ROBERT KOCH INSTITUT

Specific Inhibition of the PKR-Mediated Antiviral Response by the Murine Cytomegalovirus Proteins m142 and m143

Matthias Budt,[1] Lars Niederstadt,[1] Ralitsa S. Valchanova,[1] Stipan Jonjic,[2] and Wolfram Brune[1]

[1] Division of Viral Infections, Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany
[2] Department of Histology and Embryology, Faculty of Medicine, University of Rijeka, Brace Branchetta 20, 51000 Rijeka, Croatia

Abstract

Double-stranded RNA (dsRNA) produced during viral infection activates several cellular antiviral responses. Among the best characterized is the shutoff of protein synthesis mediated by the dsRNA-dependent protein kinase (PKR) and the oligoadenylate synthetase (OAS)/RNase L system. As viral replication depends on protein synthesis, many viruses have evolved mechanisms for counteracting the PKR and OAS/RNase L pathways. The murine cytomegalovirus (MCMV) proteins m142 and m143 have been characterized as dsRNA binding proteins that inhibit PKR activation, phosphorylation of the translation initiation factor eIF2α, and a subsequent protein synthesis shutoff. In the present study we analyzed the contribution of the PKR- and the OAS-dependent pathways to the control of MCMV replication in the absence or presence of m142 and m143. We show that the induction of eIF2α phosphorylation during infection with an m142- and m143-deficient MCMV is specifically mediated by PKR, not by the related eIF2α kinases PERK or GCN2. PKR antagonists of vaccinia virus (E3L) or herpes simplex virus (γ34.5) rescued the replication defect of anMCMVstrain with deletions of both m142 and m143. Moreover, m142 and m143 bound to each other and interacted with PKR. By contrast, an activation of the OAS/RNase L pathway by MCMV was not detected in the presence or absence of m142 and m143, suggesting that these viral proteins have little or no influence on this pathway. Consistently, an m142- and m143-deficient MCMV strain replicated to high titers in fibroblasts lacking PKR but did not replicate in cells lacking RNase L. Hence, the PKR-mediated antiviral response is responsible for the essentiality of m142 and m143.

The intrusion of an infectious agent is noticed by target cells through specific receptors that recognize pathogen-associated molecular patterns (32). These sensors trigger the induction of an antimicrobial response aimed at elimination of the pathogen. Many different structural features of microbes can activate such a response, among them virus-associated nucleic acids such as long double-stranded RNA (dsRNA), which is absent from uninfected cells (51). dsRNA not only constitutes the genetic material of dsRNA viruses but is also produced in infected cells by positive-strand RNA viruses and some DNA viruses, especially those with large genomes and genes arranged on both strands of the viral DNA genome (63).

Toll-like receptor 3 (TLR3) and the RNA helicases RIG-I and MDA5 serve as sensors for dsRNA. Upon activation, they induce signaling cascades culminating in the expression of type I interferons (IFNs) (58). These IFNs induce the expression of a plethora of antiviral genes, which can interfere with the viral replication cycle (54). The IFN-inducible gene products comprise the dsRNA-dependent protein kinase (PKR) and oligoadenylate synthetases (OAS). Both PKR and OAS are directly activated by dsRNA. Hence, dsRNA induces the expression of these antiviral effector proteins and is also necessary for their activation.

Upon binding to dsRNA, PKR dimerizes and undergoes autophosphorylation to gain full catalytic activity (30, 46, 59). Once activated, PKR phosphorylates the eukaryotic translation initiation factor eIF2α (10, 39). In its phosphorylated state, eIF2α forms a stable complex with the nucleotide exchange factor eIF2B, which is then no longer recycled for initiation of protein translation by GDP/GTP exchange (57). Consequently, PKR activation leads to a global block to protein synthesis in the infected cell, which can hamper the production of virus progeny. However, it is important to note that while eIF2α is inactivated by PKR, it also constitutes an important cellular stress checkpoint

utilized by three additional signaling pathways. These are activated by different cellular malfunctions, all of which require a temporary halt of protein synthesis to overcome the cause of stress. The PKR-related endoplasmic reticulum kinase (PERK) responds to protein overload in the endoplasmic reticulum (18), while the kinase GCN2 (general control non-derepressible 2) reacts to dysregulation of amino acid metabolism (64) or to UV light (12). Finally, the heme-regulated inhibitor HRI functions as a checkpoint for hemoglobin biosynthesis in reticulocytes (34). So far, antiviral functions have been reported only for the eIF2α kinases PKR, PERK, and GCN2 (3, 15, 26), and only these will be considered in the present study.

The members of the OAS protein family are encoded by several cellular genes and possess a very specific catalytic activity in being able to condense ATP molecules via unusual 2'-5'-phosphodiester linkages (23, 49). The resulting oligomers of variable length bind and activate the latent RNase L enzyme, which then cleaves different RNA species, among them viral RNAs, mRNAs, and rRNAs (14, 55, 65). As a result, synthesis of viral proteins is inhibited, and viral RNA genomes are directly destroyed.

The prominent antiviral role of PKR is underscored by the fact that many different viruses have evolved proteins that in one way or another prevent the activation of the PKR signalling cascade. For instance, the vaccinia virus (VV) E3L, the herpes simplex virus type 1 (HSV-1) US11, and influenza virus NS1 proteins bind dsRNA and PKR and inhibit its activation (7, 35, 42). The γ34.5 protein, also from HSV-1, functions as a regulatory subunit of the cellular PP1 phosphatase, directing it to dephosphorylate eIF2α, thereby terminating the PKR-induced signal (20).

Murine cytomegalovirus (MCMV) is a representative of the *Herpesviridae* and possesses several properties that should make it vulnerable to the antiviral host response: a large DNA genome with genes encoded on both strands, harboring the potential for extensive dsRNA formation; a prolonged replication cycle allowing enough time for the cell to mount an effective defense; and finally the establishment of a latent state in an organism with an activated immune response (56). Therefore, it is not surprising that MCMV encodes several different proteins that interfere with various host defense mechanisms.

We have recently shown that the MCMV proteins m142 and m143 are essential for MCMV replication and are both required to inhibit PKR activation, eIF2α phosphorylation, and a shutdown of protein synthesis (62). Child and colleagues showed that the two proteins can be coimmunoprecipitated from lysates of infected cells and function together to bind dsRNA (9). Thus, MCMV's system differs from other viral systems (such as E3L, NS1, or US11) in that a synergism of two different proteins is required to prevent PKR activation. However, whether these viral proteins also inhibit other dsRNAdependent host defenses and whether other eIF2α kinases contribute to the protein synthesis shutoff have not been determined.

In the present study, we show that the function of m142 and m143 is specifically directed against the PKR pathway. When cells were infected by an MCMV strain lacking these genes, eIF2α was phosphorylated specifically by PKR, not by the related eIF2α kinases PERK or GCN2. The OAS/RNase L pathway, by contrast, was not detectably activated during MCMV infection. Consistent with these findings, an m142- and m143-deficient MCMV replicated to titers equivalent to the wild-type (wt) virus in PKR-deficient cells but did not grow in RNase L-deficient cells. These results suggested that PKR is the predominant (if not the only) target of m142 and m143.

Materials and Methods

Virus mutagenesis.
All mutant viruses were constructed on the basis of an MCMV variant expressing the green fluorescent protein (MCMV-GFP), which has been used in previous studies (4, 27). The MCMV-GFP genome, cloned as a bacterial artificial chromosome (BAC), was modified in the *Escherichia coli* strain DY380 as described previously (4, 5). For generation of an MCMV mutant
with a deletion of both m142 and m143 (MCMVΔΔm142/m143), a zeocin resistance gene (*zeo*) was PCR amplified with primers that contained 50-nucleotide sequences homologous to positions 199621 to 199670 and 202594 to 202643, respectively, and used for homologous recombination. For generation of a revertant virus, a fragment spanning the genomic region of m142 and m143 (nucleotides 195201 to 200853 of the MCMV genome [MCMV Rev m142/m143]) was excised with

EcoRI and SspI from a pUC19 plasmid containing the cloned MCMV strain Smith HindIII/I region (kindly provided by M. Messerle, Hannover Medical School, Hannover, Germany). NIH 3T3 cells were transfected with the purified fragment and superinfected with MCMVΔΔm142/m143 2 h posttransfection. Since wt NIH 3T3 cells do not support replication of the MCMVΔΔm142/m143 mutant virus, only revertant virus was recovered.

