Myxoma virus M156 is a specific inhibitor of rabbit PKR but contains a loss-of-function mutation in Australian virus isolates

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Myxoma virus (MYXV) is a rabbit-specific poxvirus, which is highly virulent in European rabbits. The attenuation of MYXV and the increased resistance of rabbits following the release of MYXV in Australia is one of the best-documented examples of host–pathogen coevolution. To elucidate the molecular mechanisms that contribute to the restriction of MYXV infection to rabbits and MYXV attenuation in the field, we have studied the interaction of the MYXV protein M156 with the host antiviral protein kinase R (PKR). In yeast and cell-culture transfection assays, M156 only inhibited rabbit PKR but not PKR from other tested mammalian species. Infection assays with human HeLa PKR knock-down cells, which were stably transfected with human or rabbit PKR, revealed that only human but not rabbit PKR was able to restrict MYXV infection, whereas both PKRs were able to restrict replication of a vaccinia virus (VACV) strain that lacks the PKR inhibitors E3 and K3. Inactivation of M156R led to MYXV virus attenuation in rabbit cells, which was rescued by the ectopic expression of VACV E3 and K3. We further show that a mutation in the M156 encoding gene that was identified in more than 50% of MYXV field isolates from Australia resulted in an M156 variant that lost its ability to inhibit rabbit PKR and led to virus attenuation. The species-specific inhibition of rabbit PKR by M156 and the M156 loss-of-function in Australian MYXV field isolates might thus contribute to the species specificity of MYXV and to the attenuation in the field, respectively.

poxvirus | myxoma virus | PKR | translational regulation | host–pathogen interaction

Poxviruses are large double-stranded DNA viruses that exclusively replicate in the cytoplasm of infected cells. Members of the *Poxviridae* family can productively infect a wide variety of animal hosts. Interestingly, the binding and entry of poxviruses into cells is largely independent of host species after which virus replication is initiated. The successful completion of virus replication, however, depends on the effective subversion of the host cell's innate immune responses (1). Even closely related poxviruses can exhibit drastic differences in their host ranges. Whereas some poxviruses have only one host species, such as variola virus, the causative agent of smallpox, which is restricted to humans, others, such as cowpox and monkeypox viruses, can infect many different species and thus display very broad host ranges (2). A number of poxvirus genes have been discovered that influence the host range and cell tropism of poxviruses and have therefore been termed “host range genes” (3, 4). Although the molecular mechanisms responsible for their host range functions have not been elucidated in detail, it is clear that most poxviral host range proteins interact with components of the host immune system and that host species-specific interactions likely play a major role.

Myxoma virus (MYXV) is a poxvirus that belongs to the genus *leporipoxvirus* and shows a restricted host range infecting only leporids (rabbits and hares). MYXV is highly lethal to European (E.) rabbits causing case fatality rates (CFRs) of close to 100%. Since 1950, MYXV was repeatedly introduced into Australia to combat the invasive feral E. rabbit population, which has caused

ecological and economical havoc. Shortly after the release of the MYXV standard laboratory strain (SLS), which caused a CFR of 99.8% in laboratory rabbits (grade 1 virulence), attenuated virus strains began to appear in the wild and started to outcompete the more virulent parental strain. The predominant strains found in the field are of grade 3 and grade 4 virulence and exhibit a CFR in laboratory rabbits between 70–95% and 50–70%, respectively. Concomitantly, rabbits evolved increased resistance to MYXV infection. Evolution of attenuated MYXV and increased resistance of rabbits to infection were also observed after the illegal release of MYXV in Europe (reviewed in ref. 5). The molecular mechanisms of the attenuation of MYXV and the increased resistance of rabbits to infection are unknown. Recently, the complete genomes of 24 MYXV strains that were collected in the field in Australia were reported. Although a number of mutations were discovered, it was not immediately clear which mutations led to changes in MYXV virulence (6, 7). Two candidate genes that might contribute to changes in virulence are *M029L* and *M156R*, the MYXV orthologs of vaccinia virus (VACV) *E3L* and *K3L*, respectively. VACV *E3L* and *K3L* are virulence and host range genes, and their protein products E3 and K3 inhibit the activation and activity of PKR (8, 9). PKR is an antiviral protein that is found in most vertebrates. It is constitutively expressed at moderate levels and can be induced by type I interferons. PKR is composed of two N-terminal double-stranded RNA (dsRNA) binding domains that sense viral dsRNA, and a C-terminal kinase domain. Upon binding to dsRNA, two inactive PKR monomers dimerize and undergo autophosphorylation.

