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The MC159 Protein from the Molluscum Contagiosum Poxvirus Inhibits NF-κB Activation by Interacting with the IκB Kinase Complex

Crystal M. H. Randall,* Janet A. Jokela,† and Joanna L. Shisler*

Molluscum contagiosum virus (MCV) causes persistent neoplasms in healthy and immunocompromised people. Its ability to persist likely is due to its arsenal of viral immunoevasion proteins. For example, the MCV MC159 protein inhibits TNF-R1–induced NF-κB activation and apoptosis. The MCV protein is a viral FLIP and, as such, possesses two tandem death effector domains (DEDs). We show in this article that, in human embryonic kidney 293 T cells, the expression of wild-type MC159 or a mutant MC159 protein containing the first DED (MC159 A) inhibited TNF-induced NF-κB, or NF-κB activated by PMA or MyD88 overexpression, whereas a mutant protein lacking the first DED (MC159 B) did not. We hypothesized that the MC159 protein targeted the IκB kinase (IKK) complex to inhibit these diverse signaling events. Indeed, the MC159 protein, but not MC159 B, coimmunoprecipitated with IKKγ. MC159 communoprecipitated with IKKγ when using mouse embryonic fibroblasts that lack either IKKα or IKKβ, suggesting that the MC159 protein interacted directly with IKKγ. MC159-IKKγ communoprecipitations were detected during infection of cells with either MCV isolated from human lesions or with a recombinant MCV-expressing vaccinia virus. MC159 also interacts with TRAF2, a signaling molecule involved in NF-κB activation. However, mutational analysis of MC159 failed to reveal a correlation between MC159–TRAF2 interactions and MC159’s inhibitory function. We propose that MC159–IKK interactions, but not MC159–TRAF2 interactions, are responsible for inhibiting NF-κB activation.

*Department of Microbiology, College of Medicine, University of Illinois, Urbana, IL 61801; and †Department of Internal Medicine, College of Medicine, University of Illinois, Urbana, IL 61801

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Address correspondence and reprint requests to Dr. Joanna L. Shisler, Department of Microbiology, B103 Chemical and Life Sciences Labs, 601 South Goodwin Avenue, Urbana, IL 61801. E-mail address: jshisler@illinois.edu

Abbreviations used in this article: DED, death effector domain; HA, hemagglutinin; IKK, IκB kinase; McK, molluscum contagiosum; MCV, molluscum contagiosum virus; MEF, mouse embryonic fibroblast; MOL, multiplicity of infection; p, plasmid; PLB, passive lysis buffer; PVDF, polyvinylidene difluoride; WT, wild-type.

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tions, we demonstrate that the MC159 protein coimmunoprecipitates with the IKKγ subunit of the IKK complex. Previous studies in our laboratory revealed that the N-terminal DED (DED(A) of MC159 was sufficient to block NF-κB activation and also mediates MC159–TRAF2 interactions (19). In this study, using MC159 proteins containing substitution mutations within DED(A), data failed to reveal a correlation between MC159–TRAF2 interactions and MC159’s inhibitory function, suggesting that only MC159–IKK interactions are relevant for this inhibitory function of MC159.

Materials and Methods

Cell culture, plasmids, and viruses

Human embryonic kidney 293 T cells and human lung fibroblast cells (MRC-5) were obtained from the American Type Culture Collection. Wild-type (WT), IKKα−/−, and IKKβ−/− mouse embryonic fibroblast (MEF) cells were obtained from Dr. Michael May at the University of Pennsylvania (20). IKKγ−/− MEFs were obtained from Dr. Tak Mak. All cells were cultured in Eagle’s MEM supplemented with 2 mM L-glutamine, 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Plasmid pMCM159 consists of the MC159 gene inserted into vector pCI (Promega). pMC159 A encodes a MC159 gene that lacks the C-terminal DED (DED(B)) (21). Plasmid pMC159 B encodes a MC159 gene that lacks the N-terminal DED (DED(A)) (22). Plasmid pMC159 C contains a MC159 gene with substitution mutations where residues L72, L73 and L74 were altered to alanine (23). Plasmid pMC159 D contains a MC159 gene where residue L31 was altered to an alanine (23). Plasmid pCMV tag 2A-HA was provided by Dr. Michael May at the University of Pennsylvania (21). Plasmid pMC159 A encodes a MC159 gene that lacks the C-terminal DED (23). Plasmid pMC159 D contains a MC159 gene where residue L31 was altered to an alanine (23). Thus, as a control, some cells were infected with vcrmA. All luciferase activities were measured as relative light units using a BioTek luminometer and the Dual Luciferase Reporter Assay System (Promega), with the exception of luciferase assays shown in Fig. 1, in which the Luminoskan Microplate Luminometer (Labsystems) was used. All experiments were performed in triplicate. Relative luciferase activity ratios were calculated by dividing firefly luciferase activities by sea pansy luciferase activities. Luciferase activity ratios were then normalized to that of pCI-transfected cells incubated in regular medium, and this value was set as 1. Statistical significance was calculated using a Student t test where p < 0.05. Cellular lysates from luciferase assays were analyzed for protein expression by immunoblotting (see later).