The US11 and γ34.5 genes from HSV-1 and E3L from VV were introduced into the region of m02 to m06 (m02-m06) of MCMVΔΔm142/m143 by homologous recombination by means of the pReplacer plasmid, as previously described (27). The VV E3L gene was purchased as a synthetic, codon-optimized (for expression in mouse cells) version from Geneart (Regensburg, Germany). The synthetic E3L sequence is as follows:
5'-
ATGAGCAAGATCTACATCGACGAGAGAAGCGACGCCGAGATCGTGTGCGCCGCCATCAAGAACA
TCGGCATCGAGGGCGCCACAGCCGCCCAGCTGACCAGGCAGCTGAACATGGAGAAGCGGG
AGGTGAACAAGGCCCTGTACGACCTGCAGAGAAGCGCCATGGTGTACAGCAGCGACGACATCC
CCCCCAGATGGTTCATGACCACCGAGGCCGACAAGCCCGACGCCGACGCCATGGCCGACGTGA
TCATCGACGACGTGAGCCGGGAGAAGAGCATGAGAGAGGACCACAAGAGCTTCGACGATGTGAT
CCCCGCCAAGAAGATCATCGACTGGAAGGACGCCAACCCCGTGACCATCATCAACGAGTACTGC
CAGATCACCAAGAGGGACTGGTCCTTCAGGATCGAGAGCGTGGGACCCAGCAACAGCCCCACC
TTCTACGCCTGCGTGGACATCGACGGCAGGGTGTTCGACAAGGCCGACGGCAAGAGCAAGAGG
GACGCCAAGAACAACGCCGCCAAGCTGGCCGTGGACAAGCTGCTGGGCTACGTGATCATCAGG
TTCTGA-3'. The HSV-1 US11 gene was amplified by PCR from pCIneo-US11 (kindly provided by I. Mohr, New York University, NY), introducing a hemagglutinin (HA) epitope tag at the 5' end, and the γ34.5 gene was excised from pRC-CMV-γ34.5. The three genes were inserted into the multiple cloning site of pReplacer (27). A fragment containing a *kan* selection marker, the viral gene driven by a phosphoglycerate kinase promoter, and homology arms for homologous recombination was excised from the pReplacer plasmid with KpnI and SacI and used for inserting the sequence into the m02-m06 region of theMCMVΔΔm142/m143 BAC as described previously (27). The resulting BACs were purified with Nucleobond PC100 columns (Macherey and Nagel, Düren, Germany) and transfected into immortalized PKR-/- fibroblasts, which support full replication of m142- and m143-deficient viruses.

Viral DNA from MCMV Rev m142/m143 was isolated from supernatants of infected cells essentially as described previously (48). Genomic integrity was confirmed by EcoRI digestion and separation on ethidium bromide-stained 0.6% agarose gels and by sequencing the mutated sites.

Cells and viruses.
NIH 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% newborn calf serum. Murine 10.1 fibroblasts (19) and human 293A cells (Invitrogen) were grown in DMEM with 5% fetal calf serum. Primary murine embryonic fibroblasts (MEFs) from BALB/c mice were used at passage 3 for experiments. Primary PKR-/- MEFs (66) were provided by U. Kalinke (Paul Ehrlich Institute, Langen, Germany). Immortalized 3T3-like fibroblasts were generated from these cells by continuous passaging, as described previously (66). Immortalized RNase L-/- fibroblasts and corresponding wt fibroblasts (67) were provided by R. H. Silverman (Cleveland Clinic, Cleveland, OH). PERK-/- GCN2-/- double mutant cells (24) were obtained from D. Ron (New York University, New York, NY). All primary and immortalized MEFs were cultured in DMEM with 10% fetal calf serum, 100 μM β-mercaptoethanol, and nonessential amino acids (PAA Laboratories, Pasching, Austria).

For generation of E3L-expressing cells, the E3L gene was cloned in the pMSCV-puro plasmid (Clontech). Production of retroviral vectors using the Phoenix packaging cell line and transduction of NIH 3T3 cells were done as described previously (4). Transduced cells were selected with 4 μg/ml puromycin and grown as bulk cultures without clonal selection.

All virus stocks of viruses deficient in both m142 and m143 were produced on PKR-/- cells. Virus was harvested from the supernatant by ultracentrifugation, resuspended in complete medium, and titrated on the same cells according to the 50% tissue culture infective dose (TCID50) method (37). For analysis of virus replication, cells were seeded in six-well dishes and infected with different MCMV strains. The supernatants were harvested daily, and fresh medium was added. Viral titers were determined on PKR-/- MEFs as described above. All growth kinetics experiments were done in triplicate, and the means and standard deviations were used for the diagrams.

Immunoprecipitation and Western blotting.
In the expression plasmid pcDNA-m142HA (62), the HA tag was replaced by a FLAG tag by PCR with the forward primer 5'-AAA *GAA TTC* CAC CAT GGA CGC CCT GTG CGC GGC-3' and the reverse primer 5'-AAA *CTC GAG* TCA CTT ATC ATC GTC ATC CTT GTA GTC GTC GTC ATC GTC GGC GTC CGC-3'. The Cterminal FLAG tag introduced by the reverse primer is underlined, and the restriction sites are in italics. The PCR product was cloned in the pcDNA3 vector with EcoRI and XhoI. pcDNAm143FLAG was generated by the same method from pcDNA-m143HA (62) with the forward primer 5'-AAA *GGA TCC* ACC ATG TCT TGG GTG ACC GGA GAT-3', the reverse primer 5'-AAA *GAA TTC* TCA CTT ATC ATC GTC ATC CTT GTA GTC CGC GTC GGT CGC TCT CTC GTC-3', and cloning with BamHI and EcoRI. pcDNA-HA-US11 was generated from pReplacer-HA-US11 by HindIII and EcoRI cloning.

For analysis of the interaction of m142, m143, and PKR, 293A or NIH 3T3 cells grown in 10-cm dishes were transfected with HA- or FLAG-tagged m142 and/or m143 expression plasmids for 48 h. Cells were then lysed on ice in cold immunoprecipitation lysis buffer (140 mM NaCl, 5 mM MgCl2, 20 mM Tris, pH 7.6, and 1% [vol/vol] Triton X-100 with complete protease inhibitor cocktail [Roche]). Insoluble material was removed by centrifugation. Equal amounts of protein were subjected to immunoprecipitation using either rabbit anti-HA or anti-FLAG antiserum (Sigma) and protein G-Sepharose (Amersham). Precipitates were washed twice with washing buffer 1 (0.15MNaCl, 10 mM Tris, pH 7.6, 2 mM EDTA, 0.2% Triton X-100), twice with washing buffer 2 (0.5 M NaCl, 10 mM Tris, pH 7.6, 2 mM EDTA, 0.2% Triton X-100), and once with washing buffer 3 (20 mM Tris, pH 8.0), and precipitated proteins were released by boiling in reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDSPAGE) sample buffer. Proteins were separated by SDS-PAGE, blotted on nitrocellulose membranes, and detected by immunoblotting as described previously (62). RNase III digestion of immunoprecipitates was done by resuspending the precipitated complexes in 100 µl of RNase III reaction buffer (Ambion) and incubation with or without 2 U of dsRNA-specific RNase III (Ambion) for 30 min at 37°C. Samples were then washed and analyzed as above.

For analysis of eIF2α phosphorylation, cells were treated with 1 µM thapsigargin ([TG] Calbiochem) for 1 h or were infected with 3 TCID50/cell of the MCMV strains indicated in the figures, with centrifugal enhancement (two rounds of centrifugation at 2,000 g for 15 min, with 180° inversion of the plates in between). After 16 h, cells were lysed on ice in cold radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% [vol/vol] Triton X-100, 0.1% [wt/vol] SDS, and 1% [wt/vol] sodium deoxycholate with complete protease inhibitor cocktail), lysates were cleared by centrifugation, and equal amounts of protein were separated by SDS-PAGE and blotted as above. The following antibodies were used for detection in immunoblotting analysis: rabbit anti-HA (mouse monoclonal antibody [MAb] 3F10; Sigma), anti-FLAG (mouse MAb M2; Sigma), anti-phospho-eIFa, anti-total eIF2α (both from Cell Signaling Technology), anti-MCMV immediate-early antigen 1 ([IE1] MAb CROMA 101), anti-MCMV early antigen 1 ([E1] MAb CROMA103), anti-MCMV glycoprotein B ([gB] MAb SN 1.07), and anti-PKR (mouse MAb B10; Santa Cruz Biotechnology).

RNA analysis.
Total RNA was isolated from six-well dishes using an RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA samples (1.5 µg each) were stained with ethidium bromide, heat denatured in 25 µl of loading buffer (50% formamide, 6% [vol/vol] formaldehyde, 5% [vol/vol] glycerol in MOPS buffer [20 mM morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0]) for 15 min at 75°C, and separated on 1% agarose formaldehyde gels in MOPS buffer at 50 V for 3 h.

OAS activation assay.
Detection of ATP oligomerization to 2',5'-linked oligoadenylates (2-5A) by activated OAS enzyme was performed as described previously (52). Briefly, cell lysates were incubated with ATP and [α^{32}P]ATP, and the resulting oligomers were separated on 20% acrylamide–7 M urea gels in a sequencing gel apparatus (Bethesda Research Laboratories) and subjected to autoradiography.

Immunofluorescence.
Murine 10.1 cells were seeded on gelatin-coated glass coverslips and infected 24 h later with intact or UV-inactivated MCMV or with encephalomyocarditis virus ([EMCV] strain Maus Elberfeld, kindly provided by A. Nitsche, Robert Koch Institute) for 16 h. Cells were fixed with 3% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.5% Triton X-100, and stained with either anti-dsRNA MAb J2 (English and Scientific Consulting, Szirák, Hungary) or an isotype-matched control antibody and with an antimouse- Alexa594 conjugate (Invitrogen). Nuclei were counterstained with DAPI (4',6'-diamidino-2-phenylindole). Images were acquired using an epifluorescence microscope.

Results

Accumulation of dsRNA during MCMV infection.
Although CMVs are DNA viruses whose replication cycle does not include a dsRNA intermediate, their genomic organization, with gene expression occurring from both strands, harbors the potential for dsRNA formation inside the infected cell. To test
whether dsRNA is detectable in MCMV-infected cells, we stained MCMV-infected and control cells with a MAb specifically recognizing dsRNA (63) or an isotype-matched control antibody. By immunofluorescence analysis (Fig. 1), dsRNA was detected in MCMV-infected cells although the signal was not as strong as in cells infected with EMCV. By contrast, mock-infected cells and cells infected with UV-inactivated MCMV were negative, indicating that viral gene expression and/or viral replication are required for the production of dsRNA. MCMV-encoded expression of GFP was used as an infection control. Cells transfected with polyinosinic:poly(C) [poly(I:C)] showed a coarsely speckled pattern after staining with the J2 MAb, similar to what has was seen in a previous study (63). The detection of dsRNA in MCMV-infected cells suggested that the virus needs to respond to dsRNA-induced host antiviral defenses.

