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Double-Stranded RNA Binding by a Heterodimeric Complex of Murine Cytomegalovirus m142 and m143 Proteins

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In response to viral infection, cells activate a variety of antiviral responses, including several that are triggered by double-stranded (ds) RNA. Among these are the protein kinase R and oligoadenylate synthetase/RNase L pathways, both of which result in the shutoff of protein synthesis. Many viruses, including human cytomegalovirus, encode dsRNA-binding proteins that prevent the activation of these pathways and thereby enable continued protein synthesis and viral replication. We have extended these analyses to another member of the β subfamily of herpesviruses, murine cytomegalovirus (MCMV), and now report that products of the m142 and m143 genes together bind dsRNA. Coimmunoprecipitation experiments demonstrate that these two proteins interact in infected cells, consistent with their previously reported colocalization. Jointly, but not individually, the proteins rescue replication of a vaccinia virus mutant with a deletion of the dsRNA-binding protein gene E3L (VVΔE3L). Like the human cytomegalovirus dsRNA-binding protein genes TRS1 and IRS1, m142 and m143 are members of the US22 gene family. We also found that two other members of the MCMV US22 family, M23 and M24, encode dsRNA-binding proteins, but they do not rescue VVΔE3L replication. These results reveal that MCMV, like many other viruses, encodes dsRNA-binding proteins, at least two of which can inhibit dsRNA-activated antiviral pathways. However, unlike other well-studied examples, the MCMV proteins appear to act in a heterodimeric complex.

Double-stranded RNA (dsRNA) produced during viral infection activates several cellular antiviral responses (31, 36, 42, 47). Among the best characterized of these is the shutoff of protein synthesis mediated by protein kinase R (PKR) and oligoadenylate synthetase (OAS)/RNase L. Since viral replication depends on protein synthesis, many viruses have evolved mechanisms for counteracting the PKR and OAS/RNase L pathways (36). One such mechanism is the sequestration of dsRNA by a viral dsRNA-binding protein, such as pE3L, the product of the vaccinia virus (VV) E3L gene (22). Infection with VV lacking the E3L gene (VVΔE3L) results in activation of the PKR and OAS/RNase L pathways, shutoff of protein synthesis, little or no viral replication in most cell types, and very reduced virulence in infected animals (2–4, 9, 27).

We previously reported that human cytomegalovirus (HCMV) can rescue replication of VVΔE3L by blocking activation of the PKR and OAS/RNase L pathways (15, 16). Either of two related HCMV genes, TRS1 or IRS1, complements the replication defect of VVΔE3L. They also rescue a herpes simplex virus type 1 mutant lacking the γ34.5 gene, the product of which counteracts the PKR pathway by cooperating with protein phosphatase 1α to dephosphorylate eIF2α phosphate (11). TRS1 and IRS1 contain an unconventional dsRNA-binding domain at their identical amino termini that is necessary for rescuing VVΔE3L (19). However, the dsRNA-binding domain is not sufficient for this activity; a carboxy-terminal domain with unknown biochemical activity is also required.

In an effort to better understand the mechanisms and significance of evasion of dsRNA-mediated antiviral responses by herpesviruses of the β subfamily, we initiated a search for murine cytomegalovirus (MCMV) genes that could block dsRNA-activated antiviral cellular responses. Here, we report the identification of two such genes, m142 and m143, the products of which bind dsRNA and rescue replication of VVΔE3L. In contrast to other well-characterized viral dsRNA-binding proteins, m142 and m143 act in a complex to bind dsRNA and rescue VVΔE3L replication. Consistent with our results, Valchanova et al. report that m142 and m143 are each necessary to block PKR activation and enable ongoing protein synthesis necessary for MCMV replication (43).

MATERIALS AND METHODS

Cells and virus. BHK, NIH 3T3, and 293T cells were propagated as previously described (15, 20). VVΔE3L (2), obtained from Bertram Jacobs (Arizona State University), was propagated in BHK cells. MCMV strain Smith (VR-194) and MCMV MC.55, a recombinant of a strain derived from MCMV K181 that expresses green fluorescent protein (GFP) (provided by Jeff Vieira, University of Washington [44]) were propagated in NIH 3T3 cells. Infections were performed without centrifugal enhancement.

Plasmids. Plasmids expressing the MCMV US22 family members with optimal-context ATG codons and carboxy-terminal His tags (except for pEQ985 and pEQ939, which do not contain the His tag) were made by PCR amplifying each gene from the indicated MCMV cosmids (derived from MCMV strain Smith [VR-1399] and kindly provided by Barry Holwerda [18]) with the indicated oligonucleotide primers (Table 1). The resulting PCR products were cloned into pcDNA3.1/V5-His-TOPO using the TOPO-TA cloning system (Invitrogen). To generate pEQ1073, a plasmid that expresses pm142 with a carboxy-terminal His

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TABLE 1. Primers used to construct selected MCMV gene expression plasmids

