M135R Is a Novel Cell Surface Virulence Factor of Myxoma Virus

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Myxoma virus (MV) encodes a cell surface protein (M135R) that is predicted to mimic the host alpha/beta interferon receptor (IFN-α/β-R) and thus prevent IFN-α/β from triggering a host antiviral response. This prediction is based on sequence similarity to B18R, the viral IFN-α/β-R from vaccinia virus (VV), which has been demonstrated to bind and inhibit type I interferons. However, M135R is only half the size of VV B18R. All other poxvirus-encoded IFN-α/β-R homologs align only to the amino-terminal half of M135R. Peptide antibodies raised against M135R were used for immunoblotting and immunofluorescence and indicate that M135R is expressed as an early gene and that the product is a cell surface N-linked glycoprotein that is not secreted. In contrast to the predicted properties of M135R as an inhibitor of type I interferon, all binding and inhibition assays designed to demonstrate whether M135R can interact with IFN-α/β have been negative. However, pathogenesis studies with a targeted M135-knockout MV construct (vMyx135KO) indicate that the deletion of M135R severely attenuates MV pathogenesis in the European rabbit. We propose that M135R is an important immunomodulatory virulence factor for myxomatosis but that the target immune ligand is not from the predicted type I interferon family and remains to be identified.

Myxoma virus (MV) is a rabbit-specific virus that causes a lethal disease called myxomatosis in the European rabbit (Oryctolagus cuniculus). Myxomatosis is highly infectious and overwhelms the host immune system, resulting in systemic cellular disruption leading to death within 9 to 12 days. In contrast, infection of the MV evolutionary host, Sylvilagus sp. of North and South America, results in the production of a small, localized, dermal lesion at the site of inoculation that resolves slowly (16). A suite of MV virulence genes that subvert the host immune response to the virus infection mediate the ability of MV to infect and kill O. cuniculus. The genes required for MV to induce the disease syndrome of myxomatosis are known as the viral virulence genes and include gene products that modulate host response and control virus replication in host tissues and virus spread. A pathogenic virus such as MV has evolved multiple strategies to defend its ability to replicate in the face of the host immune defenses.

Two major cytokine arms of the innate mammalian antiviral response are the interferon (IFN)- and tumor necrosis factor (TNF)-mediated responses (13). IFNs are a heterogeneous family of cytokines that include the type I IFNs (alpha IFN [IFN-α] subtypes, IFN-β, IFN-ω, IFN-κ, and IFN-τ), which bind to the common cellular receptor IFN-α/β-R. The single type II IFN (IFN-γ) binds to a different cellular receptor (IFN-γ-R), and most recently the type III IFN (IFN-λ) has been shown to interact through a receptor complex that includes IFN-λ1-R1 and interleukin-10 (IL-10) receptor 2 (17). In contrast, the TNF-mediated arm of the host cellular defense plays a key role in apoptosis and cell survival as well as in inflammation and immunity (29).

Poxviruses have acquired a large group of wide-ranging gene products to combat these host IFN and TNF defenses (15, 24). For example, MV encodes a secreted viral IFN-γ-R (vIFN-γ-R) (M-T7) that inhibits the interaction between IFN-γ and its cellular receptor (28). MV also encodes a secreted inhibitor of TNF (M-T2) that can block the ability of rabbit TNF to bind to its cognate cell surface TNF receptors. Other poxvirus genera have acquired other strategies; for instance, the yatapoxviruses encode a high-affinity TNF binding protein (2L) (8, 22), while the orthopoxviruses encode extracellular inhibitors, including several TNF binding proteins (Crm family [21], viral IFN-γ-Rs [B8R family], and vIFN-α/β-R [B18R] [3, 10, 25]). Many poxviruses also encode multiple intracellular inhibitors which include homologs of vaccinia virus E3L, which inhibits the α subunit of eukaryotic initiation factor 2, and K3L, an inhibitor of protein kinase R (1, 4, 24). Although many of these virally encoded factors, which modulate the host immune response, have been identified based on their sequence similarities to cellular molecules, animal studies in which the viral gene has been deleted or insertionally inactivated has allowed for the characterization of the gene function in vivo. Surprisingly, knockout studies have demonstrated that the deletion of many of these individual open reading frames (ORFs) has not resulted in the complete attenuation of the virus, reflecting the complex interaction of the viral arsenal and the host immune system (15).

