have observed that coexpression of the MC160 and MC159 proteins reduces TNF-α-induced luciferase activity to a greater degree than expression of either protein alone (data not shown), a detailed analysis of NF-κB-directed transcriptional activity in cells coexpressing both viral proteins is needed to further test this idea.

It appears that blocking NF-κB activation of virus-infected cells is important for poxvirus pathogenesis. In this regard, elimination of the M150R ORF from the myxoma virus genome created a mutant that causes limited disease in rabbits, unlike its extremely virulent progenitor (6). Similarly, mice infected with vaccinia virus whose DNA lacked either the A52R or A46R ORFs, which encode proteins capable of inhibiting Toll-like receptor-induced NF-κB activation, exhibited a decreased rate of virus-induced illness compared to animals receiving the genetically unaltered parent (19, 48). While no MCV animal model is currently available, we predict that in vivo infection with MC160 and/or MC159 ORF deletion mutants would result in increased TNF-RI-mediated signal transduction through TNF-RI, culminating in increased inflammation and presumably more rapid resolution of MCV-infected lesions.

ACKNOWLEDGMENTS

This research was supported by a grant to J.L.S. (AI055530) from the National Institutes of Health and by funds provided by the School of Molecular and Cellular Biology at the University of Illinois, Urbana-Champaign.

REFERENCES

1. **Baldi, L., K. Brown, G. Franzoso, and U. Siebenlist.** 1996. Critical role for lysines 21 and 22 in signal-induced, ubiquitin-mediated proteolysis of IκB-α. J. Biol. Chem. **271:**376–379.
2. **Barnhart, B. C., J. C. Lee, E. C. Alappat, and M. E. Peter.** 2003. The death effector domain protein family. Oncogene **22:**8634–8644.
3. **Blonska, M., Y. You, R. Geleziunas, and X. Lin.** 2004. Restoration of NF-κB