Gene	Plasmid name	PCR template	5′ primer		3′ primer	
			No.	Sequence	No.	Sequence
m128	pEQ1039	pBH341	542	GGATCCACCATGGAGCGTGTTCGGGGAGCT	543	CTGAATCTTCTTCCTGACGGT
m139	pEQ1040	pBH341	544	GGATCCACCATGTGGTCTCTCCGCGGCGCC	545	GCGATCGGAGCCGGTTTCCAT
m140	pEQ1041	pBH341	378	ACCATGGACAGCACACTGTGGGAG	379	CGGTCTGCAGAGTCTGAGCGG
m141	pEQ1042	pBH341	546	GGATCCACCATGGAGAGGCCGGTGACGGCG	547	GGTCTCCGAGGGACCCGGCC
m142	pEQ985	pBH341	461	ACCATGGACGCCCTGTGCGC	462	GTCAGTCGTCATCGTCGGCGT
m143	pEQ939	pBH341	410	ACCATGTCTTGGGTGACCGGAGAT	411	GAGAGATGACATGTCGTCACA
M23	pEQ1045	pBH303	550	GGATCCACCATGGCGCCTCCGGAAGCTCTC	551	CCTCCTTTTGATGGAGCGCAT
M24	pEQ1046	pBH303	552	GGATCCACCATGGATTCGACGGGGAAAATC	553	GAACTCGCTGAGGCGCGGGG
m25.1	pEQ1047	pBH303	554	GGATCCACCATGTGGGGACGACAGGTTCTT	555	GTGTCTGACTTTATTGATATC
m25.2	pEQ1048	pBH303	556	GGATCCACCATGCCTGCTTCATCATGTACG	557	GACCGCTTTCAAAAAGAACCT
M36	pEQ1049	pBH400	558	GGATCCACCATGTATGAGCAAGAGGAACAA	559	TCGATATCCCCGTGTCATCTT
M43	pEQ1050	pBH400	560	GGATCCACCATGACAACGACGTTGACGGGG	561	TTGATGTCGGCAACACACCGT

tag, the m142 open reading frame was PCR amplified from pEQ985 using primers 461 (Table 1) and 548 (GTCGTCATCGTCGGCGTCCGC) and cloned as described above. Following cloning of the m142 insert, the vector was digested with XhoI and XbaI, and a biotinylation signal (5) was introduced by ligating in annealed oligonucleotides 519 (TCGATGGCTGGTGGCCTCAACGACATCT TCGAGGCCCAGAAGATCGAGTGGCATGAA) and 520 (CTAGTTCATG CCACTCGATCTTCTGGGCCTCGAAGATGTCGTTGAGGCCACCAGCCA). Plasmid pEQ985 was used in some experiments (see Fig. 2, 4, and 6); pEQ1073 was used in others (see Fig. 3).

Due to a difference we detected in the sequence of m143 compared to the published sequence (see below), our first attempt at producing a vector expressing His-tagged pm143 yielded pEQ1072 (see Fig. 3), a plasmid that expresses untagged pm143, similar to that expressed by pEQ939 (see Fig. 2, 3, and 6).

Two different VV E3L expression vectors were used as positive controls. Plasmid pMTE3L (13), used in VVΔE3L rescue experiments, was kindly provided by Bertram Jacobs. For dsRNA-binding experiments, we utilized pEQ843, which was generated by PCR amplification of E3L from VV wild-type DNA using primers 270 (AGTTCTCTACCACCATGGCTAAGATCTA) and 271 (G ATAACTAGAATCAGAATCT) and then cloning the insert as described above.

The HCMV TRS1 protein was also used as a positive control in these experiments. Three different TRS1-expressing vectors were utilized, each of which yielded similar results in all assays. Plasmid pEQ902 (see Fig. 2), which encodes a full-length, untagged version of TRS1, was PCR amplified from HCMV rTowne-1 (1) genomic DNA using oligonucleotides 356 (GCCTCGACGTCGG ATCCGTCCGGCGGCCATGGCC) and 357 (CACAGAATTCTCGTAAGCA TGTTGACAACTG) and cloned as described above. Plasmid pEQ876 (see Fig. 6), which contains the same version of TRS1 PCR amplified from a cloned 3-kb fragment of HCMV rTowne-1 has been described previously (19). Plasmid pEQ1069 (see Fig. 3) encodes a fully functional His-tagged carboxy-terminal truncation of TRS1 (missing codons 710 to 795) that also possesses a biotinylation signal (see above).

The negative control plasmids included pEQ879 (19), which served as an empty vector control, and pEQ1100, which expresses GFP containing both a biotinylation signal and a carboxy-terminal His tag. This plasmid was constructed by removing GFP from pEGFP-1 (Clontech) using restriction enzymes HindIII and BsrGI (blunted with Klenow) and cloning the insert into pcDNA3.1/V5-His-TOPO (containing the biotinylation signal described above) after digestion with HindIII and EcoRV.

Transient transfection and rescue of VVΔE3L replication. Triplicate wells of 293T cells at 70 to 90% confluence in 24-well plates were transfected with plasmid DNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. At 24 h posttransfection, the cells were infected with VVΔE3L (multiplicity of infection [MOI] = 3), which contains a late promoter: *lacZ* cassette in place of the E3L gene, enabling viral replication to be measured by β-galactosidase (β-Gal) activity. At 24 h postinfection, β-Gal activity was measured by a 4-methylumbelliferyl β-D-galactoside fluorometric cleavage assay by replacing the cell medium with medium containing 4-methylumbelliferyl β-D-galactoside (0.15 mg/ml) and detecting fluorescent cleavage products on a Fluoroskan Ascent plate reader (ThermoLabsystems).