Myxoma virus encodes a putative cell surface protein (M135R) which was predicted to mimic the host IFN-α/β-R and act to block the host antiviral response. Here we describe the characterization of M135R as a cell surface virulence factor critical for manifesting MV virulence in O. cuniculus. When the M135R gene was deleted, MV was attenuated in vivo, and myxomatosis did not develop. Additionally, we demonstrate

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7 Published ahead of print on 25 October 2006.
that MV is dramatically resistant to treatment with exogenous rabbit type I IFN, regardless of whether M135R is expressed or not. This suggests that the predicted role of M135R is not as a type I IFN binding protein, although its evolutionary origin and the target immune ligand remain to be identified. Finally, the observations reported lead to the expectation that full-length VV B18R is another example of a poxvirus-encoded immunomodulatory molecule that has evolved with dual functions. We speculate that B18R’s ability to bind type I IFN resides in its C-terminal half of the protein, while the N-terminal half shares an as-yet-undefined function with M135R.

MATERIALS AND METHODS

Viruses and cells. Several strains of MV were employed, including untagged wild-type MV: strain Lausanne (vMyxLau); vMyxL, which expresses β-galactosidase under a late viral promoter (19, 20); and vMyxgfp, which expresses enhanced green fluorescent protein (EGFP) under a synthetic early/late promoter inserted in the intergenic region between M135 and M136 (14). The M135 knockout virus (vMyx135KO) and the revertant (vMyx135Rev) are described within the text. Vesicular stomatitis virus expressing green fluorescent protein (VSV:gfp) was a gift of John Bell (University of Ottawa). All viruses were amplified and passed on baby green monkey kidney (BGMK) cells and rabbit kidney cells (RK13; ATCC CCL-37) in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Primary rabbit embryo fibroblasts (pREFs) were established from 21-day-old New Zealand White rabbit embryos by fine mincing of the whole embryos. Further disaggregation was achieved by incubation with 0.25% trypsin. The trypsin was removed by centrifugation and the disaggregated cells were resuspended in Dulbecco’s modified Eagle medium with 10% fetal bovine serum and antibiotics.

Generation of recombinant viruses. Tagged wild-type viruses vMyxLac and vMyxgfp have been described previously (14, 19). Regions flanking the M135R gene were amplified in the intergenic region between M135 and M136 (14). The M135 KO construct contains an amplicon of the region between M134 and M136. The left flank was amplified using primers JB08.00 (5′-CGACCGTATGAATTG-3′) and JB09.00 (5′-TGTGGATCCACCTCGCCATTTTAGC-3′). The SpeI sites are underlined. Between the flanking regions was inserted a cassette that expressed EGFP under the VV synthetic early/late promoter and guanine phosphoribosyltransferase (gpt) under the VV P7.5K promoter. The transfer plasmid was transfected into MV (strain Lausanne)-infected BGMK cells by use of LipoTAXI transfection reagent (Stratagene). The resultant M135R knockout virus (vMyx135KO) produced fluorescent green foci and was purified by three rounds of selection under mycoplasma-selective antibiotic medium. The tagged wild-type virus (vMyx135Rev) was produced by amplification of the correct region of the intergenic region between M135 and M136 by use of primers JB08.00 and JB10.00 (see Fig. 4A). The resultant 1.5-kb fragment was cloned into pBluescript, and this recombinant vector was transfected into vMyx135KO virus-infected BGMK cells. The revertant virus was purified by selection of colorless foci, and this recombinant vector was transfected into vMyx135KO virus-infected BGMK cells. The revertant virus was produced by amplification of the right flank using primers JB10.00 (5′-GTGGGAACATTTAGGATACCTG GTC-3′) and JB09.00 (5′-CAGGAACTAGTCCACCAAATAC-3′). The SpeI sites are underlined. The right flank was amplified using primers JB08.00 (5′-CGACCGTATGAATTG-3′) and JB09.00 (5′-TGTGGATCCACCTCGCCATTTTAGC-3′) and JB07.00 (5′-GTGGGAACATTTAGGATACCTG GTC-3′). The BamHI sites are underlined. Between the flanking regions was inserted a cassette that expressed EGFP under the VV synthetic early/late promoter and guanine phosphoribosyltransferase (gpt) under the VV P7.5K promoter. The transfer plasmid was transfected into MV (strain Lausanne)-infected BGMK cells by use of LipoTAXI transfection reagent (Stratagene). The resultant M135R knockout virus (vMyx135KO) produced fluorescent green foci and was purified by three rounds of selection under mycoplasma-selective antibiotic medium. The tagged wild-type virus (vMyx135Rev) was produced by amplification of the correct region of the intergenic region between M135 and M136 by use of primers JB08.00 and JB10.00 (see Fig. 4A). The resultant 1.5-kb fragment was cloned into pBluescript, and this recombinant vector was transfected into vMyx135KO virus-infected BGMK cells. The revertant virus was purified by selection of colorless foci, the result of homologous recombination between the M135KO-gpt-EGFP fragment of vMyx135KO and the intact M135R coding region of the revertant plasmid.