Significance

The virulence and host range of viruses is controlled by the interaction of the host innate immune system with viral molecules. This interaction is an important driver for the evolution of both the host and the virus. The attenuation of myxoma virus, a rabbit-specific poxvirus, after its deliberate release to control European rabbit populations, and the increased resistance of the rabbits, is one of the best-known examples for host–virus coevolution on the population level. We show that the myxoma virus protein M156 specifically inhibited the antiviral protein kinase R (PKR) from rabbits but not PKR from other mammals, that PKR inhibition correlated with virus replication during infection, and that M156 contains a loss-of-function mutation in Australian field isolates.

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Activated PKR subsequently phosphorylates the alpha subunit of eukaryotic translation initiation factor 2 (eIF2), which leads to the general suppression of protein translation and inhibition of virus replication. During vertebrate evolution PKR has evolved rapidly, likely as a consequence of positive selective pressure exerted by viral PKR antagonists. We and others previously showed that VACV K3 inhibits PKR in a species-specific manner; e.g., whereas mouse PKR was sensitive to K3 inhibition, human PKR was largely resistant (10, 11). MYXV 156 is a homolog of eIF2 α and was previously tested for its ability to inhibit human PKR in a heterologous yeast assay in which it showed no inhibition of human PKR activity (12). Here we explored the hypothesis that *M156R* evolved to inhibit rabbit PKR and that species-specific inhibition of PKR contributes to the restricted host range of MYXV to rabbits. We further tested whether variations found in MYXV field isolates affected the inhibitory potential of M156 and M029 against rabbit PKR.

Results

Predominant Expression of a Short M156 Form. *M156R* is located at the 3' end of the genome and partly overlaps with the inverted terminal repeat (ITR) region in the reference Lausanne (Lu) strain (13). Among all known poxvirus K3 orthologs, M156 is unique, because it contains a predicted N-terminal extension, based on an elongated ORF (open reading frame) (Fig. 1*A* and Fig. S1*A*). In MYXV strains that descended from South America, *M156R* is annotated to encode a 102-amino acid protein with a predicted molecular mass of 12 kDa (13). The M156 orthologs of the closest relatives, rabbit fibroma virus and the Californian MYXV MSW strain, lack this putative extension, but contain a putative start codon

that encodes for 78- and 77-aa-long ORFs, respectively, with predicted molecular masses of 9 kDa (Fig. S1*A*) (14, 15). A putative start codon at the corresponding position is also found in the South American-derived MYXV strains. In the solution structure of M156, for which the long *M156R* ORF was used, the first 32 amino acids were unstructured (12). A predicted poxvirus early promoter motif is absent in the 500 bp 5' to the first start codon, but is present at nucleotide position -42 relative to the second predicted start codon (Fig. S1*B*). To determine the authentic ORF of *M156R*, we performed rapid amplification of cDNA ends (RACE) PCR to determine the transcriptional start sites (TSSs) using total RNA from MYXV-Lu-infected RK13 cells 4, 12, or 24 h postinfection (hpi). Prominent bands were observed at about 120 bp at all time points but not in the uninfected control (Fig. S1*C*). PCR products were randomly cloned and sequenced. TSSs of 17 out of 18 clones started within the extended ORF, with the majority (82%) of sequences starting at positions -15 to -7 relative to the second start codon (Figs. S1*B* and S2). None of the discovered TSSs are predicted to lead to the translation of the long M156 isoform. To analyze the expression pattern of M156 and compare its molecular mass to that of transfected M156, we performed Western blot analyses of RK13 cells infected with Lu MYXV at various time points (Fig. S1*D*). M156 expression was detected as early as 1 hpi and was most strongly expressed after 4–24 hpi and thus showed comparable expression to M-T7, an early expressed MYXV protein. In contrast, expression of the late protein M130 was not observed until 8 hpi. We also transfected RK13 cells with plasmids encoding the short or long M156 isoforms and analyzed their expression using anti-M156 serum. The short M156 isoform (Fig. S1*D*, lane 7) was detected at a comparable size as M156 in MYXV-infected cells. We were unable to detect the expression of the long M156 isoform under any conditions using our M156 antiserum. In conclusion, our data indicate that the short form of M156 is the predominant isoform in the tested cells, and we therefore used this version in subsequent experiments. To avoid confusion with *M156R* annotations of MYXV genomes, we kept the numbering of the long isoform when referring to specific amino acids.

