1   Binding and Relocalization of PKR by Murine Cytomegalovirus

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24   **ABSTRACT**

25   Many viruses have evolved mechanisms to evade the repression of translation

26   mediated by protein kinase R (PKR). In the case of murine cytomegalovirus (MCMV),

27   the protein products of two essential genes, m142 and m143, bind to dsRNA and block

28   phosphorylation of PKR and eukaryotic initiation factor 2α. A distinctive feature of

29   MCMV is that two proteins are required to block PKR activation whereas other viral

30   dsRNA-binding proteins that prevent PKR activation contain all the necessary functions

31   in a single protein. In order to better understand the mechanism by which MCMV evades

32   the PKR response, we investigated the associations of pm142 and pm143 with each other

33   and with PKR. Both pm142 and pm143 interact with PKR in infected and transfected

34   cells. However, the ~200 kDa pm142:pm143 complex that forms in these cells does not

35   contain substantial amounts of PKR, suggesting that the interactions between

36   pm142:pm143 and PKR are unstable or transient. The stable, soluble pm142:pm143

37   complex appears to be a heterotetramer consisting of two molecules of pm142 associated

38   with each other and each one binding to and stabilizing a monomer of pm143. MCMV

39   infection also causes relocalization of PKR into the nucleus and to an insoluble

40   cytoplasmic compartment. These results suggest a model in which the pm142:pm143

41   multimer interacts with PKR and causes its sequestration in cellular compartments where

42   it is unable to shut off translation and repress viral replication.

INTRODUCTION

As a consequence of the threat viruses pose to the survival and fitness of their hosts, an array of cellular defenses have evolved to repress viral infection. In mammals, the interferon system mediates multi-faceted innate immune responses to viral infections (25). Among the important effectors of this system is the interferon-stimulated double stranded RNA (dsRNA)-activated protein kinase R (PKR). dsRNA, which is produced during many viral infections (29), binds to PKR, causing a conformational change, dimerization, and autophosphorylation followed by phosphorylation of the alpha subunit of initiation factor 2 (eIF2α). Phosphorylated eIF2α inhibits the activity of the guanine nucleotide exchange factor eIF2B, resulting in repression of translation initiation (8).

Because viral replication depends on ongoing translation, viruses have evolved a variety of mechanisms for evading the PKR response (22). One such mechanism utilizes virally encoded dsRNA-binding proteins such as the E3L protein of vaccinia virus and the NS1 protein of influenza virus. The observation that vaccinia virus lacking E3L (VVΔE3L) has a very limited host range in cell culture and is avirulent in animals but does replicate in PKR-deficient cells illustrates the importance of blocking the PKR response (3, 30, 31). Many viral dsRNA-binding proteins form homodimers and bind to PKR, but the relative contributions of the various interactions to anti-PKR functions vary. For example, in the case of E3L, binding to and sequestering dsRNA may be sufficient to prevent PKR activation (5), while in the case of NS1, direct binding to PKR appears to be more critical than binding to dsRNA (20).

In previous studies, we identified dsRNA-binding proteins encoded by two β herpesviruses, human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV)

66 (6, 7). The TRS1 and IRS1 genes of HCMV each encode proteins that block PKR

67 activation by a mechanism that appears to depend on binding to both dsRNA and PKR

68 (10, 11). These proteins also have the unusual property that they cause accumulation of

69 PKR in the nucleus.

70 In the case of MCMV, the m142 and m143 genes function by blocking PKR

71 activation. Valchanova and coworkers reported that the failure of mutant viruses lacking

72 either m142 or m143 to replicate is linked to activation of PKR and inhibition of protein

73 synthesis (27). We found that the protein products of these two genes (pm142 and

74 pm143) co-immunoprecipitate in infected cells and function together to bind dsRNA and

75 to enable replication of VVΔE3L (7). Thus, the MCMV system differs from other viral

76 systems studied thus far in that two different proteins are required to block PKR

77 activation.

78 The limited understanding of how dsRNA binding proteins actually block PKR

79 function coupled with the unusual features of the MCMV system led us to undertake

80 studies aiming to clarify the interactions among pm142, pm143 and PKR. We found that

81 like other well-studied viral dsRNA binding proteins, pm142 and pm143 each can bind to

82 PKR. Pm142 and pm143 appear to form a stable soluble heterotetrameric complex,

83 which, interestingly, does not contain substantial amounts of PKR. Similar to HCMV,

84 MCMV infection also causes relocalization of PKR to the nucleus, as well as to insoluble

85 cytoplasmic complexes. These results suggest a model in which interaction with pm142

86 and pm143 results in PKR sequestration into compartments where it is unable to shut off

87 protein synthesis.

88

MATERIALS and METHODS

90 **Cells, virus, and infections.** NIH 3T3 cells, PKR null mouse fibroblasts (provided by

91 Bryan Williams, Cleveland Clinic Foundation), and HeLa cells were maintained in

92 Dulbecco's modified Eagle's medium supplemented with 10% NuSerum (Collaborative

93 Biomedical) as previously described (6). MCMV strain K181 and MCMV MC.55, a

94 recombinant of a strain derived from K181 that expresses green fluorescent protein (GFP)

95 (provided by Jeff Vieira, University of Washington (28)) were propagated in NIH 3T3

96 cells. Infections were performed at a multiplicity of infection (MOI) of 3.