Immunoprecipitation (IP) and immunoblot analysis. For immunoblot analyses to detect pm142 and pm143 from mock- or MCMV-infected (MOI = 1) NIH 3T3 cells, or from 293T cells transfected with pm142- and pm143-expressing constructs and then infected with VVΔE3L at 24 h posttransfection, cell lysates were harvested at 24 h postinfection in buffer A (100 mM KCl, 20 mM HEPES [pH 7.5], 10% glycerol, 5 mM MgOAc, 1 mM dithiothreitol, 1 mM benzamidine [Sigma]) plus 1% NP-40, and the nuclei were removed by centrifugation at 500 × *g* for 3 min. Equivalent amounts of cytoplasmic lysates before or after binding to dsRNA agarose (see below) were separated on 10% or 12% polyacrylamide gels and then transferred to either nitrocellulose or polyvinylidene difluoride (PVDF) membranes by electroblotting. The membranes were incubated with rabbit polyclonal antisera directed against pm142 and pm143 (20). Proteins were detected using the Western-Star chemiluminescent detection system (TROPIX, Inc.) according to the manufacturer's recommendations.

Harvesting and detection of His-tagged proteins from 293T cells transfected with the indicated expression vectors and infected with VVΔE3L 24 h later were carried out by harvesting cells in 2% sodium dodecyl sulfate, separation of equivalent amounts of protein on a 12% polyacrylamide gel, transfer to a PVDF membrane, and chemiluminescent detection as described above using Penta · His antibody (QIAGEN) according to the manufacturer's recommendations.

For immunoprecipitation followed by immunoblot analysis, NIH 3T3 cells were mock infected or infected with MCMV (MOI = 2). At 24 h postinfection, the cells were washed and harvested in IP lysis buffer as described previously (20). Immunoprecipitations were performed with the indicated rabbit antisera and TrueBlot anti-Rabbit Ig IP beads (eBioscience, San Diego, CA) using precipitation and wash conditions as described previously (20). The washed complexes were separated on a 12.5% polyacrylamide gel and analyzed by immunoblot assays carried out using the Rabbit IgG TrueBlot kit (eBioscience) according to the manufacturer's directions. Proteins were detected using the Immun-Star Chemiluminescent kit (Bio-Rad).

Radiolabeling of cell proteins. NIH 3T3 cells were mock infected or infected with MCMV (MOI = 1). At 24 h postinfection, the cells were [^{35}S]methionine labeled (Translabel; 100 μCi/ml; MP Biomedicals, Inc.) for 4 h in medium lacking methionine. The cells were then washed in phosphate-buffered saline and lysed in buffer A (see above) plus 1% NP-40. The nuclei were removed by centrifugation at 500 × *g* for 3 min, and the resulting cytoplasmic extracts were used for dsRNA-binding assays.

In vitro transcription and translation. Plasmids encoding MCMV US22 family members or the indicated controls were linearized with XhoI or XbaI and transcribed using the Ampliscribe T7 Transcription kit (Epicenter Technologies). The resulting RNAs (and a luciferase control RNA from the Ampliscribe kit) were in vitro translated using Promega's Rabbit Reticulocyte Lysate System in the presence of 1 mCi/ml [^{35}S]methionine (Translabel; MP Biomedicals, Inc.) according to the manufacturer's recommendations.

Double-stranded RNA-binding assays. dsRNA [poly(rI · rC)] agarose beads were prepared as described previously (28). Radiolabeled cell extracts (50 μl) or in vitro-translated proteins (5 μl) in 250 μl total volume of buffer A plus 1% NP-40 (see above) were incubated with dsRNA agarose beads (plus carrier Sepharose CL-6B [Sigma-Aldrich]) or with Sepharose CL-6B alone as a binding control for 1 h at 4°C on a rotating mixer. After binding, the beads were pelleted (16,000 × *g*; 3 min) and washed in buffer A (~1 ml) four times. For competition assays, extracts were preincubated in buffer A with the indicated free competitor (50 to 100 μg) for 30 min on a rotating mixer prior to addition of poly(I · C) agarose or Sepharose CL-6B beads.

All samples were brought up to 20 μl final volume in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, denatured at 95°C for 5 min, and separated on 12% polyacrylamide gels; the gels were dried, and the proteins

were visualized by autoradiography. Lysate corresponding to 1/10 the amount used for each binding reaction was analyzed alongside each set of binding reactions.

RESULTS

Identification of pm142 and pm143 as dsRNA-binding proteins. Having previously identified two HCMV dsRNA-binding proteins that counteract the important host cell antiviral pathways (15, 19), we initiated a search for MCMV genes that serve a similar function. We used a dsRNA agarose pull-down assay that has been used to demonstrate dsRNA binding by proteins such as pE3L and pTRS1 (19, 28). In MCMV-infected cells, we detected two prominent proteins that bound poly(I · C) agarose beads (Fig. 1). In competition assays, preincubating the extracts with unbound poly(I · C) (Fig. 1) reduced the binding of these proteins to the dsRNA agarose. In contrast, preincubation with dsDNA did not reduce the binding (data not shown). Thus, these proteins appear to bind selectivity to dsRNA. The absence of similar proteins in uninfected cells indicated that the two are either encoded or induced by MCMV.

The two HCMV dsRNA-binding protein genes, TRS1 and IRS1, have been classified as members of the US22 gene family, a group of 12 genes present in both HCMV and MCMV that share one or more short sequence motifs (14, 32, 38). Based on similarities between HCMV and MCMV US22 family members (20, 32) and comparisons of the predicted sizes of the MCMV US22 family members with the masses of the observed dsRNA-binding proteins (~50 kDa and ~62 kDa), we hypothesized that the dsRNA-binding proteins might be products of the m142 and m143 genes (pm142 and pm143, respectively). In support of this hypothesis, we found that pm142 and pm143 synthesized by in vitro transcription and translation comigrated with the dsRNA-binding proteins in MCMV-infected cells (Fig. 1A).