Host-Specific PKR Inhibition by M156. Yeast-based assays have been previously used to analyze interactions of PKR and viral inhibitors (10, 11, 16–18). In one of these assays, M156 failed to inhibit human PKR, whereas its VACV ortholog K3 showed PKR inhibition (12). We hypothesized that *M156R* evolved to inhibit rabbit PKR. To test this hypothesis, we generated isogenic yeast strains that were stably transformed with empty vector (control), human PKR, or E. rabbit PKR under the control of a galactose-inducible promoter (18). These strains were subsequently transformed with plasmids encoding VACV K3, MYXV M156, or empty vector (control), which are also under the control of the galactose-inducible promoter. Under inducing conditions (galactose-containing agar plates), all transformants of the control strain expressing no PKR showed comparable growth, indicating that K3 or M156 alone had no effect on yeast growth (Fig. 1*B*). Induction of both human and E. rabbit PKR expression was toxic in the vector-transformed strains, whereas this toxicity was suppressed by K3 (Fig. 1*C* and *D*). Expression of M156 had no effect on human PKR toxicity, whereas it reduced toxicity of E. rabbit PKR, as indicated by no growth and growth, respectively (Fig. 1*C* and *D*). The transformants were also grown in liquid media to measure eIF2 α phosphorylation levels 4 h after galactose induction by Western blot analysis. No eIF2 α phosphorylation was observed in the absence of PKR (Fig. 1*E*). Both human and E. rabbit PKR induced eIF2 α phosphorylation, which was reduced by the expression of K3 for both PKR. Expression of M156 only inhibited eIF2 α phosphorylation induced by E. rabbit PKR, but not that mediated by human PKR. These results demonstrate that M156 is an inhibitor of E. rabbit PKR but not of human PKR in

Fig. 1. M156 inhibits rabbit but not human PKR in yeast. (A) Multiple sequence alignment of MYXV-Lu M156, VACV K3, and human (h) eIF2 α . Conserved residues are highlighted in yellow (100% conservation) or purple (identical with M156). The methionine encoded by the putative start codon of the predominant M156 isoform is shown in red. An asterisk indicates L98 in MYXV-Lu M156. Plasmids encoding VACV K3, MYXV M156, or empty vector under the control of a yeast GAL-CYC1 hybrid promoter were transformed into isogenic yeast strains, which have either empty vector (control) (B), human PKR (C), or E. rabbit PKR (D) stably integrated at the LEU2 locus under the control of the GAL-CYC1 promoter. Transformants were colony purified and grown under inducing conditions at 30 °C for 4 d. Results shown are representative of four independent transformants for each plasmid. (E) Transformants described above were grown in liquid SC-Gal medium for 4 h to induce expression. Whole cell protein extracts were obtained from equal numbers of cells and subjected to Western blot analyses. The blots were probed with phospho-specific antibodies against Ser51 eIF2 α (eIF2 α -P), then stripped and probed with polyclonal antiserum against total eIF2 α .

Fig. 3. Human PKR but not rabbit PKR suppresses MYXV replication. (A) Control and congenic HeLa-PKR^{kd} HeLa cell lines that were stably transfected with vector, human PKR, or E. rabbit PKR were infected with VC-R2 [VACVΔE3L(EGFP-d2)/ΔK3L] or MYXV-EGFP at a MOI of 0.1 or 1, respectively. Representative images taken 48 hpi at 100× magnification are shown. EGFP levels in the two viruses cannot be compared directly, because in VC-R2, a destabilized EGFPd2 is driven by the natural E3L promoter, whereas in MYXV, unmodified EGFP is driven by a synthetic early-late poxvirus promoter. (B) Indicated HeLa cell lines were infected with VC-R2 or MYXV-EGFP at a MOI of 0.1 or 1, respectively. Viruses were collected at 0, 12, 24, 48, and 72 hpi and titered on RK13 cells, which stably express VACV E3 and K3. (C) Total protein lysates were collected from mock (–), VC-R2, or MYXV-EGFP-infected HeLa cells at 8 hpi to measure phosphorylated and total eIF2α by Western blot analyses. Lysates shown were run on the same gel in a different order and lanes were reordered for clarity (Fig. S7 shows original).