Single-step growth curve. BGMK and RK13 cells were seeded into six-well dishes, and confluent monolayers (6 × 10E5 cells) were infected with either vMyxL or vMyx135KO at a multiplicity of infection (MOI) of 5 for 1 h. The inoculum was removed, and the cell monolayer was washed three times with complete medium. Samples were collected at various times postinfection (0, 4, 8, 12, 24, and 48 hours postinfection [hpi]), and the virus was collected and then titrated back onto BGMK monolayers by serial dilution in triplicate. Fluorescent foci were scored as 48 hpi and plotted using Excel.

Production of anti-M135R sera. Two predicted antigenic peptides (29–FQKR YNELS0PIKR43 and 90–WRFERETTEDVESDP–104) were synthesized (Invitrogen). Peptides were conjugated to the carrier protein keyhole limpet hemocyanin using an Immune Immunogen EDC conjugation kit (Pierce) according to the manufacturer’s instructions. The conjugated complex was suspended in complete medium, and the conjugated peptides were resuspended in complete medium and incubated at room temperature for 5 min. Cells were pelleted and washed with 2 × SSC (1 × SSC = 0.15 M NaCl plus 0.015 M sodium citrate). This was repeated. The surface-biotinylated cells were then used for immunoprecipitation experiments.

Immunoprecipitation and immunoblot analysis. Immunoprecipitation was performed on 500 μg of total cellular proteins from cell lysates. Streptavidin agarose beads (Sigma) were mixed with cell lysates for 45 min. The beads were washed three times with lysis buffer and then run on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Fifty-microgram portions of total protein from cell lysates were run as expression controls.

Cells (6 × 10E5 BGMK or RK13) were infected with various viruses at an MOI of 3 for 1 h at 37°C. Following adsorption, the inoculum was removed and the cells were washed once with complete medium. At various times postinfection, cells were collected and lysed in a small volume of lysis buffer (20 mM Tris [pH 7.5], 2 mM EDTA, 137 mM NaCl, 1% NP-40, 10% glycerol). Protein concentrations of each sample were determined by use of the Bradford assay (6a). Samples were analyzed by SDS-PAGE and transferred to Hybond-C Extra (Amersham) by semidry blotting. The membranes were blocked in Tris-buffered saline–Tween 20 (TBST)–5% powdered milk for 1 h at room temperature. The membrane was incubated for 1 h overnight with a 1:50 dilution of the affinity-purified anti-M135R antibody in TBST–5% milk powder, followed by a single brief wash in TBST for 10 min. The membrane was incubated at room temperature for 1 h with goat anti-rabbit horseradish peroxidase (1:5,000 dilution; Jackson ImmunoResearch) in TBST–5% milk powder and subsequently washed three times for 10 min each wash in TBST, and then the bands were detected by use of Western Lightning chemiluminescence reagent (Perkin-Elmer).

Animal studies. New Zealand White (Oryctolagus cuniculus) female rabbits were housed in the level II bioccontainment facilities per Health Canada’s Laboratory Safety Guidelines, 2nd edition (13a), and the Canadian Food Inspection Agency guidelines in Containment Standards for Veterinary Facilities (9a). All protocols were in accordance with the regulations of the Animals for Research Act (Ontario) (5a) and the guidelines and policy statements of the Canadian Council on Animal Care (18a). Each rabbit was injected intradermally with 1,000 PFU of virus into each hind flank. The rabbits were monitored twice daily by research and veterinary staff for symptoms of myxomatosis as well as for food and water intake. Weight changes, core body temperature, and waste production were measured daily. Moribund animals were immediately sacrificed by intravenous injection with euthanol administered following anesthesia. Four rabbits were inoculated with wild-type MV (strain Lausanne); three rabbits were inoculated with the tagged wild-type virus, vMyxgfp; six rabbits were inoculated with vMyx135KO; and three animals were inoculated with the revertant, vMyx135Rev. Disease progression was monitored for 28 days or until euthanasia was required.