Immunoprecipitations

To detect MC159–TRAF2 interactions, we cotransfected 1 well of a 6-well tissue culture plate of 293 T cells with 1000 ng pha-TRAF2 and 1000 ng pCI, pMC159, pMC159 B, pMC159 21, or pMC159 24. At 24 h posttransfection, cells from each well were removed from the plate by scraping and collected by centrifugation (1000 × g for 10 min). Cellular pellets were resuspended in 500 μl DED lysis buffer for 30 min at 4C (23). Cellular lysates were centrifuged (14,000 × g for 10 min). Clarified supernatants were collected. A total of 50 μl of the supernatants was saved, and 30 μg protein from this sample (∼15 μl) was used to assess expression levels of proteins. The remaining 450 μl of clarified lysates was incubated overnight with 1 μg monoclonal mouse anti-HA (Sigma Aldrich) and 50 μg protein G-Sepharose (Invitrogen) beads with constant rotation at 4C to detect MC159–TRAF2 interactions. To detect MC159–IKKγ interactions, 1 well of a 6-well plate of 293 T cells was transfected with 1000 ng of either pCI or pha-MC159. At 24 h posttransfection, cells from each well were removed from the plate by scraping and collected by centrifugation (1000 × g for 10 min). Cellular pellets were resuspended in 500 μl DED lysis buffer for 30 min at 4C (23). A total of 50 μl of the supernatants was set aside, and 30 μg protein from this sample (∼15 μl) was used to assess expression levels of MC159 and IKKγ. The remaining 450 μl of clarified lysates was incubated overnight with 1 μg monoclonal mouse anti-IKKγ (sc-56919; Santa Cruz) Abs to detect MC159–IKKγ interactions and 50 μl protein G-Sepharose (Invitrogen) beads with constant rotation at 4C. As a control, an identical set of clarified lysates was incubated instead with 1 μg mouse IgG (Sigma Aldrich) Abs and 50 μl protein G-Sepharose (Invitrogen) beads with constant rotation at 4C.

In other immunoprecipitation experiments in which MEFs were used, 10-cm dishes of subconfluent monolayers of WT MEFs or IKKγ−/−, or IKKβ−/− MEFs were transfected with 1000 ng pCI, pMC159, or pMC159 B. At 24 h posttransfection, cells were plated from dishes by scraping into 1 ml chilled PBS and collected by centrifugation (1000 × g for 10 min). Cellular pellets were resuspended in 500 μl DED lysis buffer for 30 min at 4C (23). Cellular lysates were centrifuged (14,000 × g for 10 min). A total of 50 μl of the supernatants was set aside, and 30 μg protein from this sample (∼5 μl) was used to assess expression levels of MC159 and IKK proteins. The remaining 100 μl of clarified lysates was incubated overnight with 2 μg polyclonal rabbit anti-IKKγ (sc-8330; Santa Cruz) Ab or 3 μg anti-IKKβ Ab (ImageTex) with constant rotation at 4C. After the incubation, 50 μl protein G-Sepharose beads was added to each of the immunoprecipitation reactions and incubated at 4C for 1 h with constant rotation.