cells infected with MYXV-M029L^{KO}, which indicates that M029 might be a negative regulator of some MYXV proteins' expression.

Loss-of-Function Mutation in M156R of Australian MYXV Isolates. Despite the availability of the full genome sequences of 24 MYXV isolates from Australia, the molecular mechanisms that underlie the attenuation of MYXV strains in the field are currently unclear. One of the identified mutations leads to a predicted leucine-to-proline amino acid substitution at position 98 (L98P) in M156R in 13 of 24 isolates (7). The corresponding leucine residue in the costructure of eIF2α with human PKR (22) is shown in Fig. 5A. We introduced this mutation into M156-encoding plasmids using site-directed mutagenesis and tested its effect on E. rabbit PKR in yeast and cell culture assays. The mutation had no effect on yeast growth in a control strain, but abolished the M156-mediated rescue of yeast growth in a strain expressing rabbit PKR (Fig. 5B and C). In correlation with this result, M156-L98P was unable to inhibit M156-mediated inhibition of eIF2α phosphorylation in yeast grown in liquid medium (Fig. 5D). The inability of M156-L98P to inhibit PKR was also confirmed in the cell-culture-based transfection assays, in which even the highest amounts of M156-L98P had no effect on PKR activity (Fig. 5E). In transfected cells, both

M156 and M156-L98P were expressed at comparable levels as determined by Western blot analysis and the loss of PKR inhibition was therefore not due to altered M156 expression (Fig. 5E). A predicted alanine-to-valine substitution at position 17 in M029, which was present in 11 of 24 isolates (7), did not result in altered PKR inhibition (Fig. S6). To test the effect of M156-L98P in MYXV infection, we inserted it or wild-type M156R (revertant) into MYXV-M029L^{KO}M156R^{KO} and infected RK13 cells with the viruses. M156 was able to restore EGFP expression, whereas M156-L98P could not (Fig. 5F). EGFP expression correlated well with virus replication, which was restored by M156 but not by M156-L98P (Fig. 5G), confirming that the latter is also a loss-of-function mutation in the context of MYXV infection. The loss of M156 function in some MYXV isolates is thus a good candidate for a contributing factor to the virulence attenuation of MYXV in Australia.

Discussion

We have shown here that M156 of MYXV is a species-specific inhibitor of rabbit PKR, that human PKR but not rabbit PKR can restrict MYXV replication in congenic HeLa cells, and that M156 deficiency leads to virus attenuation in RK13 cells. The extent of species-specific PKR inhibition by M156 is remarkable, considering its likely mode of action as a pseudosubstrate inhibitor (23). Poxvirus K3 homologs share the common S1 fold with eIF2α, the substrate of PKR and they all likely bind to the same region of PKR. In contrast to the poxvirus K3 members, which can share as little as 20% sequence identity with each other (10), eIF2α is highly conserved, e.g., 100% sequence identity between human and rabbits in the S1 domain. The finding that PKR has been evolving rapidly can be explained by the high selective pressure

Fig. 4. Deletion of M156R further attenuates MYXV that lacks M029L. RK13 cells (RK13-WT) and RK13 cells stably expressing E3 and K3 (RK13+E3L+K3L) were infected with MYXV-EGFP (WT), MYXV-M029L^{KO}, or MYXV-M029L^{KO}M156R^{KO} at a MOI of 5. (A) Fluorescent images were taken at 48 hpi at 100× magnification. (B) Viruses were collected at 0, 12, 24, and 48 hpi and titered on RK13+E3L+K3L cells. Error bars indicate SD (n = 2). (C) Total protein lysates were collected at 4 and 24 hpi to monitor expression of MYXV early (M156 and M-T7) and late (M130) proteins. β-Actin was included as loading control.

host restriction of MYXV infection to only rabbits, the prevention of productive infection in nonrabbit species (particularly humans), and the attenuation of MYXV strains currently extant in Australia. Moreover, this study shows that the choice of biologically meaningful host–virus systems is important for studying host–virus interactions. Often, studies of viral molecules or viruses are performed with molecules, cell lines, or animals of nonhost species. The results of such experiments could be misleading, if the virus molecules act in a species-specific manner as shown here for M156 and rabbit PKR.