Viral PKR antagonists rescue an m142 and m143 double mutant MCMV.
A previous study has shown that the MCMV proteins m142 and m143 bind dsRNA as a heterooligomeric complex (9). Single-gene deletion mutants failed to prevent PKR activation and a global shutdown of protein synthesis upon infection (62). To analyze the properties of MCMV in the absence of both proteins, we generated a double knockout mutant, MCMVΔΔm142/m143, lacking both genes. Using BAC technology, the region of the m142 and m143 genes was replaced with a zeocin selection cassette by homologous recombination in *E. coli* carrying the parental MCMV BAC. To exclude effects of unwanted second-site mutations, we also generated a revertant virus, MCMV Rev m142/m143. To this end, a fragment of the MCMV genome spanning the deleted region was recombined with the MCMVΔΔm142/m143 genome in NIH 3T3 fibroblasts. Since both genes, m142 and m143, are essential for MCMV replication (62), only repaired virus could grow on NIH 3T3 cells.

We also wanted to analyze whether the replication defect of the ΔΔm142/m143 mutant and, hence, the function of m142 and m143 can be compensated by well-characterized viral antagonists of the PKR signaling pathway. Therefore, we inserted genes encoding the PKR inhibitors E3L from VV and US11 from HSV-1, as well as the eIF2α phosphatase regulator γ34.5 from HSV-1 into the genome of the ΔΔm142/m143 mutant virus. The genes were inserted by homologous recombination into the m02-m06 region of the MCMV genome as described previously (27, 62). Schematic representations of the recombinant MCMV genomes and the EcoRI restriction patterns are shown in Fig. 2A and B, respectively. The mutated sites in the genome were also verified by BAC sequencing (Fig. 2C).

When we analyzed the course of infection of the virus mutants in normal fibroblasts (NIH 3T3 cells) with an intact PKR, parental and revertant viruses replicated to the same titers, while the double deletion mutant MCMVΔΔm142/m143 was unable to replicate (Fig. 3A). The PKR antagonists E3L and γ34.5 and, to a lesser extent, also US11 partially rescued the replication of the ΔΔm142/m143 mutant (Fig. 3A). Thus, expression of foreign viral PKR antagonists can overcome the replication block induced by the deletion of m142 and m143, further confirming a role of m142 and m143 in the PKR antiviral pathway. A similar partial rescue was observed when E3L was expressed in *trans* after retroviral transduction of NIH 3T3 cells. E3L-expressing but not parental NIH 3T3 cells could support replication of the ΔΔm142/m143 mutant (Fig. 3B).

Activation of PKR by dsRNA leads to phosphorylation of the translation initiation factor eIF2α and subsequent protein synthesis shutoff (43). After infection at a high multiplicity of infection ([MOI] 5 TCID50/cell), an increased amount of phosphorylated eIF2α was detected in cells infected with MCMVΔΔm142/m143 but not in cells infected with the parental or the revertant virus. As expected, eIF2α phosphorylation was antagonized by the expression of E3L, γ34.5, or US11 (Fig. 3C). Treatment of cells with TG, an activator of the eIF2α kinase PERK (61), was used as a positive control for eIF2α phosphorylation. An apparent discrepancy between the almost complete suppression of eIF2α phosphorylation compared to the only moderate rescue of virus growth by the γ34.5 and US11 proteins emerged. We therefore studied the course of the infection in more detail by analyzing the expression of the viral IE1, E1, and gB proteins. At 16 h postinfection when
eIF2α phosphorylation was measured, viral gene expression was similar in all virus mutants. By contrast, the mutants MCMVΔΔm142/m143 and MCMVΔΔm142/m143 expressing US11 showed reduced IE1 and E1 levels and completely failed to express gB at 48 h postinfection. From this we

conclude that protein expression during the early phase of infection is sustained in all complementing mutants, but US11 fails to prevent protein synthesis shutoff during the late phase.

MCMVΔΔm142/m143 induces eIF2α phosphorylation specifically through PKR, not via the related kinases PERK or GCN2.

Phosphorylation of eIF2α is a converging point of several cellular signaling pathways, which respond to different types of cellular stress. Therefore, we wanted to analyze the contribution of the eIF2α kinases PKR, PERK, and GCN2 to eIF2α phosphorylation observed after infection with MCMVΔΔm142/m143. wt, PKR-/-, and PERK-/- GCN2-/- fibroblasts were infected with MCMV or treated with the PERK activator TG. As shown in Fig. 4, the MCMVΔΔm142/m143 virus induced eIF2α phosphorylation in wt and PERK- and GCN2-deficient cells but not in PKR-deficient cells. Conversely, TG induced eIF2α phosphorylation in wt and PKR-deficient cells but not in PERK- and GCN2-deficient cells. From these results we conclude that MCMV infection in the absence of m142 and m143 selectively activates eIF2α phosphorylation by PKR and not by the related kinases PERK or GCN2.

m142 and m143 interact with each other and with PKR.

Since m142 and m143 have been shown to bind dsRNA jointly but not individually, two possible mechanisms for the inhibition of PKR are conceivable. First, the binding of m142 and m143 to dsRNA might occupy all attachment sites for PKR and sequester the activating substrate away from the kinase. In this case, a physical interaction between m142 and m143 and PKR would not be necessary. Alternatively, m142 and m143 could interact with PKR, thereby inhibiting its activation. To distinguish between the two mechanisms, we checked for an interaction of m142 and m143 with PKR in coimmunoprecipitation assays. We expressed m142 and m143 with a C-terminal HA tag (m142HA and m143HA, respectively) or a FLAG tag (m142FL and m143FL, respectively) in murine NIH 3T3 or human 293 cells by transient transfection. Cells were lysed, and equal amounts of protein were subjected to immunoprecipitation with anti-HA, anti-FLAG, or isotype-matched control antibodies coupled to protein G-Sepharose. The samples were then analyzed by immunoblotting for HA, FLAG, and endogenous PKR. After isolated expression of m142 or m143 in NIH 3T3 cells, the protein could be precipitated, but an association with PKR was not detected. In contrast, when m142HA and m143FL were cotransfected, m143FL and the endogenous cellular PKR coprecipitated with m142HA (Fig. 5A). Similarly, the m142-m143-PKR complex could be precipitated via m142FL with an anti-FLAG antibody (Fig. 5B). The same complex was also detected in lysates of transfected 293 cells. Here, immunoprecipitation with anti-HA (Fig. 5C) but not with control antibodies yielded all three components in the precipitate. Interestingly, the expression levels of m142 and m143 were increased when both proteins were coexpressed, suggesting that the m142-m143 complex is more stable than the individual proteins. In some immunoprecipitation experiments, we observed small amounts of PKR coprecipitating with the individual viral proteins, m142HA or m143FL (data not shown). However, since PKR coprecipitated much more efficiently in the presence of both viral proteins, the interactions of the individual proteins with PKR are probably weaker and of lesser relevance than the interaction of the m142-m143 complex with PKR. Combined with our previous observation that deletion of one of the two viral genes from the viral genome completely abolishes the virus's ability to inhibit PKR activation (62), we concluded that both m142 and m143 are required for a functional interaction with PKR. The results argue for an inhibition by physical interaction rather than by sequestering dsRNA away from PKR.

Interestingly, the m142-m143 complex coprecipitated endogenous PKR from human 293 cells and from murine NIH 3T3 cells even though the murine and human PKRs are only about 60% identical on the amino acid level (31). This suggested that the viral proteins interact with a highly conserved domain of PKR.

To analyze whether the complex between m142, m143, and PKR is mediated by protein interaction or bridged by dsRNA, we expressed m142HA and m143FL proteins in 293 cells and precipitated the proteins with anti-HA antibodies. The complexes were resuspended in reaction buffer for the dsRNAspecific RNase III, incubated with or without enzyme, washed extensively, and analyzed by immunoblotting (Fig. 5D). Digestion of dsRNA did not reduce the amount of PKR in the complex, indicating that the m142-m143-PKR complex does not depend on dsRNA. By contrast, RNase III digestion strongly reduced the amount of PKR coprecipitating with US11, confirming the previously described RNA dependence of the US11-PKR complex (6).

MCMV infection does not activate the OAS/RNase L pathway.
The previous experiments have shown that MCMV infection produces dsRNA and that activation of the dsRNA-dependent PKR is prevented by m142 and m143. This raised the question of whether the dsRNA-activated antiviral OAS/ RNase L pathway is also turned on by MCMV in the presence or absence of m142 and m143. To detect oligoadenylate synthetase activity, we applied an enzymatic assay, in which the substrate $[\alpha^{32}P]ATP$ is metabolized and incorporated into 2-5A (52). The reaction mixtures were then separated on polyacrylamide- urea gels and subjected to autoradiography (Fig. 6A). We were unable to detect 2-5A in MCMV-infected cells, regardless of whether the cells had been pretreated with IFN-β to induce OAS expression. Only after the addition of poly(I:C) were slower-migrating ATP derivatives (indicative of 2-5A formation) synthesized (Fig. 6A).