Type I IFN treatment and binding studies. pREFs were infected with vMyxgfp, vMyx135KO, or VSV:gfp at an MOI of 0.01. Following infection, cells were washed with medium. Complete medium containing either rabbit type I IFN or human IFN-α/β at various concentrations was applied to the infected monolayer, and fluorescent foci were counted at 24 hpi.

Five micrograms of rabbit type I IFN (Sigma) was iodinated by mixing with 1 μl of phosphate-buffered saline, a single Iodolabe, and 0.2 ml NaI214 (NEN) for 7 minutes. Unbound radioactive iodine was removed by column purification. For the binding studies, BGMK cells were left uninfected or were infected with either vMyxgfp or vMyx135KO. Infected IFN was added to the medium, and at 24 hpi the cells were washed to remove excess unbound IFN. Cells were collected and the level of 214I were measured by counting in a gamma counter (Beckman).

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RESULTS

M135R is an early gene. Myxoma virus encodes a protein (M135R) identified from the sequencing of the MV genome (9) and predicted to mimic the host IFN-α/β receptor and to prevent IFN-α/β from triggering a host antiviral response (6, 9). This prediction was based on sequence similarity to the viral IFN-α/β receptor from vaccinia virus (B18R), which has been demonstrated to employ an anti-type I interferon evasion strategy (3, 25). M135R is 23% identical to VV B18R (Table 1). However, M135R is only half the size of VV B18R and all other IFN-α/β-R homologs from sequenced poxviruses, and in all cases M135R aligns only to the amino-terminal half (Fig. 1 and Table 1).

Peptides directed against predicted immunogenic regions of M135R were synthesized and used to generate polyclonal antibodies in rabbits that were then used for Western blot analysis, immunoprecipitations, and immunofluorescence studies. Immunoblotting confirmed that M135R was expressed as an early gene. Treatment of infected cells with cytosine arabinofuranoside indicated that the synthesis of M135R was not altered by inhibition of late gene expression and that M135R was therefore an early gene product (Fig. 2A). Treatment of MV-infected cells with tunicamycin indicated that M135R was N-linked glycosylated (Fig. 2A), likely at the single site predicted from the sequence (Fig. 1B). Monensin treatment suggested that there was no O-linked glycosylation of M135R protein (Fig. 2A, lane 8).

M135R encodes a signal sequence but is not secreted. Sequence analysis of M135R pointed to the presence of a predicted N-terminal signal sequence plus a predicted transmembrane domain at the carboxy terminus (Fig. 1B). Immunoblotting of supernatants from infected BGMK cells with anti-M135R antibody indicated that M135R protein was not secreted but was readily detected in whole-cell lysates (Fig. 2B, top panel). When this membrane was stripped and reprobed with an antibody raised against M-T7, an early, strongly expressed and secreted myxoma virus protein, M-T7, was detected in both the supernatants and the lysates, confirming that both the lysates and the supernatants contained the complete complement of myxoma virus proteins (Fig. 2B, bottom panel). To test whether the signal sequence functioned to target M135R to the cell surface, we deleted the transmembrane domain and expressed the mutant M135R protein from a baculovirus expression system (Ac135TM). Comparison of AcM135R (full length)- and Ac135TM (without transmembrane)-in-

![FIG. 1. Predicted structures of and sequence comparison between M135R and VV B18R. (A) Schematic of VV B18R (above) and M135R (below). Asterisks indicate predicted N-linked glycosylation sites. The predicted signal sequence (ss) is indicated by a black box, while the predicted transmembrane domain (TM) is indicated by the hatched box. (B) Only the first 180 amino acid residues of B18R are shown in the alignment. Conserved residues are boxed. Shading within the box indicates identical residues. Boxed residues which are not shaded indicate conserved substitutions. The arrowheads indicate the predicted cleavage site of the respective signal peptides. The predicted transmembrane region of M135R is overlaid. An asterisk above residue 117 indicates the predicted N-linked glycosylation site of M135R.](http://jvi.asm.org/content/81/3/108.full)