To establish more conclusively the identities of these proteins, we performed the dsRNA agarose pull-down assays using unlabeled extracts from uninfected and MCMV-infected cells and analyzed the binding proteins using antisera specific for pm142 and pm143. These immunoblot assays revealed that pm142 and pm143 bound to dsRNA agarose beads in the absence, but not in the presence, of dsRNA competitor (Fig. 1B). As expected, no immunoreactive bands were detected in uninfected cells. These results support the conclusion that pm142 and pm143 are dsRNA-binding proteins.

Alternative m143 sequence. During the course of these studies, we were puzzled to find that plasmids designed to express m143 with a carboxy-terminal epitope tag failed to express the tagged pm143, even though they did express a protein of the size expected for the full-length untagged pm143 (data not shown). Sequence analyses revealed a discrepancy between our nucleotide sequence and that reported for MCMV strain Smith in GenBank (accession no. NC_004065). A cytidine at nucleotide 1192 (from the A of the initiator ATG) was present in our sequence but absent from the GenBank sequence (where it would correspond to a guanidine inserted between nucleotides 201402 and 201403). Thus, compared to the GenBank sequence, our m143 reading frame shifts into an alternative frame after codon 397. The termination codons

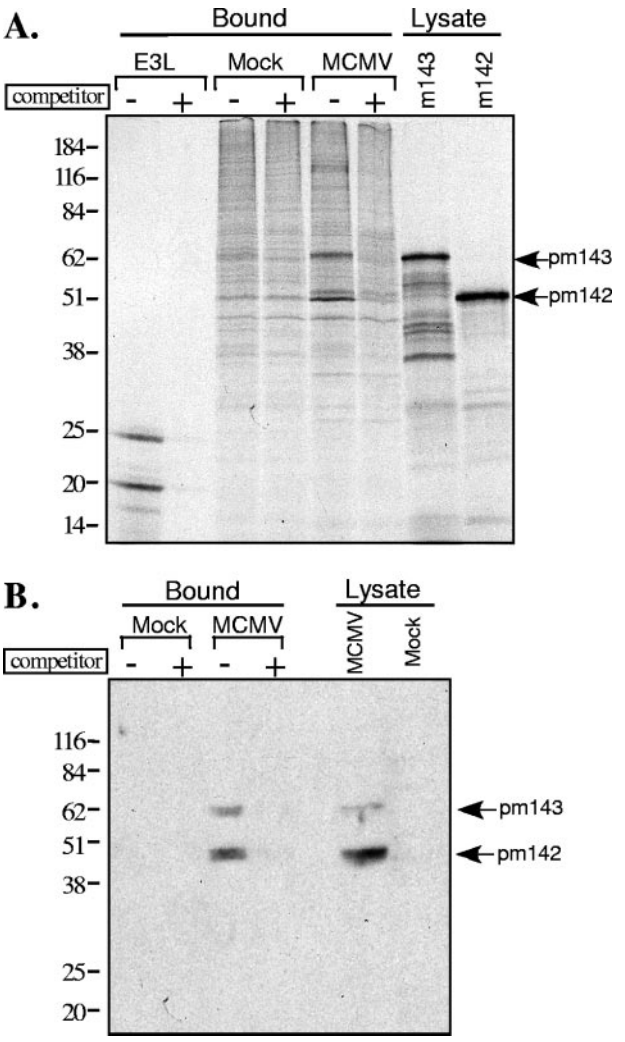

FIG. 1. MCMV pm142 and pm143 are dsRNA-binding proteins. (A) [^{35}S]methionine-labeled lysates from NIH 3T3 cells that had been mock infected (Mock) or infected with MCMV MC.55 at an MOI of 1 PFU/cell, as described in Materials and Methods, were incubated with poly(I · C) agarose in the presence or absence (+ or −) of free poly(I · C) competitor. In vitro-translated VV E3L protein was also included in the same binding assay as a positive control. After binding for 1 h, the beads were washed extensively to remove unbound protein, and the bound proteins were analyzed by gel electrophoresis and autoradiography alongside in vitro-translated pm142 and pm143, as described in Materials and Methods. (B) Unlabeled lysates from mock-infected or MCMV-infected NIH 3T3 cells were incubated with poly(I · C) agarose in the presence or absence of competitor and separated by gel electrophoresis, along with whole-cell lysates as in panel A. The samples were then transferred to nitrocellulose, and immunoblot analysis was carried out using antisera directed against pm142 and pm143, as described in Materials and Methods.

predicted from the GenBank sequence and our sequence are only 43 nucleotides apart, and the sizes of the two proteins are predicted to be quite similar, explaining the fortuitous observation that the size of the larger of the two dsRNA-binding proteins (~62 kDa) was similar to that predicted by the GenBank sequence (63.6 kDa), as well as by our sequence (61 kDa).

We detected the extra cytidine in a plasmid derived from

MCMV strain Smith (VR-194) (12), a cosmid derived from MCMV strain Smith (VR-1399), and a plaque-purified subclone of strain Smith (VR-194) (18) and in viral DNA isolated from cells infected with MCMV strain Smith (VR-194) (data not shown). Plasmids designed with the assumption that the cytidine was present expressed proteins with the expected carboxy-terminal epitope tag (data not shown). Consistent with our results, an in silico analysis of rodent CMV genomes identified an alternative reading frame at the m143 locus, suggestive of a frameshift within the m143 open reading frame (10). Valchanova et al. detected the same extra nucleotide in their strain, derived from the Smith strain (VR-194) (43), which is also the strain used to determine the nucleotide sequence of MCMV that is reported in GenBank. These findings led us to suspect that the GenBank sequence is incorrect, but we cannot rule out the possibility that m143 gene variants existed in the original MCMV Smith (VR194) stock or arose during passage of the virus in different laboratories.