To date, the only known function of 2-5A is activation of the latent RNase L enzyme, which cleaves different RNA species including the abundant rRNAs. Therefore, we also assayed the activity of RNase L in response to MCMV infection. Cells were pretreated with IFN-β and infected as indicated in Fig. 6B. PKR-/- cells were used for this assay in order to exclude any contribution of PKR. Total RNA was isolated, stained with ethidium bromide, and separated on denaturing formaldehyde-MOPS agarose gels. Consistent with the results of the OAS assay (Fig. 6A), we did not observe degradation of cellular rRNA during MCMV infection. Only when poly(I:C) was transfected into IFN-β-treated cells was rRNA degradation detected as a ladder of degradation products (Fig. 6B). Under the same conditions, rRNA degradation was not detected in RNase L-/- cells, confirming that this effect is mediated specifically by RNase L (Fig. 6C).

The failure to observe RNase L activity during MCMV infection could be attributed to a lack of a strong activating stimulus (as suggested by the results shown in Fig. 6A) or to an active inhibition of this pathway by MCMV. To test for the latter, we analyzed MCMV's ability to inhibit RNase L activation induced by an exogenous stimulus. To this end, cells were pretreated with IFN, infected with MCMV, and subsequently transfected with poly(I:C). As shown in Fig. 6D, MCMV infection did not significantly affect poly(I:C)-induced rRNA degradation, regardless of the presence or absence of m142 and m143. Taken together, these results suggested that the OAS/RNase L pathway is neither activated nor actively inhibited by MCMV and that the dsRNA-binding proteins m142 and m143 do not play an important role in controlling this dsRNA-activated pathway.

PKR, but not RNase L, controls replication of MCMVΔΔ m142/m143.
The results of the previous experiments showed that MCMV infection in the absence of m142 and m143 activates PKR, whereas activation of the OAS/RNase L pathway was not detectable. This suggested that the PKR pathway, but not the OAS/RNase L pathway, inhibits replication of MCMVΔΔm142/m143. We tested this hypothesis by analyzing the replication kinetics of this virus in wt fibroblasts and in fibroblasts derived from PKR or RNase L knockout mice. As expected, MCMVΔΔm142/m143 did not replicate in wt and RNase L-deficient cells (Fig. 7), nor did MCMV mutants lacking only one of the two genes (data not shown). However, the MCMVΔΔm142/m143 mutant grew to similar titers as the parental virus and the revertant virus in PKR-/- cells (Fig. 7). Hence, we concluded that PKR, but not OAS/RNase L, controls MCMV replication in the absence of m142 and m143 and that PKR is responsible for the essentiality of m142 and m143.

Discussion

Cells respond to viral infection by secreting type I IFNs, which stimulate specific receptors on infected and neighboring cells. This activates the expression of IFN-stimulated genes, many of which encode proteins with antiviral functions (54). PKR and OAS are two important representatives of these antiviral effector proteins (50). Both are activated by dsRNA and hamper viral replication by repressing protein synthesis. The importance of PKR and OAS is underscored by the fact that several viruses express gene products that inhibit the PKR or the OAS pathway (43, 55).

In MCMV, the m142 and m143 proteins play a crucial role in blocking the host antiviral response. In the absence of one or both proteins, MCMV infection leads to PKR activation, eIF2α phosphorylation, and attenuation of protein synthesis (62). The two proteins form a dsRNA-binding complex and are together able to rescue the replication of an E3L-deficient VV (9). However, m142 and m143 differ from previously characterized viral PKR inhibitors in that they are absolutely essential for virus replication in cell culture (38, 62). Two possible reasons for this essential nature are conceivable:

either the PKRmediated antiviral response is so effective against MCMV that the virus depends on an efficient inhibitory mechanism, or the two viral proteins possess an additional function beyond PKR inhibition. Such additional functions have been identified in a number of viral PKR inhibitors: human CMV (HCMV) TRS1 appears to have a role in viral DNA replication and assembly of DNA-containing capsids (1, 45), VV E3L binds Z-DNA (29), influenza virus NS1 inhibits RIG-I (47), and HSV-1 US11 prevents OAS activation (52). We also assayed the replication of the PKR antagonist-expressing virus mutants in PKR-/-cells in order to clarify whether the incomplete complementation of these proteins might be due to a dominant-negative

effect of the viral PKR antagonist on MCMV replication. However, all virus mutants grew to similar titers in PKR-/- cells, arguing against such an effect (Fig. 8).

Our first approach to delineate the functional spectrum of m142 and m143 was to complement an MCMV strain deficient in both m142 and m143 with PKR antagonists of HSV-1 and VV. These proteins reduced eIF2α phosphorylation when expressed from the genome of MCMVΔΔm142/m143 and partially restored virus replication (Fig. 3). Similar results have been obtained previously with the HCMV TRS1 gene product (62). By contrast, a knockout of the host effector protein PKR completely restored replication of the MCMVΔΔm142/m143 virus to titers indistinguishable from the parental virus (Fig. 7). This suggested that the PKR antagonists of HCMV, HSV-1, and VV inhibit murine PKR less efficiently than the MCMV proteins or that the expression levels and kinetics in our system were not adequate for a complete inhibition of PKR. Another possible explanation could be that the heterologous viral genes were inserted into the MCMV genome alone even though they synergize with a second viral protein in their natural viral context: US11 synergizes with γ34.5 in HSV-1 (44), TRS1 synergizes with IRS1 in HCMV (8), and E3L synergizes with the PKR pseudosubstrate K3L in VV (11).

We show in this study that the m142-m143 complex interacts with PKR and that the viral proteins do not bind to PKR individually or do so with lower affinity. Interestingly, the complex was not sensitive to digestion with RNase III, suggesting a protein-protein interaction of the components. It is unlikely that the short 12- to 30-bp dsRNA fragments produced by RNase III would be sufficient to maintain such a complex. However, it cannot be completely excluded that bridging dsRNA molecules are inaccessible for digestion in the m142-m143-PKR complex. It seems likely that the interaction of m142 and m143 with PKR is necessary for inhibition of PKR activation because an interaction with PKR is essential for the function of the influenza virus NS1 and the HCMV TRS1 and IRS1 proteins (17, 33). However, this hypothesis can be tested only after the dsRNA- and PKR-binding domains of m142 and m143 have been identified and mutated separately.

Another antiviral effector system that shares many similarities with the PKR-dependent pathway is the OAS/RNase L pathway. It is upregulated by type I IFNs, activated by dsRNA, and attenuates protein expression (55). Surprisingly, we found no indication that the OAS/RNase L pathway is activated during MCMV infection, regardless of whether m142 and m143 were expressed. Moreover, RNase L knockout cells did not support replication of m142- and/or m143-deficient viruses, whereas PKR knockout cells allowed replication of these viruses to titers equivalent to the parental virus (Fig. 7). Two possible explanations are conceivable. On the one hand, dsRNA levels in MCMV-infected cells could be insufficient for a robust activation of OAS. On the other hand, MCMV might express other proteins that inhibit the OAS/RNase L pathway. Candidates for such a function would be, for instance, the gene products of M23 and M24, which were shown to bind dsRNA. (9). The inability of MCMV to block rRNA degradation upon poly(I:C) transfection argues in favor of the first possibility. However, we cannot completely exclude the possibility that MCMV encodes inhibitors of the OAS/RNase L pathway that are sufficient to inhibit OAS/RNase L activities induced by endogenously produced dsRNA but insufficient to block activities triggered by transfected poly(I:C).

The lack of detectable RNase L activation by MCMV is in accordance with the observation that at least some DNA viruses are less sensitive to the OAS/RNase L pathway than RNA viruses (55). For instance, no significant activation of the OAS/RNase L pathway was detected after infection of cells with varicella zoster virus or simian virus 40 (13, 21), and the impact of this pathway on HSV-1 and VV was strongly dependent on the system investigated (55).

Besides PKR and OAS, at least two other classes of pattern recognition receptors are activated by dsRNA. TLR3 senses the presence of dsRNA in endosomes, and the RNA helicases MDA5 and (to a lesser extent) RIG-I detect dsRNA in the cytosol (53). It is unclear how the cytosolic proteins m142 and m143 could gain access to endosomes to inhibit TLR3 activation. On the other hand, it might be worth investigating the influence of m142 and m143 on RIG-I or MDA5 activation. While both helicases can be activated by poly(I:C), it has recently

become clear that RIG-I is preferentially activated by 5'-triphosphorylated single-stranded RNA (22) and that MDA5 is activated predominantly by long dsRNA (28, 53). Whether such RNA species are present in MCMV-infected cells is unknown. It has been shown, however, that MDA5 is not required for the induction of IFN-β by MCMV in vivo (16).

Viral interference with dsRNA-activated pathways does not necessarily require that the viral antagonist itself bind dsRNA. Viral proteins can interfere with signal transduction at various steps of the signaling cascades. In the case of MCMV, it has recently been shown that the M45 protein inhibits the adaptor protein RIP1 (36, 60), a protein that mediates NF-B activation in response to TLR3 and RIG-I/MDA5 stimulation (2, 40, 41). Interestingly, a study identified a role of PKR in the TLR3-dependent pathway (25), hinting at a possible indirect role for m142 and m143 in TLR3 signaling. Although the fact that MCMVΔΔm142/m143 replicated to wt levels in PKR-/- fibroblasts indicated that PKR is the primary target for m142 and m143, an influence on other signaling pathways cannot be entirely excluded. Further investigations will be necessary to reveal the full spectrum of the activities of m142 and m143 and the role of other viral proteins in subverting antiviral pathways of the host cell.