Immunoprecipitations also were performed using lysates from virus-infected cells. For these experiments, 1 well of MRC-5 cells in a 6-well plate was either mock-infected or infected with 300 μl of an MCV lesion preparation, a volume of MCV preparation that produced cytopathic effects in MRC-5 cells within 24 h. Alternatively, when using 293T cells, 1 well of cells from a 6-well plate was mock-infected or infected with vcrmA. For both experiments, at 24 h postinfection, cells were detached from plates by scraping and collected by centrifugation (1000 × g for 10 min). Cellular pellets were resuspended in 500 μl DED lysis buffer for 30 min at 4C (23). Cellular lysates were centrifuged (14,000 × g for 10 min). A total of 50 μl of the supernatants was set aside, and 30 μg protein from this sample (∼15 μl) was used to assess expression levels of MC159 and IKK proteins. The remaining 450 μl of clarified lysates was incubated with either 2 μg of a monoclonal mouse anti-IKKγ (sc-56919; Santa Cruz) Ab or mouse IgG (Sigma Aldrich) with constant rotation at 4C. After incubation with anti-IKKγ, 50 μl protein G-Sepharose beads were added and supernatants were incubated for 1 h with constant rotation at 4C.

For all immunoprecipitations, supernatant bead mixtures were pelleted by centrifugation (14,000 × g/30 s) and washed three times with DED lysis buffer. Pelleted bead–protein complexes were suspended in 30 μl of 2X Laemmli buffer containing 5% 2-ME and boiled for 5 min.

Immunoblotting and Abs

The protein concentration of each clarified cellular lysate was determined by using bicinchoninic acid assay (Pierce). Approximately 30 μg protein from each lysate sample was prepared for immunoblotting by resuspending in nonreducing lane marker sample buffer (Thermo Scientific) with 5% 2-ME and boiling for 5 min.
Immunoprecipitated samples or clarified cellular lysates were subjected to 12% SDS-PAGE, and proteins were subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Membranes were incubated with indicated primary Abs (see later), washed three times in TBST, and incubated with appropriate HRP-conjugated secondary Abs. Immunoblots were developed using Pierce super signal west chemiluminescence reagent. Primary Abs used in these experiments were polyclonal rabbit anti-IKKα (1:500, sc-8330; Santa Cruz), polyclonal rabbit anti-IKKβ (1:1000) (21), monoclonal mouse anti-HA (1:500; Sigma-Aldrich), monoclonal mouse anti-IKKα (1:500; Imgenex), or monoclonal mouse anti-IKKβ (1:500; Imgenex). Monoclonal mouse anti-E3 (1:500) Ab was a kind gift from Dr. Stuart Isaacs (University of Pennsylvania) (26). Secondary Abs conjugated to HRP were obtained from either Thermo Scientific (goat anti-mouse IgG) or Calbiochem (goat anti-rabbit IgG).

**Results**

The N-terminal DEDA of MC159 is necessary for inhibiting TNF-induced NF-κB (19), and this portion of MC159 also interacts with TRAF2, a known mediator of NF-κB activation (19). To ask whether MC159–TRAF2 interactions were indeed responsible for inhibition of NF-κB, we assayed the function of two MC159 proteins with substitution mutations in discrete motifs within DEDA: the RXDL motif (pMC159 21) and a hydrophobic patch (pMC159 24) (22, 23). Each mutant protein was assessed for its ability to inhibit TNF-induced NF-κB using a luciferase reporter assay (Fig. 1). Similar to a previous report (19), the WT MC159 protein inhibited TNF-mediated NF-κB activity, as seen by a 4-fold decrease in luciferase activity compared with cells transfected with empty vector (pCI). The RXDL motif was important in providing inhibitory function because TNF treatment of cells resulted in luciferase activity levels in pMC159 21-transfected cells that were similar to that of pCI-transfected cells. In contrast, pMC159 24-transfected cells exhibited a 10-fold decrease of luciferase activity compared with cells transfected with pCI. Differences in luciferase activity levels were independent of MC159 protein expression levels (Fig. 1, bottom panel). These data suggest that the RXDL motif, but not the hydrophobic patch in DEDA-mediated inhibition of NF-κB activation.

It was observed that MC159 expression in unstimulated cells induced relatively low levels of NF-κB-mediated luciferase activity (Figs. 1, 2). This phenotype was observed previously by our laboratory and others (19, 27). Similar to MC159, MC159 21-induced NF-κB activation was marginal when compared with luciferase expressed by TNF induction. In our hands, MC159 overexpression does not induce NF-κB activation as measured by κB degradation or induction of NF-κB–controlled genes (19), suggesting that the NF-κB activating function of MC159 is an artifact of the luciferase assay system.