### TABLE 1. Comparison of M135R to other poxviral homologs

<table>
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<tr>
<th>Homolog from:</th>
<th>Myxoma virus (178)</th>
<th>Vaccinia virus (351)</th>
<th>Variola virus (354)</th>
<th>Monkeypox virus (352)</th>
<th>Cowpox virus (351)</th>
<th>Ectromelia virus (358)</th>
<th>Camelpox virus (355)</th>
<th>YLDV (351)</th>
<th>Swinepox virus (344)</th>
<th>LSDV[a] (360)</th>
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<td>43</td>
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[a] Numbers above the diagonal (—) represent percent identity, and numbers below the diagonal represent percent similarity, between any two species. The numbers in parentheses across the top represent the numbers of amino acids in the putative proteins. The comparison was done between the predicted full-length copy of M135R (178 amino acids) and the first 178 residues of each homolog only.

[b] LSDV, lumpy skin disease virus.
fected supernatants indicated that full-length M135R was found in the cell lysate only (Fig. 2C, top panel, lane 5) whereas Ac135/TM was secreted, confirming that the signal sequence functioned to localize M135R through the Golgi-endoplasmic reticulum and into the extracellular pathway (Fig. 2C, bottom panel, lane 1).

M135R localizes to the surface of MV-infected cells. Vaccinia virus B18R was reported to be localized to the surface of infected cells (26, 27). The observation that M135R has a functional signal sequence as well as a transmembrane domain but was not secreted prompted us to assess the localization of M135R. To test whether M135R was localized to the cell surface in a manner similar to VV B18R, we labeled cell surface proteins of cells infected with either vMyxgfp or vMyx135KO with biotin and pulled down the biotinylated cell surface proteins with streptavidin agarose beads. The immunoprecipitated proteins were separated on a 15% SDS-PAGE gel and then probed with anti-M135R. Immunoprecipitation of biotinylated surface proteins and detection of M135R by Western blotting indicated that M135R was among the population of cell surface proteins residing at the surface of infected cells (Fig. 3A).

When BGMK cells were mock infected (Fig. 3B, right panels) or infected with wild-type MV (strain Lausanne, vMyxLau [MOI = 5]; Fig. 3B, left panels) for 8 or 24 h and then immunostained with affinity-purified anti-M135R antibody followed by Alexa 568 (Alex 568 [top panel])- or fluorescein isothiocyanate (FITC [bottom panel])-conjugated secondary antibody and visualized with a fluorescence microscope.

M135R is nonessential for MV replication in vitro. To test the ability of M135R to act as a virulence factor, we constructed a recombinant virus in which M135R was deleted and replaced by a cassette encoding EGFP under the VV synthetic early/late promoter and gpt under the control of VV P7.5K.
promoter (Fig. 4A). The recombinant vMyx135KO was plaque purified by selecting virus clones expressing EGFP under mycophenolic acid selection. The purity of the recombinant was confirmed by PCR (Fig. 4B). Primers JB07.00 and JB10.00 (Fig. 4A) amplified a 1.5-kb fragment from the wild-type MV template (Fig. 4B, lane 4). The product amplified from the purified recombinant viral DNA templates, using the same primers, was 3.0 kb and included the flanking regions of M135R and the EGFP/gpt cassette without any sign of the smaller wild-type product (Fig. 4B, compare lanes 2 and 3 to lane 4). Immunoblotting of BGMK cells infected with either vMyxLau or vMyx135KO confirmed that vMyx135KO was deficient in M135R expression (Fig. 4C, top panel). Detection of another MV protein (M-T7) confirmed that MV proteins were present in the lysate samples (Fig. 4C, bottom panel).

Single-step growth curves were used to test the ability of vMyx135KO to replicate in vitro. There was no replication pattern difference between vMyxgfp and vMyx135KO in either our standard nonhuman primate cell line, BGMK, or the rabbit kidney fibroblast cells, RK13 (Fig. 5). Both vMyxgfp and vMyx135KO exhibited similar replication kinetics, indicating that M135R is not required for replication in vitro.