Additional US22 family dsRNA-binding proteins. The finding that the products of four US22 genes, TRS1, IRS1, m142, and m143, all bind dsRNA led us to investigate the possibility that dsRNA binding is a shared property of the US22 gene family. We cloned each of the US22 family members from MCMV into an expression vector, transcribed and translated the proteins in vitro, and analyzed their dsRNA-binding activities using the dsRNA pull-down assay. In these experiments, we used agarose beads lacking dsRNA as a control for nonspecific binding. As expected, the controls pTRS1 and pE3L bound to dsRNA (Fig. 2). The MCMV proteins pm128, pm139, pm140, pm141, pm142, pm25.1, pm25.2, and pM36 each bound minimally or undetectably, similar to the negative control luciferase. In this experiment, pm143 and pM43 bound to dsRNA-agarose beads, but compared to the input, the fraction that bound was considerably less than that of the pE3L or pTRS1 control. Two proteins, pM23 and pM24, bound to dsRNA to almost the same extent as the positive controls. These results reveal that a subset, but not all, of the US22 family members bind dsRNA, and an unexpectedly small fraction of pm142 and pm143 bound under these conditions.

Cooperative dsRNA binding by pm142 and pm143. The apparent discrepancy in the results shown in Fig. 1 (in which pm142 and pm143, expressed in MCMV-infected cells, bound well to dsRNA) and Fig. 2 (where the same proteins bound poorly after synthesis by in vitro transcription and translation) could be due to several differences in the experiments. For example, the proteins made in infected cells might be posttranslationally modified or alternatively folded compared to those made by cell-free translation. The observation that the proteins made by both methods comigrated (Fig. 1A) suggested that there were not large differences in posttranslational modifications. Another possibility, suggested by the previously reported colocalization of m142 and m143 in confocal-immunofluorescence studies (20), was that the two proteins bound to dsRNA in a cooperative manner.

To investigate this possibility, we translated m142 and m143 RNAs in cell extracts either individually or together. Translating them as a mixture greatly enhanced the fraction of the input that bound to dsRNA compared to the proteins synthesized individually (Fig. 3). Similar results were obtained by mixing pm142 and pm143 after they were first synthesized in

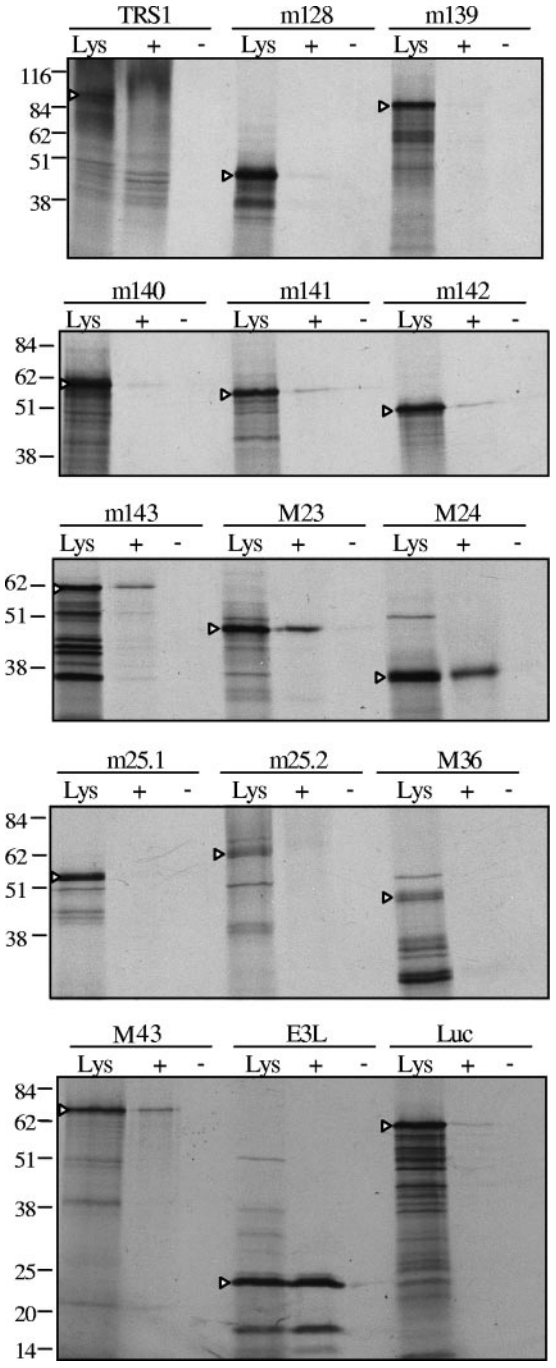

FIG. 2. Analysis of dsRNA-binding by MCMV US22 family members. The MCMV US22 family members were cloned into a T7 expression vector, in vitro transcribed and translated in the presence of [^{35}S]methionine, and tested for the ability to bind dsRNA by incubation with poly(I · C) agarose (+) or unconjugated agarose beads (−) as a negative control, as described in Materials and Methods. TRS1 and VV E3L were used as positive binding controls, and luciferase (Luc) was used as a negative control for these assays. After being extensively washed to remove unbound protein, the resulting bound samples were analyzed by gel electrophoresis and autoradiography, along with lysate (Lys) corresponding to 1/10 the amount of sample used for binding. The arrowheads indicate the expected size of each protein.