Materials and Methods

Plasmids, Yeast Strains, and Cell Lines. PKR and viral genes were cloned into pSG5 for expression in mammalian cells. Generation of yeast strains, stably transformed with empty vector (J673) or human PKR (J983) under the control of a yeast GAL-CYC1 hybrid promoter, was described (18). A yeast strain stably transformed with E. rabbit PKR (O8) was generated using the same methods. VACV K3L and M156R were cloned into the vector pYX113, which contains the GAL-CYC1 hybrid promoter. HeLa-PKR^{kd} cells (24), kindly provided by Charles Samuel, were stably transfected with knock-down resistant human PKR and E. rabbit PKR under the control of the human PKR promoter (19). The generation of RK13+E3L+K3L cells was described (21). See *SI Materials and Methods* for details.

RACE PCR. E. rabbit RK13 cells were infected with MYXV-EGFP at a multiplicity of infection (MOI) of 1. Total RNA was collected with TRIzol reagent at 4, 12, and 24 h postinfection. RACE PCR was performed with the GeneRacer Core Kit (Invitrogen) according to the manufacturer's instruction. See *SI Materials and Methods* for details.

Yeast Growth and eIF2 α Phosphorylation Assays. Experiments were performed as previously described (18, 25). See *SI Materials and Methods* for details.

Luciferase Assay. The 5×10^4 HeLa-PKR^{kd} cells were seeded in 24-well plates 1 day before transfection. For each transfection, firefly luciferase (pGL3

promoter, 0.05 μ g, Promega), and pSG5 plasmids encoding PKR (0.2 μ g), M156, or M029 (0.4 μ g) were transfected using GenJet-HeLa (Signagen). For titration experiments, amounts of transfected plasmids are indicated in the figures. For controls, empty pSG5 vector was transfected using the same amount. Each transfection was conducted in triplicate. After 48 h, cell lysates were harvested using mammalian lysis buffer (Goldbio) and luciferase activity was determined using luciferase detection reagents (Promega) in a luminometer (Berthold).

Construction of Recombinant Viruses. The construction of VC-R2 and MYXV-EGFP were described previously (20, 26) and the construction of M156R mutant viruses is illustrated in Fig. S5 and described in *SI Materials and Methods*.

Infection Assays. HeLa-control, HeLa-PKR^{kd}, HeLa-PKR^{kd+vector}, HeLa-PKR^{kd+hPKR}, and HeLa-PKR^{kd+E. rabbit PKR} cells were seeded into six-well plates and confluent monolayers (1×10^6 cells) were mock infected with PBS or infected with VC-R2 and MYXV-EGFP at an MOI of 0.1 and 1, respectively. RK13 cells were infected with MYXV-EGFP or MYXV-EGFP-derived recombinant viruses at an MOI of 5. Fluorescent pictures were taken with an inverted fluorescent microscope (Leica) and viruses were collected at 0, 24, 48, and 72 hpi for titration in RK13 cells that stably express E3 and K3. See *SI Materials and Methods* for details.

Western Blot Analyses. Protein lysates from RK13 cells that were infected with MYXV-EGFP (MOI = 10) were blotted on PVDF membranes and incubated with anti-M156 (27), and after stripping, reprobed with antiserum against M-T7 (28), M130 (29), and anti- β -actin. For eIF2 α phosphorylation assays, PKR-expressing cells were infected with VC-R2 or MYXV-EGFP at an MOI of 5. Total protein lysates were collected 8 hpi and eIF2 α was detected with phospho-specific eIF2 α and total eIF2 α . See *SI Materials and Methods* for details.

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