activation by tumor necrosis factor alpha receptor complex-targeted MEKK3 in receptor-interacting protein-deficient cells. Mol. Cell. Biol. **24:**10757–10765.
4. **Bonizzi, G., and M. Karin.** 2004. The two NF-κB activation pathways and their role in innate and adaptive immunity. Trends Immunol. **25:**280–288.
5. **Bowie, A., E. Kiss-Toth, J. A. Symons, G. L. Smith, S. K. Dower, and L. A. O'Neill.** 2000. A46R and A52R from vaccinia virus are antagonists of host IL-1 and toll-like receptor signaling. Proc. Natl. Acad. Sci. USA **97:**10162–10167.
6. **Camus-Bouclainville, C., L. Fiette, S. Bouchiha, B. Pignolet, D. Counor, C. Filipe, J. Gelfi, and F. Messud-Petit.** 2004. A virulence factor of myxoma virus colocalizes with NF-κB in the nucleus and interferes with inflammation. J. Virol. **78:**2510–2516.
7. **Chaudhary, P. M., A. Jasmin, M. T. Eby, and L. Hood.** 1999. Modulation of the NF-kappa B pathway by virally encoded death effector domains-containing proteins. Oncogene **18:**5738–5746.
8. **Chen, G., and D. V. Goeddel.** 2002. TNF-R1 signaling: a beautiful pathway. Science **296:**1634–1635.
9. **Chen, Z., J. Hagler, V. J. Palombella, F. Melandri, D. Scherer, D. Ballard, and T. Maniatis.** 1995. Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. Genes Dev. **9:**1586–1597.
10. **Devin, A., A. Cook, Y. Lin, Y. Rodriguez, M. Kelliher, and Z. Liu.** 2000. The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. Immunity **12:**419–429.
11. **DiDonato, J. A., M. Hayakawa, D. M. Rothwarf, E. Zandi, and M. Karin.** 1997. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. Nature **388:**548–554.
12. **DiPerna, G., J. Stack, A. G. Bowie, A. Boyd, G. Kotwal, Z. Zhang, S. Arvikar, E. Latz, K. A. Fitzgerald, and W. L. Marshall.** 2004. Poxvirus protein N1L targets the I-κB kinase complex, inhibits signaling to NF-κB by the tumor necrosis factor superfamily of receptors, and inhibits NF-κB and IRF3 signaling by toll-like receptors. J. Biol. Chem. **279:**36570–36578.
13. **Ducut Sigala, J. L., V. Bottero, D. B. Young, A. Shevchenko, and F. Mercurio.** 2004. Activation of transcription factor NF-κB requries ELKS, an IκB kinase regulatory subunit. Science **304:**1963–1967.
14. **Esposito, J., and F. Fenner.** 2001. Poxviruses, p. 2885–2921. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, Pa.
15. **Fiorentino, L., C. Stehlik, V. Oliveira, M. E. Ariza, A. Godzik, and J. C. Reed.** 2002. A novel PAAD-containing protein that modulates NF-kappa B induction by cytokines tumor necrosis factor-alpha and interleukin-1β. J. Biol. Chem. **277:**35333–35340.
16. **Goltsev, Y. V., A. V. Kovalenko, E. Arnold, E. E. Varfolomeev, V. M. Brodianskii, and D. Wallach.** 1997. CASH, a novel caspase homologue with death effector domains. J. Biol. Chem. **272:**19641–19644.
17. **Gottlieb, S. L., and P. L. Myskowski.** 1994. Molluscum contagiosum. Int. J. Dermatol. **33:**453–461.
18. **Han, D. K. M., P. T. Chaudhary, M. E. Wright, C. Friedman, B. J. Trask, R. T. Riedel, D. G. Baskin, S. M. Schwartz, and L. Hood.** 1997. MRIT, a novel death-effector domain-containing protein, interacts with caspases and Bcl-Xl and initiates cell death. Proc. Natl. Acad. Sci. USA **94:**11333–11338.
19. **Harte, M. T., I. R. Haga, G. Maloney, P. Gray, P. C. Reading, N. W. Bartlett, G. L. Smith, A. Bowie, and L. A. O'Neill.** 2003. The poxvirus protein A52R targets Toll-like receptor signaling complexes to suppress host defense. J. Exp. Med. **197:**343–351.
20. **Hayden, M. S., and S. Ghosh.** 2004. Signaling to NF-κB. Genes Dev. **18:**2195–2224.
21. **Hiscott, J., H. Kwon, and P. Genin.** 2001. Hostile takeovers: viral appropriation of the NF-κB pathway. J. Clin. Investig. **107:**143–151.
22. **Hoffmann, A., T. H. Leung, and D. Baltimore.** 2003. Genetic analysis of NF-κB/Rel transcription factors defines functional specificities. EMBO J. **22:**5530–5539.
23. **Hoffmann, A., A. Levchenko, M. L. Scott, and D. Baltimore.** 2002. The IκB-NF-κB signaling module: temporal control and selective gene activation. Science **298:**1241–1245.
24. **Hsu, H., J. Huang, H. B. Shu, V. Baichwal, and D. V. Goeddel.** 1996. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity **4:**387–396.
25. **Hsu, H., H. B. Shu, M. G. Pan, and D. V. Goeddel.** 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell **84:**299–308.
26. **Hsu, H., J. Xiong, and D. V. Goeddel.** 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell **81:**495–504.
27. **Inohara, N., T. Koseki, Y. Hu, S. Chen, and G. Nunez.** 1997. CLARP, a death effector domain-containing protein interacts with caspase-8 and regulates apoptosis. Proc. Natl. Acad. Sci. USA **94:**10717–10722.
28. **Irmler, M., M. Thome, M. Hahne, P. Schneider, K. Hofmann, V. Steiner, J. L. Bodmer, M. Schroter, K. Burns, C. Mattmann, D. Rimoldi, L. E. French, and J. Tschopp.** 1997. Inhibition of death receptor signals by cellular FLIP. Nature **388:**190–195.