Acknowledgements

References

1. Adamo, J. E., J. Schroer, and T. Shenk. 2004. Human cytomegalovirus TRS1 protein is required for efficient assembly of DNA-containing capsids. J. Virol. 78:10221–10229.
2. Balachandran, S., E. Thomas, and G. N. Barber. 2004. A FADD-dependent innate immune mechanism in mammalian cells. Nature 432:401–405.
3. Berlanga, J. J., I. Ventoso, H. P. Harding, J. Deng, D. Ron, N. Sonenberg, L. Carrasco, and C. de Haro. 2006. Antiviral effect of the mammalian translation initiation factor 2α kinase GCN2 against RNA viruses. EMBO J. 25: 1730–1740.
4. Brune, W., M. Nevels, and T. Shenk. 2003. Murine cytomegalovirus m41 open reading frame encodes a Golgi-localized antiapoptotic protein. J. Virol. 77:11633–11643.
5. Brune, W., M. Wagner, and M. Messerle. 2006. Manipulating cytomegalovirus genomes by BAC mutagenesis: strategies and applications, p. 61–89. In M. J. Reddehase (ed.), Cytomegaloviruses: molecular biology and immunology. Caister Academic Press, Wymondham, United Kingdom.
6. Cassady, K. A., and M. Gross. 2002. The herpes simplex virus type 1 U(S)11 protein interacts with protein kinase R in infected cells and requires a 30-amino-acid sequence adjacent to a kinase substrate domain. J. Virol. 76:2029–2035.
7. Chang, H. W., J. C. Watson, and B. L. Jacobs. 1992. The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, doublestranded RNA-dependent protein kinase. Proc. Natl. Acad. Sci. USA 89: 4825–4829.
8. Child, S. J., M. Hakki, K. L. De Niro, and A. P. Geballe. 2004. Evasion of cellular antiviral responses by human cytomegalovirus TRS1 and IRS1. J. Virol. 78:197–205.
9. Child, S. J., L. K. Hanson, C. E. Brown, D. M. Janzen, and A. P. Geballe. 2006. Double-stranded RNA binding by a heterodimeric complex of murine cytomegalovirus m142 and m143 proteins. J. Virol. 80:10173–10180.
10. Clemens, M. J., and A. Elia. 1997. The double-stranded RNA-dependent protein kinase PKR: structure and function. J. Interferon Cytokine Res. 17:503–524.
11. Davies, M. V., H. W. Chang, B. L. Jacobs, and R. J. Kaufman. 1993. The E3L and K3L vaccinia virus gene products stimulate translation through inhibition of the double-stranded RNA-dependent protein kinase by different mechanisms. J. Virol. 67:1688–1692.
12. Deng, J., H. P. Harding, B. Raught, A. C. Gingras, J. J. Berlanga, D. Scheuner, R. J. Kaufman, D. Ron, and N. Sonenberg. 2002. Activation of GCN2 in UV-irradiated cells inhibits translation. Curr. Biol. 12:1279–1286.

13. Desloges, N., M. Rahaus, and M. H. Wolff. 2005. Varicella-zoster virus does not significantly induce cell defence mechanism mediated by the 2-5A/ RNase L pathway during its replication cycle. Med. Microbiol. Immunol. 194:25–31.

14. Floyd-Smith, G., E. Slattery, and P. Lengyel. 1981. Interferon action: RNA cleavage pattern of a (2'-5')oligoadenylate-dependent endonuclease. Science 212:1030–1032.

15. Garcia, M. A., E. F. Meurs, and M. Esteban. 2007. The dsRNA protein kinase PKR: virus and cell control. Biochimie 89:799–811.

16. Gitlin, L., W. Barchet, S. Gilfillan, M. Cella, B. Beutler, R. A. Flavell, M. S. Diamond, and M. Colonna. 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc. Natl. Acad. Sci. USA 103:8459–8464.

17. Hakki, M., E. E. Marshall, K. L. De Niro, and A. P. Geballe. 2006. Binding and nuclear relocalization of protein kinase R by human cytomegalovirus TRS1. J. Virol. 80:11817–11826.

18. Harding, H. P., Y. Zhang, and D. Ron. 1999. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 397:271–274.

19. Harvey, D. M., and A. J. Levine. 1991. p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. Genes Dev. 5:2375–2385.

20. He, B., M. Gross, and B. Roizman. 1997. The $\gamma_1 34.5$ protein of herpes simplex virus 1 complexes with protein phosphatase 1α to dephosphorylate the α subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA 94:843–848.

21. Hersh, C. L., R. E. Brown, W. K. Roberts, E. A. Swyryd, I. M. Kerr, and G. R. Stark. 1984. Simian virus 40-infected, interferon-treated cells contain 2',5'- oligoadenylates which do not activate cleavage of RNA. J. Biol. Chem. 259:1731–1737.

22. Hornung, V., J. Ellegast, S. Kim, K. Brzozka, A. Jung, H. Kato, H. Poeck, S. Akira, K. K. Conzelmann, M. Schlee, S. Endres, and G. Hartmann. 2006. 5'-Triphosphate RNA is the ligand for RIG-I. Science 314:994–997.

23. Hovanessian, A. G., and J. Justesen. 2007. The human 2'-5'oligoadenylate synthetase family: unique interferon-inducible enzymes catalyzing 2'-5' instead of 3'-5' phosphodiester bond formation. Biochimie 89:779–788.

24. Jiang, H.-Y., S. A. Wek, B. C. McGrath, D. Lu, T. Hai, H. P. Harding, X. Wang, D. Ron, D. R. Cavener, and R. C. Wek. 2004. Activating transcription factor 3 is integral to the eukaryotic initiation factor 2 kinase stress response. Mol. Cell. Biol. 24:1365–1377.

25. Jiang, Z., M. Zamanian-Daryoush, H. Nie, A. M. Silva, B. R. Williams, and X. Li. 2003. Poly(I-C)-induced Toll-like receptor 3 (TLR3)-mediated activation of NFB and MAP kinase is through an interleukin-1 receptorassociated kinase (IRAK)-independent pathway employing the signaling components TLR3-TRAF6-TAK1-TAB2-PKR. J. Biol. Chem. 278:16713–16719.

26. Jordan, R., L. Wang, T. M. Graczyk, T. M. Block, and P. R. Romano. 2002. Replication of a cytopathic strain of bovine viral diarrhea virus activates PERK and induces endoplasmic reticulum stress-mediated apoptosis of MDBK cells. J. Virol. 76:9588–9599.

27. Jurak, I., and W. Brune. 2006. Induction of apoptosis limits cytomegalovirus cross-species infection. EMBO J. 25:2634–2642.

28. Kato, H., O. Takeuchi, E. Mikamo-Satoh, R. Hirai, T. Kawai, K. Matsushita, A. Hiiragi, T. S. Dermody, T. Fujita, and S. Akira. 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J. Exp. Med. 205: 1601–1610.

29. Kim, Y. G., M. Muralinath, T. Brandt, M. Pearcy, K. Hauns, K. Lowenhaupt, B. L. Jacobs, and A. Rich. 2003. A role for Z-DNA binding in vaccinia virus pathogenesis. Proc. Natl. Acad. Sci. USA 100:6974–6979.

30. Kostura, M., and M. B. Mathews. 1989. Purification and activation of the double-stranded RNA-dependent eIF-2 kinase DAI. Mol. Cell. Biol. 9:1576–1586.

31. Kuhen, K. L., X. Shen, E. R. Carlisle, A. L. Richardson, H. U. Weier, H. Tanaka, and C. E. Samuel. 1996. Structural organization of the human gene (PKR) encoding an interferon-inducible RNA-dependent protein kinase (PKR) and differences from its mouse homolog. Genomics 36:197–201.

32. Lee, M. S., and Y. J. Kim. 2007. Pattern-recognition receptor signalling initiated from extracellular, membrane, and cytoplasmic space. Mol. Cells 23:1–10.

33. Li, S., J. Y. Min, R. M. Krug, and G. C. Sen. 2006. Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. Virology 349:13–21.

34. Lu, L., A. P. Han, and J. J. Chen. 2001. Translation initiation control by heme-regulated eukaryotic initiation factor 2α kinase in erythroid cells under cytoplasmic stresses. Mol. Cell. Biol. 21:7971–7980.

35. Lu, Y., M. Wambach, M. G. Katze, and R. M. Krug. 1995. Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the eIF-2 translation initiation factor. Virology 214:222–228.

36. Mack, C., A. Sickmann, D. Lembo, and W. Brune. 2008. Inhibition of proinflammatory and innate immune signaling pathways by a cytomegalovirus RIP1-interacting protein. Proc. Natl. Acad. Sci. USA 105:3094–3099.

37. Mahy, B. W. J., and H. O. Kangro. 1996. Virology methods manual. Academic Press, San Diego, CA.

38. Menard, C., M. Wagner, Z. Ruzsics, K. Holak, W. Brune, A. E. Campbell, and U. H. Koszinowski. 2003. Role of murine cytomegalovirus US22 gene family members in replication in macrophages. J. Virol. 77:5557–5570.

39. Meurs, E. F., Y. Watanabe, S. Kadereit, G. N. Barber, M. G. Katze, K. Chong, B. R. Williams, and A. G. Hovanessian. 1992. Constitutive expression of human double-stranded RNA-activated p68 kinase in murine cells mediates phosphorylation of eukaryotic initiation factor 2 and partial resistance to encephalomyocarditis virus growth. J. Virol. 66:5805–5814.

40. Meylan, E., K. Burns, K. Hofmann, V. Blancheteau, F. Martinon, M. Kelliher, and J. Tschopp. 2004. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-B activation. Nat. Immunol. 5:503–507.

41. Michallet, M. C., E. Meylan, M. A. Ermolaeva, J. Vazquez, M. Rebsamen, J. Curran, H. Poeck, M. Bscheider, G. Hartmann, M. Konig, U. Kalinke, M. Pasparakis, and J. Tschopp. 2008. TRADD protein is an essential component of the RIG-like helicase antiviral pathway. Immunity 28:651–661.