The regions of MC159 responsible for interactions with either TRAF2 or TRAF3 are dispensable for NF-κB inhibitory function

Because the MC159 DEDA is sufficient to inhibit NF-κB activation, and MC159 A also immunoprecipitates with TRAF2, a TNF-R1 signaling adaptor protein, this suggests that MC159–TRAF2 interactions are required for the MC159 inhibitory function (19). To test this hypothesis, we assayed the mutant MC159 21 and MC159 24 proteins for their ability to coassociate with TRAF2 using immunoprecipitations (Fig. 3). Similar to the WT MC159 protein, the MC159 21 and MC159 24 proteins coassociated with HA-TRAF2 (Fig. 3, top panel), indicating that MC159–TRAF2 interactions did not correlate with inhibition of NF-κB. The MC159 B protein, which lacks DEDA, no longer immunoprecipitated with TRAF2, as previously reported. Similar levels of expression of the TRAF2 and MC159 proteins were detected by immunoblotting (Fig. 3, middle and bottom panels, respectively). The MC159 protein was not detected from samples in which the HA-TRAF2 protein was absent, suggesting that the MC159 protein does not nonspecifically bind to protein G-Sepharose beads.

**FIGURE 1.** The MC159 RXDL motif confers resistance to TNF-induced NF-κB activation. Subconfluent 293T cellular monolayers were transfected with pNF-κB Luc, pRL-null, and either pCI, pMC159, pMC159 21, or pMC159 24. At 24 h later, cells were incubated with regular medium or medium containing TNF (10 ng/ml). After an additional 12-h incubation, cells were lysed and luciferase activities were measured. Statistically significant data for MC159 inhibition of NF-κB–mediated luciferase activity (p < 0.05) are indicated with an asterisk. Lysates also were analyzed by immunoblotting. Proteins were subjected to SDS-12% PAGE, transferred to PVDF membranes, and incubated with an anti-MC159 antisem
An earlier report by Thurau et al. (25) established that TRAF3–MC159 interactions stabilized TRAF2–MC159 interactions. If TRAF3 stabilizes MC159–TRAF2 interactions to aid in the inhibition of NF-κB, then we hypothesized that MC159 proteins that do not bind to TRAF3 would not inhibit NF-κB. Accordingly, two mutant MC159 proteins (MC159 DM and MC159 D) that are deficient in TRAF3 binding (25) were assessed by a luciferase reporter assay for their ability to inhibit TNF-induced NF-κB activation (Fig. 2, graph). The inhibition of NF-κB activation by either MC159 or MC159 DM was statistically significant (p < 0.05), with WT MC159 inhibiting luciferase activity 3.4-fold, and MC159 DM inhibiting 3.4-fold. Although luciferase activity levels in MC159 Δ-expressing cells were lower than in pCI-transfected cells, the difference in levels was not statistically significant. The similar expression levels of each MC159 protein were confirmed by immunoblotting of the cellular lysates (Fig. 2, bottom panel). Unfortunately, the MC159 Δ protein lacks the epitope recognized by the anti-MC159 Ab (21) and, therefore, was not detectable by this method. Our results indicate that TRAF2 and TRAF3 interactions are insufficient to mediate inhibition of NF-κB by the MC159 protein.

MC159 DEDA inhibits TNF-, PMA- or MyD88-induced NF-κB activation

Although many external stimuli trigger different upstream NF-κB activation pathways, most NF-κB pathways converge on IKK activation. We observed that the WT MC159 protein inhibited luciferase activity in response to three stimuli (TNF, PMA, and MyD88 overexpression) that use different upstream signal transduction pathways to stimulate IKK and NF-κB (Fig. 4). In this case, the MC159 inhibitory function was about a 2.8-fold decrease from pCI-transfected cells, in comparison with Fig. 1, in which MC159 inhibited 4-fold as compared with pCI-transfected cells. MC159 A also decreased luciferase activity in response to TNF (1.8-fold), PMA (2.3-fold), or MyD88 (2.3-fold) (Fig. 4A–C, respectively), albeit not to the extent of WT MC159. However, the MC159 B protein did not significantly decrease luciferase activity under any condition tested. A similar level of MC159 protein expression was observed for all three experiments as shown by immunoblotting (Fig. 4). Thus, the MC159 protein used DEDA to inhibit NF-κB activation by inhibiting an event that is conserved among all three signaling pathways.
Most inducers of canonical NF-κB signaling require IKK activation (15). Because MC159 inhibited several NF-κB inducers, we hypothesized that the MC159 protein interacts with one or more of the components of the IKK complex. MC159 was reported to not coimmunoprecipitate with either IKKa or IKKβ (28). Moreover, the Kaposi’s sarcoma herpes virus K13 protein, an MC159 homolog, was shown to bind to IKKγ (29). As a result of these data, we queried whether MC159 interacted with IKKγ, evaluating whether MC159 and IKKγ could be coimmunoprecipitated in MC159-expressing cells. As shown in Fig. 5A, the IKKγ protein coassociated with MC159 in pHA-MC159-transfected cells but was not immunoprecipitated from cells transfected with empty vector (pCI; Fig. 5A). Similarly, immunoprecipitations using an anti-IKKγ Ab resulted in MC159-IKKγ coassociations from pMC159-transfected cells, but not pCI-transfected cells (Fig. 5B). If an Ab that did not detect MC159 or IKKγ was used for immunoprecipitations, the MC159 protein was no longer detected in immunoprecipitated samples (Fig. 5C), indicating that the MC159-IKKγ coimmunoprecipitations were specific.