29. **Kock, A., T. Schwarz, R. Kirnbauer, A. Urbanski, P. Perry, J. C. Ansel, and T. A. Luger.** 1990. Human keratinocytes are a source for tumor necrosis factor alpha: evidence for synthesis and release upon stimulation with endotoxin or ultraviolet light. J. Exp. Med. **172:**1609–1614.
30. **Li, X., Y. Yang, and J. D. Ashwell.** 2002. TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. Nature **416:**345–347.
31. **Ling, L., Z. Cao, and D. V. Goeddel.** 1998. NF-κB-inducing kinase activates IKK-alpha by phosphorylation of Ser-176. Proc. Natl. Acad. Sci. USA **95:**3792–3797.
32. **Lisby, S., K. M. Mullser, C. V. Jongeneel, J. H. Saurat, and C. Hauser.** 1995. Nickel and skin irritants up-regulate tumor necrosis factor-alpha mRNA in keratinocytes by different but potentially synergistic mechansism. Int. Immunol. **7:**343–352.
33. **Liu, L., M. T. Eby, N. Rathore, S. K. Sinha, A. Kumar, and P. M. Chaudhary.** 2002. The human herpes virus 8-encoded viral FLICE inhibitory protein physically associates with and persistently activates the I-κ B kinase complex. J. Biol. Chem. **277:**13745–13751.
34. **Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh, and C. A. Janeway, Jr.** 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol. Cell **2:**253–258.
35. **Mercurio, F., H. Zhu, B. W. Murray, A. Shevchenko, B. L. Bennett, J. Li, D. B. Young, M. Barbosa, M. Mann, A. Manning, and A. Rao.** 1997. IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. Science **278:**860–866.
36. **Moss, B., J. L. Shisler, Y. Xiang, and T. Senkevich.** 2000. Immune-defense molecules of molluscum contagiosum virus: a human poxvirus. Trends Microbiol. **8:**473–477.
37. **Muller, J. R., and U. Siebenlist.** 2003. Lymphotoxin beta receptor induces sequential activation of distinct NF-kappa B factors via separate signaling pathways. J. Biol. Chem. **278:**12006–12012.
38. **Murao, L., and J. Shisler.** 2005. The MCV MC159 protein inhibits late, but not early, events of TNF-alpha induced NF-κB activation. Virology **340:**255–264.
39. **Nakano, H., M. Shindo, S. Sakon, S. Nishinaka, M. Mihara, H. Yagita, and K. Okumura.** 1998. Differential regulation of IκB kinase alpha and beta by two upstream kinases, NF-κB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc. Natl. Acad. Sci. USA **95:**3537–3542.
40. **Oie, K. L., and D. J. Pickup.** 2001. Cowpox virus and other members of the orthopoxvirus genus interfere with the regulation of NF-κB activation. Virology **288:**175–187.
41. **Pomerantz, J. L., and D. Baltimore.** 1999. NF-κB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase. EMBO J. **18:**6694–6704.
42. **Scherer, D. C., J. A. Brockman, Z. Chen, T. Maniatis, and D. W. Ballard.** 1995. Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. Proc. Natl. Acad. Sci. USA **92:**11259–11263.
43. **Schmidt, C., B. Peng, Z. Li, G. M. Sclabas, S. Fujioka, J. Niu, M. Schmidt-Supprian, D. B. Evans, J. L. Abbruzzese, and P. J. Chiao.** 2003. Mechanisms of proinflammatory cytokine-induced biphasic NF-κB activation. Mol. Cell **12:**1287–1300.
44. **Senkevich, T. G., E. V. Koonin, J. J. Bugert, G. Darai, and B. Moss.** 1997. The genome of molluscum contagiosum virus: analysis and comparison with other poxviruses. Virology **233:**19–42.
45. **Shisler, J. L., and X. L. Jin.** 2004. The vaccinia virus K1L gene product inhibits host NF-κB activation by preventing IκBα degradation. J. Virol. **78:**3553–3560.
46. **Shisler, J. L., and B. Moss.** 2001. Molluscum contagiosum virus inhibitors of apoptosis: the MC159 v-FLIP protein blocks Fas-induced activation of procaspases and degradation of the related MC160 protein. Virology **282:**14–25.
47. **Shu, H.-B., D. Halpin, and D. V. Goeddel.** 1997. Casper is a FADD- and caspase-related inducer of apoptosis. Immunity **6:**751–763.
48. **Stack, J., I. R. Haga, M. Schroder, N. W. Bartlett, G. Maloney, P. C. Reading, K. A. Fitzgerald, G. L. Smith, and A. G. Bowie.** 2005. Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. J. Exp. Med. **201:**1007–1018.
49. **Thompson, J. E., R. J. Phillips, H. Erdjument-Bromage, P. Tempst, and S. Ghosh.** 1995. I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B. Cell **80:**573–582.
50. **Tschopp, J., M. Irmler, and M. Thome.** 1998. Inhibition of fas death signals by FLIPs. Curr. Opin. Immunol. **10:**552–558.
51. **Viac, J., and Y. Chardonnet.** 1990. Immunocompetent cells and epithelial cell modifications in molluscum contagiosum. J. Cutan. Pathol. **17:**202–205.
52. **Zhou, A., S. Scoggin, R. B. Gaynor, and N. S. Williams.** 2003. Identification of NF-kappa B-regulated genes induced by TNFα utilizing expression profiling and RNA interference. Oncogene **22:**2054–2064.
53. **Zhou, L., A. Tan, S. Iasvovskaia, J. Li, A. Lin, and M. B. Hershenson.** 2003. Ras and mitogen-activated protein kinase kinase kinase-1 coregulate activator protein-1- and nuclear factor-κB-mediated gene expression in airway epithelial cells. Am. J. Respir. Cell Mol. Biol. **28:**762–769.