M135R is a critical virulence factor for myxomatosis in European rabbits. We next tested the ability of vMyx135KO to produce myxomatosis in lab rabbits. In contrast to the animals injected with vMyxLau or vMyxgfp, which developed full-blown myxomatosis and had to be euthanized between days 9 and 10 postinjection, the rabbits injected with vMyx135KO developed only moderate symptoms and recovered completely (Table 2). The initial clinical manifestations of myxomatosis were delayed compared to what was seen for wild-type MV-
infected rabbits. Additionally, vMyx135KO was unable to efficiently spread throughout the rabbit, as evidenced by the lack of satellite lesions and the reduced secondary lesions on the ears and eyes (Table 2). Finally, soon after the controls were euthanized due to the severity of the symptoms, the animals infected with vMyx135KO began to exhibit signs of healing and recovery. These animals continued to improve over the remainder of the study.

To confirm that the loss of M135R caused the attenuation of vMyx135KO, we generated a revertant virus in which M135R was restored, and we tested the ability of this revertant (vMyx135Rev) to restore the ability to produce myxomatosis. All four treated groups of rabbits responded in a similar manner for the first 6 days following the injection of the respective viruses (Table 2). We observed a large red, raised lesion at the site of injection in all treatment groups by 4 days postinfection. However, beginning at day 6 and continuing over the next 3 to 4 days, the differences between the vMyx135KO and vMyx135Rev viruses became evident. Those animals injected with either the untagged, wild-type (vMyxLau) or EGFP-tagged (vMyxgfp) or revertant (vMyx135Rev) virus had numerous secondary lesions in the ears, eyes, and nose, which were not observed for the animals injected with vMyx135KO (Table 2). We conclude that the loss of M135R drastically attenuated MV in the European rabbit myxomatosis model and that M135R is a critical virulence factor in vivo.

The temperatures of rabbits were taken daily for the 5 days preceding the study and considered as the baseline body temperatures of the animals. We continued to take the temperature of each animal daily for the duration of the study. However, there was no difference in body temperature profiles between the treatment groups (Fig. 6). This suggests that M135R does not play a role in the febrile response of infected animals, which is thought to be controlled by host IL-1beta responses, suggesting that M135R does not perturb this particular cytokine.

M135R protein does not bind or inhibit IFN-α/β. The sequence of M135R is similar to that of the vaccinia virus B18R, an IFN-α/β receptor mimic (2, 4, 10, 25). Therefore, we next tested the ability of M135R to bind rabbit type I IFN. We first iodinated rabbit IFN and tested the ability of vMyx135KO-infected cells to bind 125I-rabbit IFN in comparison to that of cells infected with vMyxgfp. We found that the deletion of M135R did not affect IFN-α/β binding to MV-infected cells, and we did not observe any difference in the amounts of IFN bound to the cell surface of either RK13 or BGMK cells infected with MV or vMyx135KO (Fig. 7A). Additionally, treatment of RK13 or BGMK cells with exogenous rabbit type I IFN following virus adsorption did not affect the infection of cells by MV or vMyx135KO (data not shown). This was also true when cells were pretreated with rabbit IFN 24 h before MV infection to induce an antiviral state in the cell. We also noted no significant difference in the MV focus sizes or numbers formed following low-multiplicity infection in either RK13 or BGMK cells (data not shown). This inability to inhibit MV (vMyxgfp) or vMyx135KO was also true if cells were treated with human IFN-αA/D, which is known to be active in rabbit species and humans (data not shown). Finally, we were unable to observe any protein-protein interactions when Ac135ΔTM supernatants were applied to rabbit IFN-α/β adhered to a BIAcore chip (data not shown).