42. Mohr, I., and Y. Gluzman. 1996. A herpesvirus genetic element which affects translation in the absence of the viral GADD34 function. EMBO J. 15:4759–4766.

43. Mohr, I. J., T. Pe'ery, and M. B. Mathews. 2007. Protein synthesis and translational control during viral infection, p. 545–599. *In* M. B. Mathews, N. Sonenberg, and J. W. B. Hershey (ed.), Translational control in biology and medicine. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

44. Mulvey, M., J. Poppers, D. Sternberg, and I. Mohr. 2003. Regulation of eIF2α phosphorylation by different functions that act during discrete phases in the herpes simplex virus type 1 life cycle. J. Virol. 77:10917–10928.

45. Pari, G. S., and D. G. Anders. 1993. Eleven loci encoding *trans*-acting factors are required for transient complementation of human cytomegalovirus *ori-* Lyt-dependent DNA replication. J. Virol. 67:6979–6988.

46. Patel, R. C., P. Stanton, N. M. McMillan, B. R. Williams, and G. C. Sen. 1995. The interferon-inducible double-stranded RNA-activated protein kinase self-associates in vitro and in vivo. Proc. Natl. Acad. Sci. USA 92:8283–8287.

47. Pichlmair, A., O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to singlestranded RNA bearing 5'-phosphates. Science 314:997–1001.

48. Redwood, A. J., M. Messerle, N. L. Harvey, C. M. Hardy, U. H. Koszinowski, M. A. Lawson, and G. R. Shellam. 2005. Use of a murine cytomegalovirus K181-derived bacterial artificial chromosome as a vaccine vector for immunocontraception. J. Virol. 79:2998–3008.

49. Roberts, W. K., A. Hovanessian, R. E. Brown, M. J. Clemens, and I. M. Kerr. 1976. Interferon-mediated protein kinase and low-molecular-weight inhibitor of protein synthesis. Nature 264:477–480.

50. Sadler, A. J., and B. R. Williams. 2008. Interferon-inducible antiviral effectors. Nat. Rev. Immunol. 8:559–568.

51. Saito, T., and M. Gale, Jr. 2007. Principles of intracellular viral recognition. Curr. Opin. Immunol. 19:17–23.

52. Sanchez, R., and I. Mohr. 2007. Inhibition of cellular 2'-5' oligoadenylate synthetase by the herpes simplex virus type 1 Us11 protein. J. Virol. 81:3455–3464.

53. Schlee, M., W. Barchet, V. Hornung, and G. Hartmann. 2007. Beyond double-stranded RNA-type I IFN induction by 3pRNA and other viral nucleic acids. Curr. Top. Microbiol. Immunol. 316:207–230.

54. Sen, G. C., and S. N. Sarkar. 2007. The interferon-stimulated genes: targets of direct signaling by interferons, double-stranded RNA, and viruses. Curr. Top. Microbiol. Immunol. 316:233–250.

55. Silverman, R. H. 2007. Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response. J. Virol. 81:12720–12729.

56. Simon, C. O., C. K. Seckert, N. K. A. Grizmek, and M. J. Reddehase. 2006. Murine model of cytomegalovirus latency and reactivation: the silencing/ desilencing and immune sensing hypothesis, p. 483–500. *In* M. J. Reddehase (ed.), Cytomegaloviruses: molecular biology and immunology. Caister Academic Press, Wymondham, United Kingdom.

57. Sudhakar, A., A. Ramachandran, S. Ghosh, S. E. Hasnain, R. J. Kaufman, and K. V. Ramaiah. 2000. Phosphorylation of serine 51 in initiation factor 2α (eIF2α) promotes complex formation between

eIF2 alpha(P) and eIF2B and causes inhibition in the guanine nucleotide exchange activity of eIF2B. Biochemistry 39:12929–12938.

58. Takeuchi, O., and S. Akira. 2007. Recognition of viruses by innate immunity. Immunol. Rev. 220:214–224.

59. Thomis, D. C., and C. E. Samuel. 1995. Mechanism of interferon action: characterization of the intermolecular autophosphorylation of PKR, the interferon-inducible, RNA-dependent protein kinase. J. Virol. 69:5195– 5198.

60. Upton, J. W., W. J. Kaiser, and E. S. Mocarski. 2008. Cytomegalovirus M45 cell death suppression requires receptor-interacting protein (RIP) homotypic interaction motif (RHIM)-dependent interaction with RIP1. J. Biol. Chem. 283:16966–16970.

61. Urano, F., X. Wang, A. Bertolotti, Y. Zhang, P. Chung, H. P. Harding, and D. Ron. 2000. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science 287:664–666.

62. Valchanova, R. S., M. Picard-Maureau, M. Budt, and W. Brune. 2006. Murine cytomegalovirus m142 and m143 are both required to block protein kinase R-mediated shutdown of protein synthesis. J. Virol. 80:10181–10190.

63. Weber, F., V. Wagner, S. B. Rasmussen, R. Hartmann, and S. R. Paludan. 2006. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J. Virol. 80:5059–5064.

64. Wek, S. A., S. Zhu, and R. C. Wek. 1995. The histidyl-tRNA synthetaserelated sequence in the eIF-2α protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids. Mol. Cell. Biol. 15:4497–4506.

65. Wreschner, D. H., J. W. McCauley, J. J. Skehel, and I. M. Kerr. 1981. Interferon action—sequence specificity of the ppp(A2'p)nA-dependent ribonuclease. Nature 289:414–417.

66. Yang, Y. L., L. F. Reis, J. Pavlovic, A. Aguzzi, R. Schafer, A. Kumar, B. R. Williams, M. Aguet, and C. Weissmann. 1995. Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. EMBO J. 14: 6095–6106.