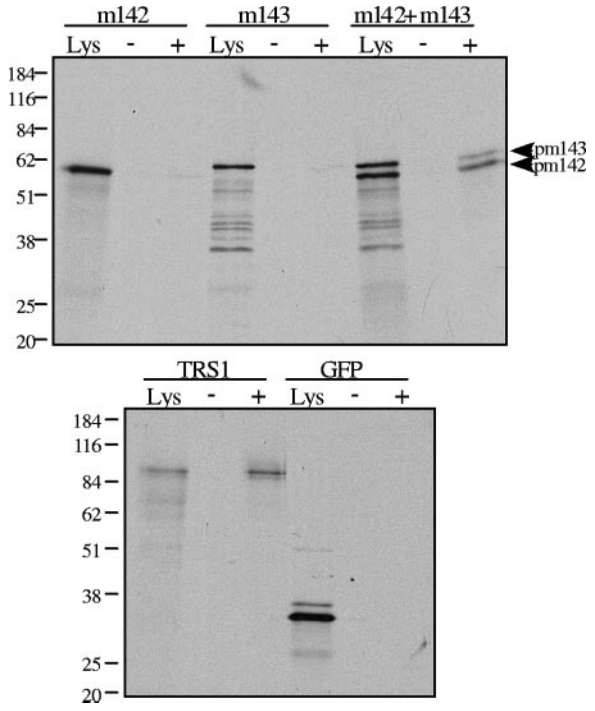

FIG. 3. Binding of pm142 and pm143 to dsRNA is cooperative. Plasmids containing m142 and m143 were in vitro transcribed and then translated, either singly or in combination, and tested for the ability to bind poly(I · C) agarose (+) or unconjugated agarose beads (−; negative control), as described in Materials and Methods. The samples were then separated by gel electrophoresis and visualized by autoradiography. Lysate (Lys) lanes contained 1/10 the amount of sample used for binding. TRS1 was used as a positive control and GFP as a negative control.

independent reactions (data not shown). As expected, pTRS1 bound and pGFP did not bind dsRNA-agarose under these conditions.

We evaluated the nucleic acid binding specificity of the combination of pm142 and pm143 by adding competitor nucleic acids prior to adding the poly(I · C) beads. Incubation of the proteins with a mixture of poly(I) and poly(C) or poly(A) and poly(U) greatly reduced the binding of pm142 and pm143 to dsRNA-agarose, while none of the single homopolymers competed (Fig. 4). We observed a puzzling but reproducible increase in pm142 plus pm143 binding following addition of poly(I) compared to no competitor in this and several other experiments. Although we do not know the basis for this observation, it might result from the added soluble poly(I) hybridizing to poly(C) on the beads that might otherwise be in excess and partially single stranded, thereby increasing the total amount of dsRNA on the beads. dsDNA also did not compete for binding. These results reveal that, like pTRS1 and pE3L, pm142 and pm143 bind selectively to dsRNA and not to single-stranded RNA or dsDNA.

pm142 and pm143 interact in vivo. Previous studies demonstrated that pm142 and pm143 colocalize in infected cells (20). Having found that the two proteins bind dsRNA much more efficiently in combination than individually, we next investigated the hypothesis that they form a complex during viral infection. Cells were mock infected or infected with MCMV.

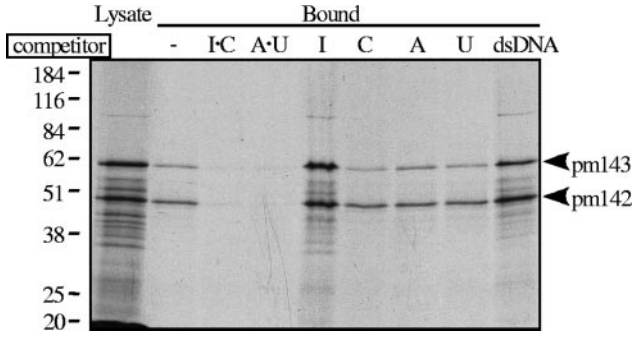

FIG. 4. The binding activities of pm142 and pm143 are specific for dsRNA. Plasmids containing m142 and m143 were in vitro transcribed and then cotranslated in the presence of [^{35}S]methionine. The resulting lysates were analyzed for the ability to bind poly(I · C) agarose in the absence (−) or presence of the indicated competitors, as described in Materials and Methods.

At 24 h postinfection, lysates were prepared and immunoprecipitated with antisera specific for pm142 or pm143, and the immunoprecipitates were analyzed by immunoblot assays. As shown in Fig. 5, immunoprecipitation with anti-pm142 serum precipitated pm143. Antiserum to pm143 also immunoprecipitated pm142. Neither preimmune serum nor antiserum directed against pm139 immunoprecipitated either pm142 or pm143. We do not know why antiserum to pm143 precipitated pm142 more completely than the low abundance of pm143 immunoprecipitated by pm142 antiserum. One possibility is that most or all of the pm142 is present in a complex with pm143 but only a small fraction of pm143 is in the complex. Regardless, these results reveal that pm142 and pm143 physically interact in infected cells.