![FIG. 6. Expression of M135R does not regulate body temperature in infected rabbits. Core body temperatures were measured daily for each rabbit. The average body temperature for each animal was determined for the 5 days before the start of the study. This average was the assumed baseline. Therefore we have plotted the change in body temperature (degree change) against the average for each rabbit. The dashed line represents data from animals infected with vMyx135KO and the solid line represents vMyxgfp-infected animals.](http://jvi.asm.org/)

<table>
<thead>
<tr>
<th>Clinical sign</th>
<th>vMyxgfp (4 animals)*</th>
<th>vMyx135KO (6 animals)</th>
<th>vMyx135REV (3 animals)</th>
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<td>Inoculation site</td>
<td>2; red visible slightly raised</td>
<td>4; 11- to 16-mm red, raised, center</td>
<td>3; small red lump, slightly raised</td>
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<tr>
<td>Satellites</td>
<td>4</td>
<td>6; just beginning, over course of infection very few observed</td>
<td>6; 5–10 visible, increasing to 30–40 visible by day 8</td>
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<tr>
<td>Conjunctival inflammation</td>
<td>None observed</td>
<td>9; 1 rabbit had discharge from eye</td>
<td>None observed</td>
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<td>7; redness, swelling</td>
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</tr>
<tr>
<td>Secondary lesions</td>
<td>6–7; first around eyes, then ears</td>
<td>7; few small red spots, not yet lesions, around ears, eyes</td>
<td>6; first observed as red areas on eyelids, clearly lesions by day 7</td>
</tr>
<tr>
<td>Respiratory difficulty</td>
<td>Little or none</td>
<td>Little or none</td>
<td>Little or none</td>
</tr>
<tr>
<td>Lesion regression</td>
<td>2 animals euthanized day 9</td>
<td>11; black, scabby 25-mm satellites losing color and becoming scabber</td>
<td>10; 3 animals euthanized due to severity of symptoms</td>
</tr>
</tbody>
</table>

* A fourth group of animals injected with the untagged wild-type virus (vMyxLau) exhibited a pathology identical to that of animals injected with vMyxgfp, and so this group was not included.
To confirm that our sample of rabbit type I IFN exhibited appropriate antiviral activity, we tested its ability to block replication of the type I IFN-sensitive VSV. pREFs were infected with VSV, vMyxgfp, or vMyx135KO at an MOI of 0.01. Following a 1-hour adsorption of the virus, the monolayer was washed, and then complete medium that contained 1,000 U of type I rabbit IFN, 1,000 U of human IFN αA/D, or no IFN was added. The addition of either type I IFN completely blocked VSV replication (Fig. 7B). This suggested that the rabbit type I IFN was biologically active. In contrast to the observation with VSV, the addition of either rabbit IFN or human IFN-αA/D had no effect on the number or appearance of the vMyxgfp or vMyx135KO foci (Fig. 7B). In an attempt to characterize the various viruses’ response to the dose of type I IFN (Fig. 7C), we infected pREFs with VSV, vMyxgfp, or vMyx135KO at an MOI of 0.01 and then supplemented the growth medium with no IFN or with 1, 10, or 100 U of rabbit type I IFN. VSV was sensitive to the addition of even 1 U of rabbit type I IFN and was completely blocked by the addition of 10 U (Fig. 7C). Neither vMyxgfp nor vMyx135KO was sensitive to the addition of rabbit type I IFN (Fig. 7C). Finally, we tested the abilities of these viruses to replicate at various MOIs following the addition of 100 U of rabbit type I IFN (Fig. 7D). The IFN-sensitive VSV was unable to replicate in medium containing 100 U of type I IFN. This is consistent with what we observed, as shown in Fig. 7B and C. However, neither MV nor 135KO exhibited any sensitivity in this assay to the addition of exogenous rabbit type I IFN (Fig. 7D).

Based on these observations, we conclude that MV must potently inhibit type I IFN, but this does not appear to be the result of the M135R gene product. The direct target of M135R in MV virulence has yet to be determined; however, the fact that M135R shares similarity to the amino half of VV B18R suggests that VV B18R may have a dual function in which the carboxy half of the protein acts to bind type I IFN and that the amino terminus has evolved to recognize distinct target(s).
**DISCUSSION**

We have demonstrated that M135R is a critical virulence factor for full-blown myxomatosis in O. cuniculus. The deletion of M135R results in a highly attenuated MV that nevertheless propagates with wild-type efficiency in vitro. The features of M135R biology include expression as an early gene product, and there is also evidence of N-linked glycosylation. M135R encodes a signal sequence and a C-terminal transmembrane domain, suggesting the ability to enter the endoplasmic reticulum and be transported to the cell surface. Immunofluorescence microscopy and biotinylation of surface proteins indicate that M135R is transited to the cell surface and that its biological action occurs at the surface of infected cells. When the C-terminal transmembrane domain was removed, expressed M135ΔTM was efficiently secreted from the baculovirus expression system. Infection of rabbits with rMx135KO resulted in attenuated myxomatosis, as exhibited by reduced viral spread and full recovery of the infected animals, which contrasted to what was seen for animals infected with the wild-type MV.