67. Zhou, A., J. Paranjape, T. L. Brown, H. Nie, S. Naik, B. Dong, A. Chang, B. Trapp, R. Fairchild, C. Colmenares, and R. H. Silverman. 1997. Interferon action and apoptosis are defective in mice devoid of 2',5'-oligoadenylatedependent RNase L. EMBO J. 16:6355–6363.
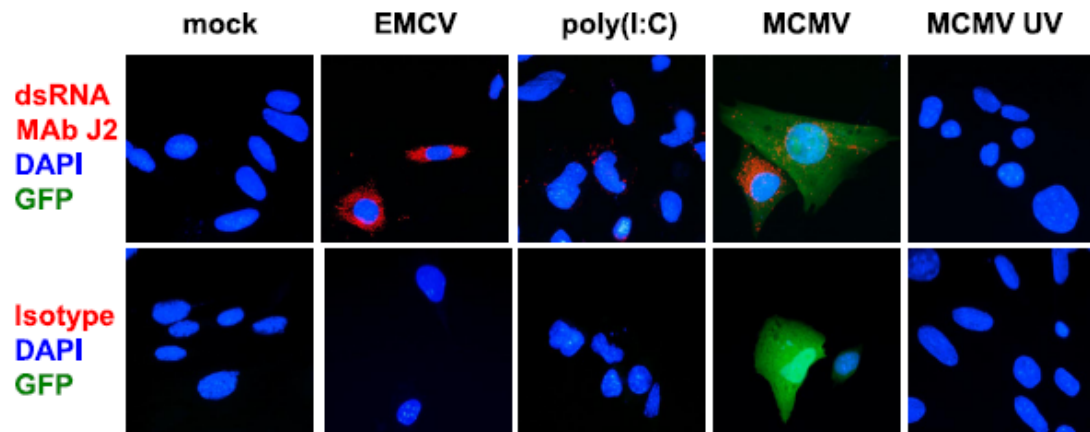
Figure 1. Accumulation of dsRNA in MCMV-infected cells. Murine 10.1 fibroblasts were mock infected or infected with the picornavirus EMCV (MOI of 0.3), MCMV (MOI of 3), or UV-inactivated MCMV for 16 h. As an additional positive control, cells were transfected with the synthetic dsRNA poly(I:C). Cells were stained with the dsRNA-recognizing MAb J2 or an isotype-matched control immunoglobulin G and an Alexa594-conjugated secondary antibody. Nuclei were counterstained with DAPI. MCMV-encoded GFP is shown in green.
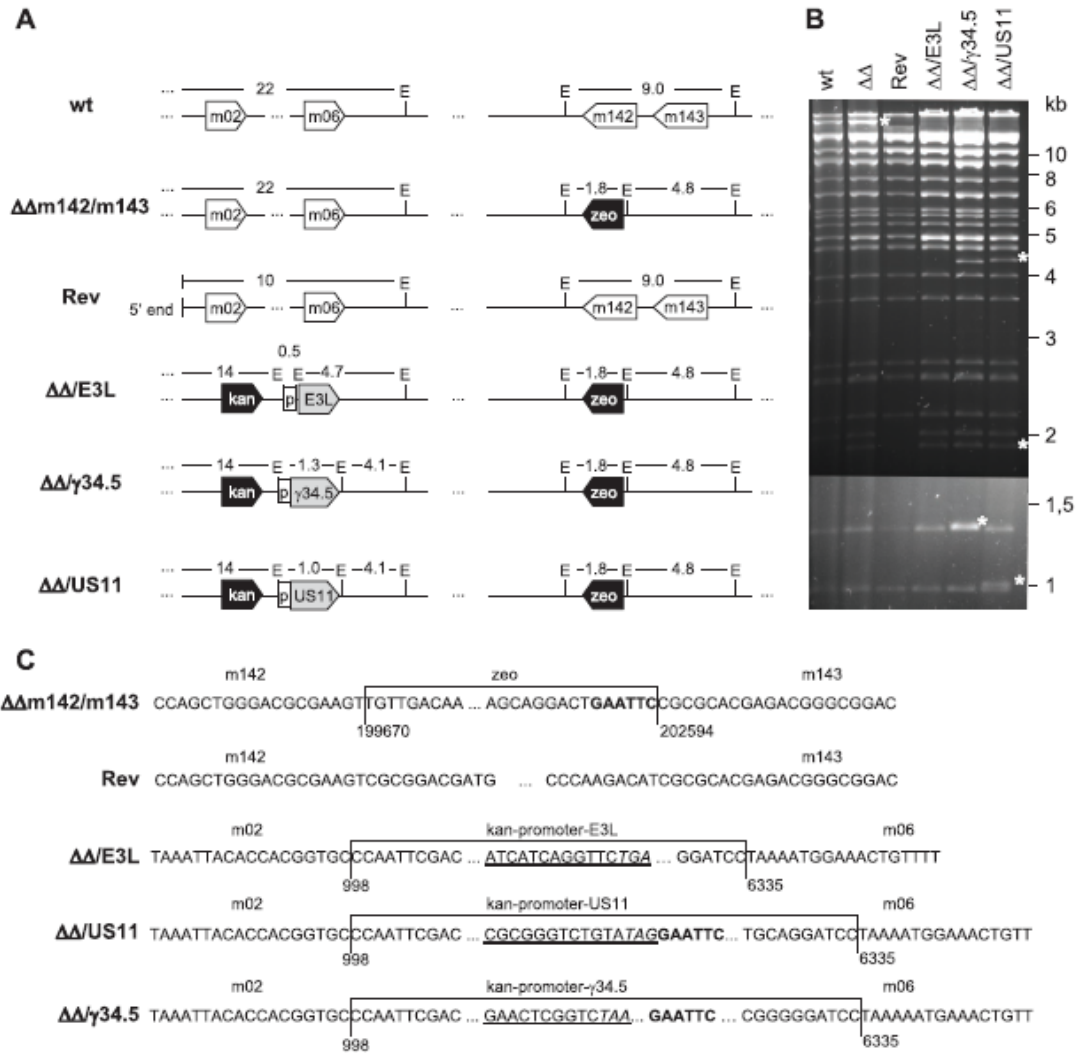
Figure 2. Construction of MCMV deletion and replacement mutants. (A) A GFP-expressing MCMV served as the parental wt virus for all mutants. Open reading frames m142 and m143 were deleted and replaced by a bacterial zeocin resistance gene (*zeo*), yielding MCMVΔΔm142/m143. The revertant virus, Rev m142/m143 (Rev), was constructed as described in Materials and Methods. The genes encoding the viral PKR antagonists E3L, γ34.5, or US11 were inserted at an ectopic location into the MCMVΔΔm142/m143 BAC, replacing open reading frames m02 to m06 and yielding MCMVΔΔm142/m143 expressing E3L, γ34.5, or US11 (ΔΔ/E3L, ΔΔ/γ34.5 or ΔΔ/US11, respectively). EcoRI (E) restriction sites and the expected fragment sizes are indicated. (B) The EcoRI restriction pattern of the parental and mutant genomes in an ethidium bromide-stained agarose gel is shown. Asterisks indicate altered fragments (shown in panel A) where they are clearly visible. Fragments of 4.8, 9, and 10 kb are difficult to identify as they comigrate with other genome fragments. Note that virion DNA was used for the revertant virus, as this virus was constructed by homologous recombination in fibroblasts. All other MCMV genomes are shown as BAC DNA. Consequently, Rev m142/m143 lacks two bands of approximately 2 and 6 kb containing the BAC cassette, which is removed during virus reconstitution. The 22-kb fragment containing the joined termini of the MCMV genome (in the wt and the ΔΔm142/m143 BAC) is also absent in Rev m142/m143 due to the linear structure of the virion DNA. The lower portion of the gel is shown as an overexposed image to visualize the smaller fragments. (C) The mutated sites of all five mutant viruses were verified by sequencing. The 3' end of the inserted genes (underlined; stop codon in italics) and the transitions between MCMV and inserted sequences are shown. Numbers indicate nucleotide positions within the MCMV genome. EcoRI restriction sites are shown in bold. p, phosphoglycerate kinase promoter.
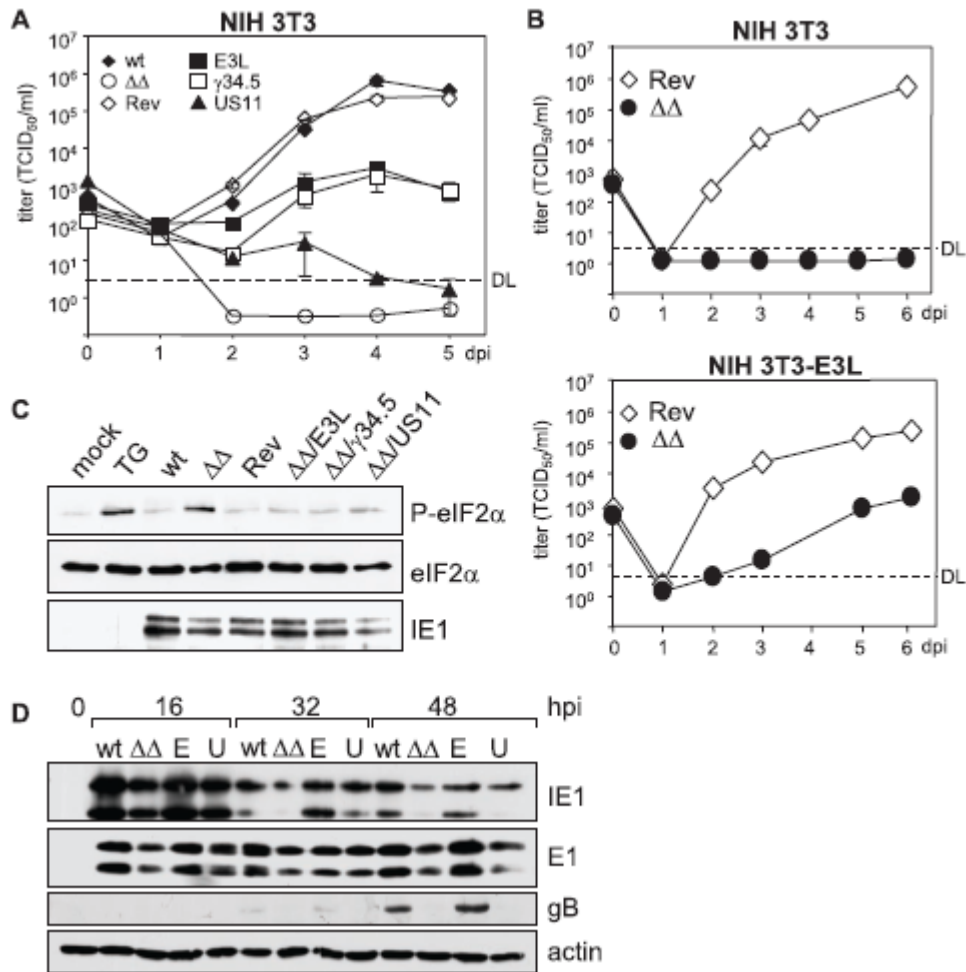
Figure 3. Rescue of MCMVΔΔm142/m143 by heterologous PKR antagonists. (A) Murine fibroblasts were infected at an MOI of 0.01 TCID50/cell with wt MCMV, MCMVΔΔm142/m143 (), MCMV Rev m142/m143 (Rev), or deletion mutants expressing E3L (ΔΔ/E3L), γ34.5 (ΔΔ/γ34.5) or US11 (ΔΔ/US11) proteins. Virus released into the supernatant was titrated on PKR-/- cells. DL, detection limit. (B) Parental NIH 3T3 cells and NIH 3T3 cells transduced with an E3L-expressing retrovirus were infected with MCMVΔΔm142/m143 or Rev m142/m143, and virus replication was analyzed as described above. (C) Murine fibroblasts were infected at an MOI of 5 TCID50/cell. Cell lysates were harvested 16 h later and probed by immunoblotting for phospho-eIF2α (P-eIF2α). Total eIF2α and the MCMV IE1 protein were detected in the lysates as loading and infection controls, respectively. (D) The expression of viral IE1, E1, and gB proteins was analyzed by immunoblotting with lysates from NIH 3T3 cells infected with 5 TCID50/cell of wt MCMV (wt), MCMVΔΔm142/m143 (ΔΔ), or the mutant expressing E3L (E) or US11 (U), respectively, for the indicated times. Actin is shown as a loading control.