The earlier experiments were performed using the 293T human cell line. We next assessed whether these same interactions occurred in MEF cells that were transiently transfected with plasmids lacking or containing the MC159 gene. As shown in Fig. 6, MC159 still coimmunoprecipitated with IKKγ. Notably, the MC159 B protein, which did inhibit NF-κB, did not coimmunoprecipitate with IKKγ, suggesting MC159 interaction with the IKK complex is critical for MC159’s inhibitory function.

Regardless of the presence or absence of MC159, both the IKKa and IKKβ proteins coimmunoprecipitated with IKKγ in WT MEFs (Fig. 6). It should be noted that, because the transfection efficiency of MEFs is <100%, it is impossible to assess whether coimmunoprecipitated IKKa and IKKβ proteins were from cells lacking or containing MC159 molecules, making it difficult to conclude whether MC159 affects IKK complex formation.

To evaluate the importance of the presence of IKKa, IKKβ, or IKKγ for coimmunoprecipitating MC159, immunoprecipitations were performed with pMC159-transfected MEFs from either IKKa (IKKa−/−), IKKβ (IKKβ−/−), or IKKγ (IKKγ−/−) knockout mice. MC159 still coimmunoprecipitated with IKKγ when probing lysates from either IKKa−/− or IKKβ−/− cells (Fig. 7A). These data suggest that either IKKa or IKKβ are not directly involved in MC159 binding, or that the absence of either IKKa or IKKβ can be compensated for. The presence of MC159 did not prevent IKKγ-IKKα or IKKγ-IKKβ coimmunoprecipitations, suggesting that MC159 did not grossly alter the IKK complex. Comparable levels of MC159, IKKa, IKKβ, and IKKγ protein

FIGURE 5. The MC159 protein coimmunoprecipitates with IKKγ. One well of subconfluent 293T cellular monolayers in a six-well plate was transfected with pCI or pHA-MC159. At 24 h posttransfection, cells were lysed in 500 μL DED lysis buffer. Immunoprecipitations (IP) were performed by incubating 450 μL clarified cellular lysates with protein G-Sepharose beads and either (A) anti-HA, (B) anti-IKKγ, or (C) anti-IKKβ Abs or IgG Abs. The remaining 50 μL of clarified cellular lysates was used for detection of IKKγ and MC159 proteins by immunoblotting. Immunoprecipitates (top) or 30 μg of corresponding clarified cellular lysates (middle and bottom panels) was subjected to SDS-12% PAGE. Immunoprecipitated samples (left panels) or clarified cellular lysates (right panels) were analyzed by immunoblotting. After SDS-12% PAGE, proteins were transferred to PVDF membranes and incubated with either mouse anti-IKKγ or anti-MC159 Abs.

FIGURE 6. IKKγ coimmunoprecipitates IKKa and IKKβ in the presence of MC159 protein. A 10-cm2 dish of subconfluent MEF cellular monolayers was transfected with pCI, pMC159, or pMC159 B. At 24 h posttransfection, cells were lysed in 150 μL DED lysis buffer. A total of 50 μL clarified lysates was collected to new tubes, and 30 μg of this sample was analyzed for MC159 and IKK proteins by immunoblotting (bottom panel). Immunoprecipitations (IP) were performed using 100 μg clarified lysates incubated with protein G-Sepharose beads and anti-IKKγ. Immunoprecipitated samples (top panel) or clarified cellular lysates (bottom panel) were analyzed by immunoblotting. After SDS-12% PAGE, proteins were transferred to PVDF membranes and incubated with either mouse anti-IKKγ, anti-IKKα, anti-IKKβ, or anti-MC159 Abs.
expression in cellular lysates were shown by immunoblotting (Fig. 7A), indicating that MC159 protein expression did not affect the expression of these proteins.

