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Myxoma virus M130R is a novel virulence factor required for lethal myxomatosis in rabbits

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ABSTRACT

Myxoma virus (MV) is a highly lethal, rabbit-specific poxvirus that induces a disease called myxomatosis in European rabbits. In an effort to understand the function of predicted immunomodulatory genes we have deleted various viral genes from MV and tested the ability of these knockout viruses to induce lethal myxomatosis. MV encodes a unique 15 kD cytoplasmic protein (M130R) that is expressed late (12 h post infection) during infection. M130R is a non-essential gene for MV replication in rabbit, monkey or human cell lines. Construction of a targeted gene knockout virus (vMyx130KO) and infection of susceptible rabbits demonstrate that the M130R knockout virus is attenuated and that loss of M130R expression allows the rabbit host immune system to effectively respond to and control the lethal effects of MV. M130R expression is a bona fide poxviral virulence factor necessary for full and lethal development of myxomatosis.

1. Introduction

Myxoma virus (MV) is a rabbit-specific poxvirus historically endemic to South America and South-Western North America but now found on all continents except Antarctica (Barrett and McFadden, 2007). MV produces a small, localized lesion in infected native rabbits of the Americas (species of the *Sylvilagus* genus) but MV infection is lethal in the European rabbit (*Oryctolagus cuniculus*). MV-infected *O. cuniculus* display a dramatic pathology that includes the production of a large, primary lesion from which replicating MV spreads to secondary sites including the host extremities (ears, paws) and mucosal orifices (nose, eyes, ano-gential) leading to a rapid dissemination of invading micro-flora and -fauna, creating respiratory problems and finally death.

Poxvirus infection involves a regulated cascade of early, intermediate and late viral gene expression that is required for a productive viral replication cycle to occur. Early gene expression occurs within the first few hours of infection and follows particle entry and uncoating within an infected cell (Moss, 2001). Viral DNA replication and intermediate gene expression are necessary for late gene expression to be initiated. Late gene expression occurs several hours following early gene expression and late gene products continue to accumulate up to 48 h post infection (hpi) (Moss, 2001).

The severity of disease, and strict species specificity of MV-induced myxomatosis in European rabbits, permits genetic analysis of individual viral open reading frames through site-directed deletion of targeted genes and monitoring the development and/or attenuation of myxomatosis (Johnston and McFadden, 2004). Disruption of immunomodulatory viral genes often result in a distinct MV-induced disease phenotype in European rabbits. As well, many knockout viruses exhibit restricted replication kinetics in cells of hematologic origin (e.g. rabbit T-lymphocyte (RL5)) but not in cells of epithelial origin (rabbit kidney (RK13))(Werden et al., 2008). Such *in vitro* host range functions can be a useful measure of gene function, and such mutants have proven to be an invaluable tool in the study of poxvirus tropism (McFadden, 2005).

During the sequencing of the myxoma virus genome we identified the small ORF M130R as exhibiting some sequence similarity to the human immunodeficiency virus trans-activating protein, Tat

Abbreviations: h, hour; hpi, hours post infection; EGFP, enhanced green fluorescent protein; MV, myxoma virus; vMyxLau, myxoma virus, strain Lausanne; vMyxgfp, myxoma virus expressing EGFP; vMyxlac, myxoma virus expressing β-galactosidase; GTPV, goatpox virus; SPPV, sheeppox virus; SPV, swinepox virus; BGMK, baby green monkey kidney; RK13, rabbit kidney 13; ATCC, American type culture collection; DMEM, Dulbecco's modified eagle media; FBS, fetal bovine serum; AraC, arabinosyl cytosine; DAPI, 4′,6-diamidino-2-phenylindole; SFM, serum free media.

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(Cameron et al., 1999). To study if there was any biological relevance to this sequence similarity, we first investigated the role of M130R in MV biology. Here we report the analysis of M130R and its role as a viral virulence factor in rabbit pathogenesis.

2. Methods

2.1. Cells and viruses

BGMK, Vero, HEK293, CCD922Sk and RK13 cells were maintained in DMEM and RL5 cells were maintained in RPMI 1640 media. All media was supplemented with 10% fetal bovine serum (heat inactivated), 100 U penicillin/ml and 100 μg/ml streptomycin. vMyxLau is the wildtype strain Lausanne of myxoma virus from ATCC. vMyxgfp is the wildtype control that expresses EGFP under the poxviral synthetic early/late promoter (Chakrabarti et al., 1997) and has been described previously (Johnston et al., 2003). vMyxlac is the wildtype control that expresses β-galactosidase under the poxviral late promoter (Opgenorth et al., 1992).