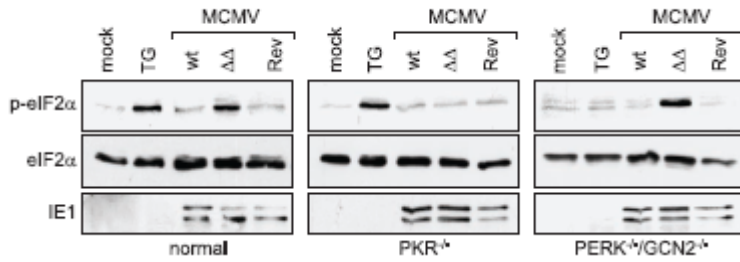Figure 4. eIF2α phosphorylation by PKR in MCMVΔΔm142/m143-infected cells. Normal, PKR-/-, or PERK-/- GCN2-/- fibroblasts were mock treated or stimulated with 1 µM TG for 1 h to induce PERK-mediated eIF2α phosphorylation (p-eIF2α). Infection of cells with wt MCMV or Rev m142/m143 (Rev) for 16 h (MOI of 5) did not induce eIF2α phosphorylation in the three cell types. Infection withMCMVΔΔm142/m143 (ΔΔ) induced eIF2α phosphorylation in normal and PERK-/- GCN2-/- cells but not in PKRdeficient fibroblasts. Total eIF2α and the MCMV IE1 protein were detected in the lysates as loading and infection controls, respectively.
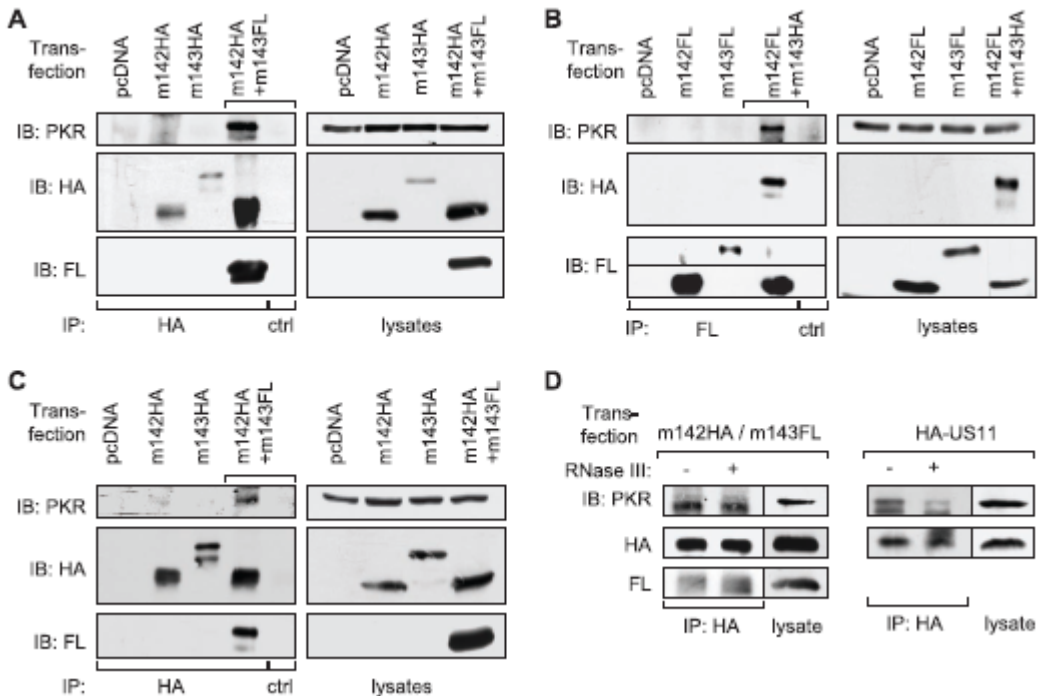

Figure 5. Interaction of m142 and m143 with PKR. Murine NIH 3T3 cells (A and B) or human 293 cells (C) were transfected with plasmids encoding HA- or FLAG-tagged m142 and/or m143. Polyclonal anti-HA (HA), anti-FLAG (FL), or control (ctrl) antibodies were used for immunoprecipitation (IP). Precipitates were analyzed by immunoblotting (IB) for the presence of m142, m143, and PKR. PKR was coprecipitated only in the presence of both the m142 and m143 proteins. (D) To analyze the dsRNA dependence of this interaction, 293 cells were either transfected with m142HA and m143FL or with HA-US11. The immunoprecipitates were incubated with or without dsRNA-specific RNase III, washed, and analyzed by immunoblotting. RNase III reduces the coprecipitation of PKR with US11 but not with the m142-m143 complex.
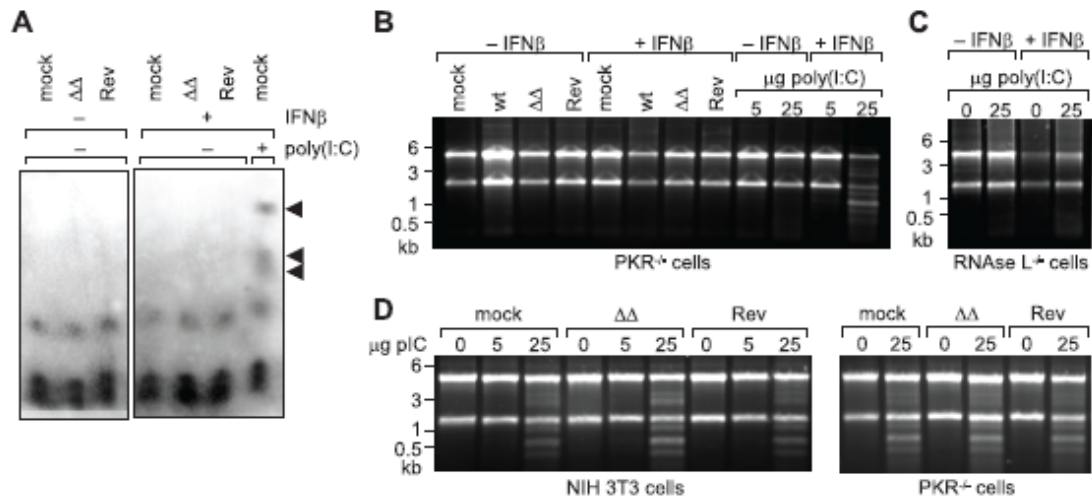
Figure 6. MCMV neither activates nor inhibits the OAS/RNase L pathway. (A) To analyze OAS activation during MCMV infection, PKR-/- fibroblasts were preincubated for 24 h with (+) or without (-) 500 U/ml IFN-β. Cells were then infected at an MOI of 5 TCID50/cell with viruses, as indicated in the figure. Cell lysates were harvested 16 h later and used in an OAS activity assay using [α^{32}P]ATP. The 2-5A enzyme was separated on a 20% polyacrylamide-urea gel and detected by autoradiography. As a positive control, OAS activity was stimulated by adding 5 µg/ml poly(I:C). (B) Poly(I:C) transfection but not MCMV infection induced cleavage of ribosomal 28S and 18S RNAs. PKR-/- cells were pretreated with IFN and infected with MCMV, as described in panel A, or transfected with poly(I:C). Total RNA was isolated and separated on denaturing formaldehyde-MOPS agarose gels. (C) rRNA was not cleaved in RNase L-/- cells, indicating that the rRNA degradation shown in panel B is RNase L specific. (D) IFN-pretreated NIH 3T3 or PKR-/- cells were infected with the indicated viruses for 16 h and transfected with different amounts of poly(I:C) (pIC). rRNA cleavage was analyzed 6.5 h after transfection. MCMV infection did not inhibit poly(I:C)-induced RNase L activation. ΔΔ, MCMVΔΔm142/m143; Rev, MCMV Rev m142/m143.
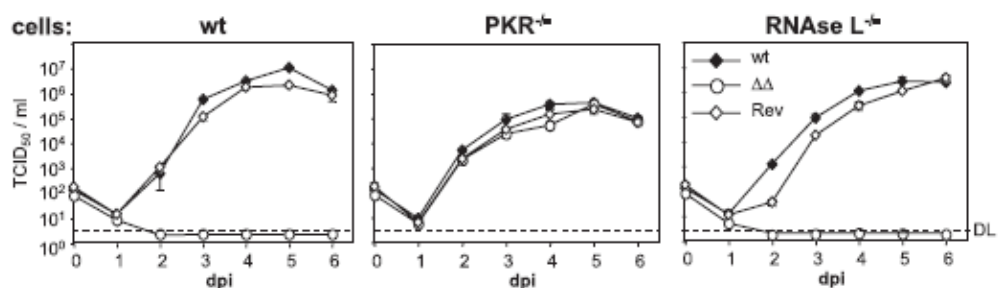

Figure 7. PKR, but not RNase L, controls replication of m142- and m143-deficient MCMV. PKR-/-, RNase L-/-, and wt fibroblasts were infected with wt MCMV, MCMVΔΔm142/m143 (ΔΔ), or MCMV Rev m142/m143 (Rev) at an MOI of 0.01 TCID50/cell. Virus titers in the supernatants were determined by titration on PKR-/- cells. DL, detection limit; dpi, days postinfection.
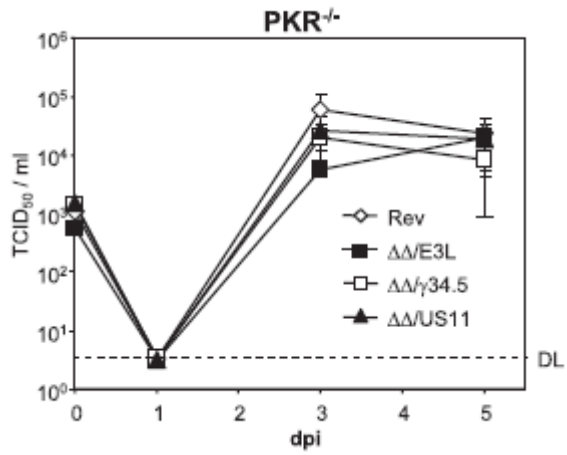
Figure 8. Normal replication of PKR antagonist-expressing MCMV in PKR-/- cells. Cells were infected with wt MCMV Rev m142/m143 (Rev) or MCMVΔΔm142/m143 expressing E3L (ΔΔ/E3L), γ34.5 (ΔΔ/γ34.5), or US11 (ΔΔ/US11) at an MOI of 0.1 TCID50/cell. Virus titers in the supernatants were determined by titration on PKR-/- cells. DL, detection limit; dpi, days postinfection.