Rescue of VVΔE3L replication by m142 and m143. Since the HCMV dsRNA-binding protein genes TRS1 and IRS1 rescue VVΔE3L replication by blocking activation of the PKR and OAS/RNase L pathways, we investigated whether m142 and/or m143 and the other MCMV US22 family members that bind dsRNA in our pull-down assays have similar functions. After transfecting expression plasmids into cells, we infected the cells with VVΔE3L. Since VVΔE3L has a *lac*Z cassette driven by a vaccinia virus late gene promoter, we measured β-Gal production to monitor the rescue of VVΔE3L (16). In initial experi-

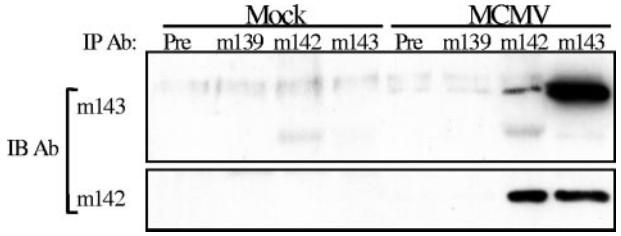

FIG. 5. Coimmunoprecipitation of pm142 and pm143. NIH 3T3 cells were mock infected or infected with MCMV strain Smith (VR-194) at an MOI of 2 PFU/cell. At 24 h postinfection, the cell lysates were harvested and immunoprecipitation was performed with the indicated antisera, as described in Materials and Methods. The samples were separated by gel electrophoresis and transferred to nitrocellulose, and immunoblot (IB) analysis was carried out using the indicated antisera directed against pm142 or pm143. Ab, antibody.

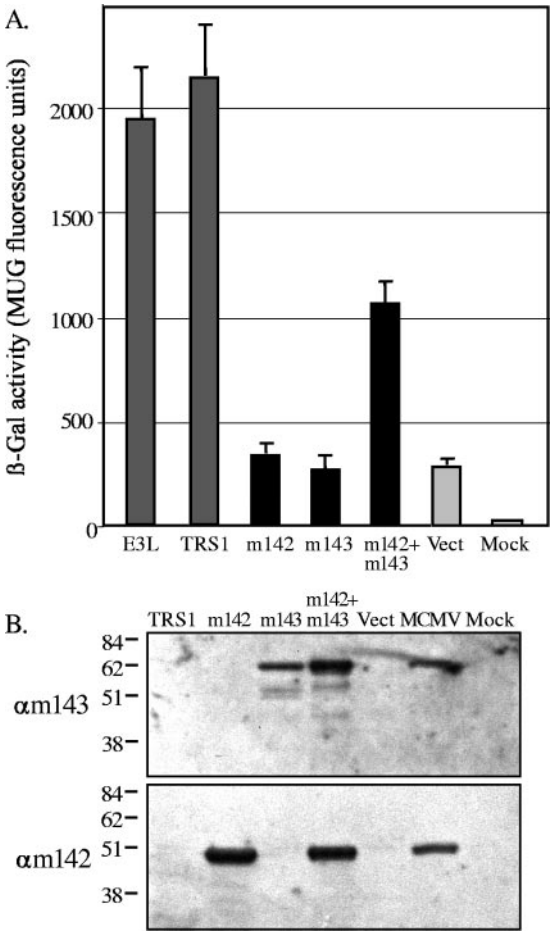

FIG. 6. Cotransfected m142 and m143 rescue VVΔE3L replication. (A) 293T cells were transfected with m142 and m143 alone or in combination and with E3L or TRS1 as positive controls and empty vector as a negative control. At 24 h posttransfection, the cells were mock infected (Mock) or infected with VVΔE3L, and at 24 h postinfection, β-Gal activity was measured as described in Materials and Methods. The means plus standard deviations for triplicate samples are shown. (B) Whole-cell lyates derived from pooled triplicate samples from panel A, along with an MCMV-infected cell lysate, were separated by gel electrophoresis and transferred to a PVDF membrane, and immunoblot analyses were carried out on parallel samples using the indicated antisera directed against pm142 and pm143, as described in Materials and Methods.

ments, we found that transfected plasmids failed to express detectable pm143 in HeLa cells (data not shown), so we performed these experiments in 293T cells.

In contrast to the TRS1 control, neither m142 or m143 rescued VVΔE3L replication when expressed individually (Fig. 6). However, in combination, m142 and m143 did rescue VVΔE3L replication. The lower rescue efficiency compared to TRS1 or E3L might be due to differences in protein expression or to some cells receiving one but not both plasmids. Alternatively, the apparent necessity for the two proteins to interact and form a functional complex may result in a lower efficiency of rescue than that achieved by the controls. The magnitudes of the rescue of VVΔE3L varied among experiments, but we detected the same effect of the combination of m142 and m143 using several independent plasmid constructs, with and without epitope tags (data not shown). The other MCMV US22 family genes that encode putative dsRNA-binding proteins, M23 and M24, did not rescue VVΔE3L either individually or in combination with m142 or m143 (data not shown). These results reveal that m142 and m143 act together to rescue VVΔE3L.

DISCUSSION

Infection by many viruses stimulates the production of dsRNA, which activates several host cell antiviral responses. The origins of the dsRNAs are thought to include genomes and replication intermediates for RNA viruses and annealed bidirectional overlapping transcripts from DNA viruses, such as herpesviruses (41, 47). Single-stranded RNAs with secondary structure, such as might be prevalent among RNAs expressed by viruses with GC-rich genomes, like cytomegaloviruses, can also activate PKR (6, 8, 34). In addition to the PKR and OAS/RNase L pathways, antiviral responses mediated by toll-like receptor 3 (TLR3), retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated gene 5 (mda-5) signaling pathways are also triggered by dsRNA (42). Short interfering RNA and microRNAs are processed from longer dsRNAs or RNAs with hairpins, which may mimic dsRNA, and in some cases these contribute to the antiviral state (29, 31).