M135R was predicted to bind and inhibit type I IFN in a manner similar to the vaccinia ortholog B18R (6, 9). The IFN arm of the mammalian host immune system’s antiviral strategy is a well-tuned and efficient inhibitor of virus infection (12), so it is not surprising that many viruses have evolved strategies to subvert or inactivate this cytokine defense pathway. For example, pseudorabies virus can disarm the infected host’s IFN-β response by blocking the phosphorylation of STAT1 (7). The influenza A virus NS1 protein blocks innate immunity by preventing type I IFN release from infected cells (11). When viruses are unable to breach the IFN response, they can be rendered unable to productively infect a host. For example, MV is unable to infect primary murine embryo fibroblast (pMEF) cells (30). Following MV infection of pMEFs, a signaling cascade is initiated that results in the activation of extracellular signal-regulated kinase and IRF3, followed by the induction of type I IFN and thus a block to productive MV infection. When wild-type mice are infected with MV, replication is highly restricted and all the mice survive, whereas MV injection into STAT1 knockout mice, where the IFN signal transduction is blocked, results in all the mice dying from the MV infection (30).

Although MV was predicted to encode a type I IFN binding protein (6, 9), it is not surprising that MV was unable to block the murine IFN response initiated in pMEFs because it is entirely possible that MV inhibition of type I IFN is species specific. Several MV characterized binding proteins, including type II viIFN-γ-R (M-T7) and vTNF-R (M-T2), have been shown to be rabbit specific (18, 23). In contrast, many VV immunomodulatory proteins exhibit broad species specificity (1). For example, VV B18R has a high affinity for human IFN-α and also binds to rabbit, bovine, rat, and mouse type I IFNs (25). Although most strains of VV express a homolog of B18R, VV strain Wyeth expresses a truncated B18R protein lacking the C-terminal immunoglobulin domain (3). In 125I-IFN binding studies, the truncated B18R ORF from VV Wyeth had a much reduced ability to bind type I IFN, exhibiting only 30% of the total binding of 125I-IFN measured for most other orthopoxviruses (3). This observation suggests that the carboxy terminus is critical for VV B18R type I IFN binding. Therefore, it is not entirely surprising that M135R, which contains only the amino-terminal half of the B18R ortholog and a larger truncation of the carboxy terminus than VV Wyeth B18R, would perform a critical function in pathogenesis that does not involve the inactivation of rabbit type I IFN signaling.

Of the poxvirus genomes sequenced to date, a homolog of VV B18R, predicted to act as a type I IFN binding protein, has been identified in members of the ortho-, sui-, and capripoxviruses and is also encoded by Yaba-like disease virus (YLDV 136), but not Yaba monkey tumor virus (24), of the yatapoxviruses. Here we have characterized the M135R version identified in MV as a putative ortholog of VV B18R. M135R is half the size of the ortholog encoded by other poxvirus members (Table 1), and the other sequenced member of the leporipoxviruses, Shope fibroma virus, has a fragmented version of even this truncated open reading frame (31). The observations that members of the para- and molluscipoxviruses do not encode an obvious functional inhibitor of type I IFNs and that the leporipoxviruses encode truncated, non-IFN-related ORFs suggest that each has evolved to block type I IFN in a manner that has diverged from that observed for other poxvirus members. In the case of MV, virus replication is completely resistant to interferon in rabbit cells, possibly due to the effects of intracellular inhibitors of interferon signaling. Thus, the inability of M135 to interact with rabbit type I interferon, or perturb the febrile effects associated with IL-1β, suggests that additional host immune targets for this class of poxvirus immunomodulators remain to be uncovered.

**ACKNOWLEDGMENTS**

We acknowledge the help of the animal care and veterinary staff of the University of Western Ontario, in particular Tracy Hill, Charla Thompson, Heather Cadieux, and Gail Jones, during this study. This work was supported by the CIHR. G.M. holds a Canada Research Chair in Molecular Virology and is an International Scholar of the Howard Hughes Medical Institute.

**REFERENCES**