2.2. Construction of knockout and revertant viruses

The M130R knockout virus (vMyx130KO) was constructed by amplifying regions flanking the M130R ORF. The left flank (555 bp) was amplified with primers JB24.03 5′-GCGGGATCCATGGCGGACG-CCGTTCAAG-3′ (BamHI underlined) and JB25.03 5′-GCGCTGCAG ATCTGTAAGAGGTCCCCCCT-3′ (Pst1 underlined). The right flank (609 bp) was amplified with primers JB26.03 5′-GCGGAATTC AAGCTTGCAACGTAAGTTAACG-3′ (EcoRI underlined) and JB27.03 5′-GCGAAGCTTTTAAGCAACTCCTATAATC-3′ (HindIII underlined) using MV genomic DNA as template. The PCR products, representing the flanking regions, were digested with BamHI and PstI (left flank) or EcoRI and HindIII (right flank), gel purified (Qiagen) and cloned directly, but sequentially into the corresponding restriction sites in pBluescript containing EGFP cassette under the poxviral synthetic early/late promoter. This transfer vector was identified as pBS:130KOgfp. BGMK cells were infected with vMyxLau at an moi of 0.1. One hour post infection the transfer plasmid (pBS:130KOgfp) was transfected (LipoTaxi, Stratagene) into the infected BGMK cells. Recombinant virus was selected by multiple rounds of focus purification of EGFP expressing recombinants.

To construct the revertant virus (vMyx130Rev) a 1.4 kb fragment, containing the complete ORFs M129R-M131R, was amplified using primers JB24.03 and JB27.03 and directly cloned into pCRII using a TOPO cloning reaction (Invitrogen). This plasmid was transfected into BGMK cells that had previously been infected with vMyx130KO virus at an moi of 0.1. Non-coloured (white foci) revertant virus foci were selected and after several rounds of purification the revertant virus (vMyx130Rev) was amplified and tested in animals.

2.3. Growth curves

Viral replication kinetics were determined by single step growth curves. CCD922Sk (human skin fibroblast), BGMK (monkey kidney), RL5 (rabbit T-lymphocyte) or RK13 (rabbit kidney) cells were infected at a high moi (moi = 10) and infected cells were collected at various times post infection. Infectious virus was titrated onto BGMK monolayers and florescent foci were enumerated 48 hpi.

2.4. Generation of polyclonal anti-M130R anti-serum

The 15-residue peptide (KPGDERKTTTEDGPPTK) selected for synthesis (Invitrogen) was located within a hydrophilic carboxy terminal region. A C-terminal cysteine was added to the peptide. This terminal cysteine permitted crosslinking of maleimide-activated mariculture keyhole limpet hemocyanin (mcKLH) to the peptide using the Imject maleimide-activated EDC conjugation kit (Pierce) according to the manufacturer's instructions. The initial injection of antigen into New Zealand white rabbits included the antigen suspended in Freund's complete adjuvant (Sigma) followed by 4 boosts in Freund's incomplete adjuvant (Sigma). Polyclonal anti-serum was confirmed by Western blot analysis of BGMK infected lysates. The generation and characterization of the M135R antibody has been described previously (Barrett et al., 2007b).

RK13 cells were seeded into 12 well dishes and infected with vMyxgfp at a moi of 0.1 or 10 with or without the addition of arabinosyl cytosine (AraC) at 40 μg/ml for 24 h. Following one-hour adsorption the cell monolayers were washed with serum free media (SFM). Infected monolayers were maintained in 0.5 ml of SFM. Supernatants and cells were collected at the time indicated. Supernatants were concentrated 10 fold using Nanosep columns with a 3K cutoff (Pall). Whole cell lysates were prepared and then lysates and concentrated supernatants were separated on 15% SDS-PAGE.

To test for N-linked glycosylation RK13 monolayers were infected with vMyxgfp at a moi of 5. One hour post adsorption infected cells were left untreated or were treated with tunicamycin (1 μg/ml) for 16 h before the infected monolayers were collected and processed as above. Following immunoblotting with anti-M130R, the membrane was stripped and immunoblotted again with anti-M135R.

2.5. Animal studies

Specific pathogen free (SPF) male New Zealand white rabbits (*O. cuniculus*) were housed in the Level II biocontainment unit at the University of Western Ontario as per Health Canada and the Canadian Food Inspection Agency (CFIA) requirements. Each rabbit was injected intradermally with 1000 pfu of the appropriate virus in each hind flank. The rabbits were monitored daily by research staff for disease progression. A scoring system of clinical signs of disease, including appearance and size of primary, secondary and satellite lesions, activity levels, food and water consumption and respiration were evaluated daily (Cameron et al., 2005a). Veterinarians were informed when an animal recorded a score of 15, and animals were monitored twice daily after that point. Animals were euthanized if scores of 20 were obtained. Animals injected with vMyx130KO were followed for 18 days and were challenged with vMyxLau and monitored for a further 14 days. Animals that had to be sacrificed were injected with euthanyl intravenously following anesthesia.

2.6. Microscopy

COS7 cells were seeded into 35 mm glass-bottom dishes (MatTek Corporation). Full length M130R (minus the stop codon) was cloned into the HindIII/XhoI sites of pcDNA3.1 myc/his (Invitrogen) so that M130R was fused to the myc/his (pcDNA3.1 M130Rmyc/his) epitope tags. Plasmids expressing an endoplasmic reticulum resident protein that expressed red fluorescent protein (pER-RFP) and a plasmid expressing β1, 4-galactosyltransferase fused to cyan fluorescent protein (pGolgi-CFP) were used to visualize the ER and golgi respectively, (McKillop et al., 2009). Cell monolayers were left uninfected or were infected with myxoma virus and then transfected with various combinations of plasmids using Lipofectamine 2000 (Invitrogen). Following a one-hour virus adsorption the monolayers were washed with complete media and infection was permitted to proceed for 24 h. Cells were stained and visualized as described previously (Wang et al., 2008).