In response to these cellular defenses, many, if not all, viruses have evolved countermeasures. Viruses from diverse families carry genes that block the PKR and OAS/RNase L pathways, and in several cases, these genes have been shown to be necessary for viral replication and pathogenesis. One of the most common and well-characterized mechanisms is the binding of dsRNA by proteins such as VV pE3L, influenza virus NS1, reovirus σ3, and herpes simplex virus type 1 pUS11 (41). Binding to these proteins appears to neutralize the ability of dsRNA to activate cellular antiviral responses.

Several members of the herpesvirus family encode dsRNA-binding proteins (25, 37). Our finding that HCMV infection could rescue VVΔE3L replication led to identification of two related dsRNA-binding proteins encoded by HCMV (15, 16). We are not sure why the analogous approach—testing whether MCMV could rescue VVΔE3L—was unsuccessful (data not shown). Dual infections with large viruses are potentially very complex. For example, MCMV infection inhibited the replication of wild-type VV (data not shown), and this effect may have obscured complementation of VVΔE3L by MCMV. Poor expression of pm143 in some cell types, as we observed in transfection studies, may also have contributed to our inability to detect the rescue of VVΔE3L by MCMV.

Analyses of MCMV-infected cells revealed the presence of two dsRNA-binding proteins, which we identified as pm142 and pm143. Notably, these proteins, when made by cell-free translation, did not bind well to dsRNA-agarose when tested individually but did when tested in combination. This cooperative binding contrasts with other viral dsRNA-binding proteins, such as NS1, σ3, and pE3L, which bind to dsRNA as homodimers (21, 35, 45, 46). In this respect, the MCMV genes also differ from the HCMV TRS1 and IRS1 genes, which function independently (15). Several additional arguments support the conclusion that pm142 and pm143 function as a complex. Previous studies using confocal fluorescence micros-

copy demonstrated that they colocalize in cells (20). We found that they also coimmunoprecipitate, and both are needed to rescue VVΔE3L replication. We do not yet know the stoichiometry of the putative pm142-pm143 complex, although optimal rescue of VVΔE3L results from transfection of equal amounts of each plasmid (data not shown). We also do not know if additional proteins are present in the complex. However, since the two proteins functionally interact in cotransfected cells and in reticulocyte lysates and colocalize in the absence of additional viral proteins (Fig. 3) (20), it seems most likely that their interaction either does not require additional partners or requires only ubiquitous factors.

The m142 and m143 genes are each essential for viral replication (32). Our results suggest the hypothesis that together they serve an essential function, which may be evasion of dsRNA-activated antiviral pathways. In support of this interpretation, Valchanova et al. found that infection with MCMV mutants lacking either m142 or m143 resulted in activation of PKR, phosphorylation of eIF2α, and inhibition of protein synthesis and viral replication (43). In the case of HCMV, neither TRS1 nor IRS1 is essential (7, 17, 23), perhaps because each one can block the PKR and OAS/RNase L pathways independently of the other. Indeed, TRS1 partially complemented the phenotypes of m142 and m143 MCMV mutants (43).

An unusual feature of the β-herpesvirus subfamily is the presence of gene families. Although precise criteria for inclusion in a family are not exact (39), TRS1, IRS1, m142, and m143 have all been considered members of the US22 family (14). Because none of the encoded proteins contain sequence signatures associated with conventional dsRNA-binding proteins and we have not yet finely mapped the dsRNA-binding domain, we could not predict whether other US22 family members might also bind dsRNA and block cellular dsRNA-activated pathways. Empirical analyses of the other MCMV US22 family members revealed that two others, pM23 and pM24, bind dsRNA under our dsRNA-agarose pull-down assay conditions. Other members of the gene family did not bind to dsRNA under these conditions, suggesting that their functions, such as blocking apoptosis in the case of M36 (32), are unlikely to be due to dsRNA-binding activity. Even though M23 and M24 bound dsRNA, they did not rescue VVΔE3L replication, either alone or in combination with m142 or m143 (data not shown). Like pm142 and pm143, at least one other set of US22 family members (m139, m140, and m141) (24) act as a complex, so it is conceivable that other combinations of US22 genes could rescue VVΔE3L replication. However, deletion of each of these other US22 genes results in viable virus (32), suggesting that none of the encoded proteins contribute to any complex that is required for replication in cell culture.

Our studies reinforce the conclusion that dsRNA-binding activity is not always sufficient for a protein to block the PKR and OAS/RNase L pathways. In addition to the carboxy-terminal dsRNA-binding domain of E3L, an amino-terminal domain that binds PKR is needed in order to restore growth of PKR-expressing yeast (40) and for full inhibition of eIF2α phosphorylation after vaccinia virus infection (26). The dsRNA-binding domains of the products of the HCMV TRS1 and IRS1 genes are necessary but not sufficient to block the cellular pathways (19). Consistent with these observations, M23 and M24 bind dsRNA but do not rescue VVΔE3L replication.

In addition to its role in blocking viral replication by activating the PKR and OAS/RNase L pathways, dsRNA can signal through TLR3, RIG-I, and mda-5 to activate cellular defenses. The dsRNA-like qualities of premicroRNAs and the possible antiviral role of the mature microRNAs raise the possibility that some dsRNA-binding members of the US22 family may operate in countering these pathways, as is known to occur with E3L and influenza NS1 (30). CMVs have a large number of genes that function in blocking antiviral responses, including cell-autonomous responses, such as apoptosis and translational shutoff, as well as innate and adaptive cellular responses (33). Thus, it would not be surprising if further studies reveal still more antiviral pathways that are targets of CMV genes.

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