Fig. 7B used IKKγ2/2 MEFs to assess the importance of IKKγ in MC159–IKK complex association. Although MC159 coimmunoprecipitated with IKKβ in WT MEFs, this interaction was no longer detected in IKKγ2/2 cells. Thus, the IKKγ molecule is necessary for MC159 to interact with the IKK complex.

**MC159 protein coimmunoprecipitates with IKK during poxvirus infection**

To evaluate MC159–IKK interactions in the context of a poxvirus infection, we performed immunoprecipitations using lysates from infected cells. Immuno-precipitations (IP) were performed using 100 μl clarified lysates incubated with protein G-Sepharose beads and anti-IKKγ. Immunoprecipitated samples (left panels) or clarified cellular lysates (right panels) were analyzed by immunoblotting. After SDS-12% PAGE, proteins were transferred to PVDF membranes and incubated with either mouse anti-IKKγ, anti-IKKα, anti-IKKβ, anti-HA, or anti-MC159 Abs.

**FIGURE 8.** The MC159 protein associates with IKKγ during poxvirus infection. (A and B) One well of a six-well plate of 293T cellular monolayers was either mock-infected or infected with vcrmA or vMC159 (MOI = 10). (C) One well of a six-well plate of MRC-5 cell monolayers was mock-infected or infected with 300 μl MCV lesion preparation. For all experiments, at 24 h postinfection, cells were lysed in 500 μl DED lysis buffer. A total of 50 μl clarified lysates was used for immunoblotting to detect the presence of IKKγ, MC159, or E3 proteins. With the remaining 450 μl of clarified lysates, immunoprecipitations (IP) were performed on clarified lysates using protein G-Sepharose beads and (A, C) anti-IKKγ antiserum or (B) IgG. Immunoprecipitated samples (top panels) or clarified cellular lysates (bottom panels) were analyzed by immunoblot. After SDS-12% PAGE, proteins were transferred to PVDF membranes and incubated with rabbit anti-IKKγ, anti-MC159, or anti-E3 Abs.
cells infected with either a recombinant vaccinia virus expressing (vMC159) or the parental vaccinia virus (vcrmA^-). MC159 co-immunoprecipitated with IKKγ in cells infected with vMC159, but not in cells infected with a virus lacking the MC159 gene (vcrmA^-) or mock-infected cells (Fig. 8A, top panel). The expression of E3, an early vaccinia virus protein, demonstrated vcrmA^- and vMC159 early protein expression in 293T cells (Fig. 8A, bottom panel). An identical set of lysates from virus-infected cells were incubated with IgG Abs that were not specific for IKKγ. MC159 proteins were no longer detected in the immunoprecipitates from these reactions (Fig. 8B), indicating that MC159 did not promiscuously bind to protein G-Sepharose beads.

It is known that MCV infection of MRC-5 cells results in abortive infection, but the early and late classes of poxviral proteins are still expressed (1, 30). To evaluate MC159–IKKγ interactions during MCV infection, MRC-5 cells were infected with an MCV lesion preparation and cell lysates were assayed for MC159–IKKγ interactions by using immunoprecipitation. MC159 coimmunoprecipitated with IKKγ, albeit in low levels, in cells infected with MCV preparations but not in mock-infected cells (Fig. 8B, top panel). In addition, MC159 and IKKγ proteins were detected in cellular lysates (Fig. 8B, bottom panel). Thus, a conclusion from this data is that the MC159 protein coimmunoprecipitates with IKKγ (Fig. 9).

Discussion

The IKK complex plays a crucial role in NF-κB activation, making it an appealing target for viruses as a means to block NF-κB-mediated activation of the proinflammatory response (31–33). Other viral proteins target the IKK complex to inhibit NF-κB activation, such as the vaccinia virus B14 protein (34) and the enterovirus 2C protein (35), each of which bind to IKKB to block IKKB phosphorylation to inhibit NF-κB activity. In this study, we show that the MC159 protein inhibited three distinct pathways that result in NF-κB activation. MC159 also coimmunoprecipitated with the IKK complex but did not require IKKα or IKKβ to coimmunoprecipitate with IKKγ. We also observed MC159–IKKγ interactions in either MCV-infected cells or cells in which MC159 was expressed by a surrogate virus (21).

The upstream signal transduction pathways triggered by TNF, MyD88, and PMA converge upon IKK (12, 36). Because MC159-inhibited NF-κB triggered through each of these effectors, we speculated that MC159 targeted the IKK complex as its mechanism. Our current model is that the MC159 protein interacts di-

![FIGURE 9.](http://www.jimmunol.org/)

A model of MC159 and MC160 inhibition of TNF-R1 signaling during MCV infection. TNF binds to the TNF-R1 receptor to initiate either apoptosis or NF-κB activation. For apoptosis, TNF-TNF-R1 interactions initiate recruitment and activation of the death-inducing signaling complex (DISC), composed of TRADD, FADD, and procaspase-8. The MC159 protein binds to FADD and prevents DISC assembly to inhibit TNF-induced apoptosis during MCV infection. NF-κB activation is initiated by TNF binding TNF-R1, which recruits TRADD, TRAF2, RIP, and MEKK, resulting in the IKK complex activation and the degradation of IκB proteins, thus enabling NF-κB activation. We propose the following model for MC159- and MC160-mediated inhibition of TNF signaling during MCV infection: during the initial stage of an MCV infection, the MC160 protein inhibits acute NF-κB activation by binding to Hsp90 to cause IκBα degradation, subsequently preventing the degradation of IκBα (52). Later during MCV infection, the MC159 protein inhibits chronic NF-κB activation by binding to the IKKγ subunit of the IKK complex, thereby inhibiting IκBβ degradation.
directly with IKKγ to inhibit NF-κB activation. This model is based on our data showing that the absence of IKKγ prevents MC159–IKKβ interactions, and that MC159 does not immunoprecipitate with either IKKe or IKKβ (28). IKKγ may have different conformations in its resting versus active states. Previously, we showed that MC159 inhibits IKKβ, but not IKKα phosphorylation (37). Whether MC159 preferentially interacts with IKKγ to prevent IKKβ phosphorylation is unknown. It is also known that IKKγ must be posttranslationally modified (ubiquitination or phosphorylation) for activity (38). Thus, another possibility is MC159 may be outcompeting another cellular IKKγ binding protein, preventing one of the previously mentioned posttranslational modification events from occurring. Regardless of the mechanism, we propose that inhibition of the MC159 protein would hamper the ability of MCV to survive and replicate in keratinocytes.

A recent study reports that the MC159 protein can function as an activator of NF-κB (27) when it is expressed at low levels. In this and a previous study, we also observed that MC159 expression itself stimulated low levels of NF-κB–controlled luciferase activity (19). It was shown that low levels of MC159 enhanced interactions between TNF-R1 signalosome components RIP1 and TRADD, but not TRAF2 (27). In this study, we found no correlation between the ability of a mutant MC159 protein to bind to TRAF2 and to either stimulate NF-κB activation autonomously or inhibit TNF-induced NF-κB activation. Thus, we conclude that MC159–TRAF2 interactions probably would not contribute to the stability of MC159–IKKγ interactions.

Analysis of the MC159 structure revealed that its tandem DEDs associate with one another in a ridged dumbbell-like structure (17, 18). Structure-based sequence analysis of the MC159 DEDs shows similarities with other DED-containing proteins (i.e., c-FLIP, procaspase-8, the HHV8 K13 protein) (39). A striking difference between MC159 and K13 is that MC159 inhibits NF-κB activation, whereas K13 stimulates it (19, 28). Recently, Bagnéris et al. (40) cocrystalized K13 with IKKγ, showing that the N-terminal DED of K13 possesses two clefts for binding IKKγ monomers. Based on a sequence alignment between K13 and MC159, Bagnéris et al. (40) suggest that the MC159 protein lacks the clefts required for IKKγ interactions, and that this loss of interaction in why the MC159 protein does not activate NF-κB. It is suggested that the residues required for formation of cleft 2 are absent in MC159, and that an extra helix in the N terminus of MC159 (H0) prevents IKKγ from binding to cleft 1. However, the H0 region of MC159 has not been successfully crystallized (17, 18), making it hard to assess whether this occlusion occurs in vivo.

The MC159 21 and MC159 24 proteins allowed and inhibited TNF-induced NF-κB activation, respectively. MC159 21 contains two mutations as residues 72 and 73, and lies within the RXDL motif in DED1 (17, 23). These two residues are buried within the DED1 structure of MC159, suggesting that they may maintain MC159 structure (17). In support of this hypothesis, MC159 21 does not inhibit apoptosis, nor form a ternary complex with Casp and FADD (18, 23). According to Bagnéris et al. (40) these residues correspond to K13 residues 68 and 69, and are predicted to not affect IKKγ binding. Despite this fact, alteration of these residues in K13 results in a mutant K13 that no longer activates NF-κB (41). Interestingly, despite having different effects on NF-κB activity, MC159 and K13 both use residues within the RXDL motif of DED1 to manipulate NF-κB signaling, suggesting that homologous viral proteins may evolve to provide different mechanisms that enable virus survival and pathogenesis.

MC159 24 retained its ability to inhibit NF-κB activation, perhaps even better than WT MC159. MC159 24 contains a single amino acid substitution at residue 31, a residue that is located at the DED interface (17, 23). This residue corresponds to K13 residue 25, and was not predicted to interact with IKKγ by Bagnéris et al. (40), suggesting that its mutation would have little effect on IKKγ binding activity. Attempts at coimmunoprecipitating MC159 21 or MC159 24 with IKKγ resulted in inconsistent results, making it difficult to draw conclusions about whether these mutant proteins associated with the IKK complex.

Motifs within the MC159 DEDs that are predicted to mediate protein–protein interactions include a surface-exposed charged region called the RXDL motif (39). Earlier studies revealed that the RXDL motifs in both DEDA and DEDB must be present in MC159 to inhibit TNF-induced apoptosis (22). Surprisingly, only the RXDL motif of DEDB was required to inhibit TNF-induced NF-κB activation (19). This suggests the MC159 protein uses different molecular mechanisms to inhibit apoptosis and NF-κB. TNF is a potent proinflammatory cytokine that initiates NF-κB activation or apoptosis via distinct signal transduction pathways (Fig. 9). It remains unclear how TNF-R1 dictates the activation of either apoptosis or NF-κB (42, 43). If this information were known, novel therapeutics could be rationally designed to precisely inhibit unwanted or enhance desired TNF-R1 signaling. Because the MC159 protein can inhibit both outcomes of TNF-R1 signaling, through distinct mechanisms, the study of MC159 can provide unique insights into how TNF-R1 initiates either apoptosis or NF-κB activation (19, 44).

TNF-induced NF-κB activation is a biphasic event (45–47). The IκBα protein is degraded during acute TNF activation, whereas the IκBβ protein is degraded after prolonged TNF signaling. Because MCV infections are persistent, it is likely that both acute and chronic TNF-induced NF-κB activation occurs in MCV-infected cells in vivo. Interestingly, it is known that MC159 inhibits degradation of IκBα where MC160 inhibits degradation of IκBβ (19, 37). Our current working model is that MC160 inhibits acute TNF-induced NF-κB activation that would occur during initial infection, and MC159 inhibits chronic TNF-induced NF-κB activation that would occur over the course of a persistent infection (Fig. 9). We also hypothesize that the complete silencing of TNF signaling via coexpression of MC159 and MC160 is critical for MCV to sustain a persistent infection whereas minimizing inflammatory responses. Unfortunately, this hypothesis is difficult to test because of a lack of animal models for MCV infections.

Recent reports have shown that IKK is constitutively active in many cancers, including melanomas (48–50). For melanomas, silencing IKK activation results in their apoptotic cell death. In these studies, IKK activation was inhibited by treatment of cells with a NEMO binding peptide, which binds to IKKγ to disrupt the IKK complex (49, 50). The MC159 protein is potentially a more useful therapeutic than NEMO binding peptide because it inhibits NF-κB activation without disrupting the IKK complex, and therefore should be less toxic. In addition, data from a clinical case report empirically indicate that MCV infection has some anticancer properties (51); a patient developed an MC lesion within a cutaneous melanoma, and histological analysis revealed the destruction of melanoma cells at the site of MC lesion. Such therapeutic potential of MC159 needs to be pursued.

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