97

98 **Plasmids.** The plasmid pCS2+BirA which expresses *E. coli* biotin ligase was obtained

99 from Bruce Clurman (Fred Hutchinson Cancer Research Center). All other expression

100 constructs used in these experiments were cloned into the pcDNA3.1/V5-His-TOPO

101 vector (Invitrogen).

102 Plasmid pEQ1100, which expresses EGFP with a C-terminal biotinylation signal

103 and adjacent 6 x His tag (hereafter designated by –B and –H, respectively) was

104 previously described (7). The plasmid pEQ1068 (TRS1[1–738]-B-H) was constructed by

105 inserting primers encoding a biotinlylation signal (7) into the *XhoI* and *XbaI* sites of

106 pEQ979 (10).

107 Construction of pEQ1073 (m142-B-H) was previously described (7). pEQ1163

108 (m142-B) was derived from pEQ1073 by digestion with *Pme*1 and *Age*1, blunting with

109 Klenow, and religating to remove the His tag. pEQ1063 (m142-H) was made by

110 amplification of pEQ985 (7) using oligos #461 (ACCATGGACGCCCTGTGCGC) and

111 #548 (GTCGTCATCGTCGGCGTCCGC) and cloning into the TOPO vector.

112   PEQ1109 (m143-H) was made by amplification of pEQ939 (7) using oligos #410

113   (ACCATGTCTTGGGTGACCGGAGAT) and #596 (CGCGTCGGTCGCTCTCTCGT)

114   and cloning into the TOPO vector . The m143 ORF was removed from pEQ1109 by

115   digestion with *Hind*III and *Eco*RV and cloned into pEQ1068 after digestion with the

116   same enzymes to construct pEQ1126 (m143-B-H). PEQ1164 (m143-B) was derived

117   from pEQ1126 by digestion with *Pme*1 and *Age*1, blunting, and religating.

118

119   **Transient transfection.** Sub-confluent HeLa cells in 12 well plates were transfected

120   with plasmid DNA using Lipofectamine 2000 reagent (Invitrogen) according to the

121   manufacturer's instructions.   Biotin (25 µM) was added to the medium following

122   transfection. At 24 to 48 h post-transfection the cells were washed with PBS at 4°C then

123   lysed in 300 µl of RIPA buffer (50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.2% SDS,

124   1% NaDOC) containing 250 mM NaCl.   After reserving a portion for subsequent

125   analysis, the resulting lysates were used for binding to avidin as described below.

126

127   **Immunoprecipitation, avidin agarose pull-down, and immunoblot analyses.** For co-

128   immunoprecipitation analyses, NIH 3T3 cells in 6 well plates were either mock-infected

129   or infected with MCMV MC.55. At 48 h post-infection the cells were washed twice with

130   PBS (4°C), then lysed in 300 µl of NP-40 lysis buffer (50 mM Tris.Cl (pH 7.5), 150 mM

131   NaCl, 1% NP-40). The cells were incubated on a rotator for 20 min., then clarified by

132   centrifugation at 16,000 x g for 10 min. at 4°C. 3% of the sample was saved for

133   assessing protein expression in the lysates. The remaining supernatants were transferred

134   to new tubes, and 5µl of the indicated rabbit polyclonal antiserum (pre-immune, anti-

135   m142, or anti-m143; provided by Laura Hanson and Ann Campbell, Eastern Virginia

136   Medical School (12)) was added. After 1-2 h on the rotator, protein A sepharose was

137   added to each tube, and rotation was continued for ~ 4 h. The samples were washed three

138   times with NP-40 lysis buffer, followed by separation on 10% polyacrylamide gels and

139   transfer to polyvinylidene difluoride (PVDF) membranes by electroblotting. The

140   membranes were probed with a mixture of m142 and m143 antisera or with mouse

141   monoclonal antibody PKR (B-10) (#sc-6282, Santa Cruz Biotechnology).

142   For avidin (AV) agarose binding assays, transfected cell lysates were incubated

143   with immobilized avidin (PIERCE) for 2-4 h, washed four times, then immunoblotted as

144   described above. Blots were probed with either Penta·His antibody (QIAGEN) or avidin-

145   AP (Avidx-AP conjugate; Applied Biosystems) according to the manufacturer's

146   recommendations.

147   For all immunoblot analyses, proteins were detected using the Western-Star™

148   chemiluminescent detection system (Applied Biosystems) according to the

149   manufacturer's recommendations. Silver staining was performed using the

150   SilverQuest™ silver staining kit (Invitrogen) according to the manufacturer's

151   recommendations.

152

153   **Metabolic labeling.** For pulse-chase analysis, HeLa cells were transfected as described

154   above with pCS2+BirA and either m143-B-H alone, or m143-B-H and m142-H. 24 h

155   later, the cells were labeled for 1 h with 100 µCi/ml [^{35}S]-methionine (Easytag™ express

156   protein labeling mix, PerkinElmer) and, after washing twice, chased by addition of

157   medium containing 1 mM cold methionine.   At the indicated times post-chase the cells

158   were lysed with 300 µl of RIPA buffer containing 250 mM NaCl, and the resulting

159   lysates incubated in the presence of AV-agarose as described above. The resulting

160   samples were separated on 10% polyacrylamide gels, dried, and subjected to

161   phosphorimager analysis on a Typhoon Trio multi-mode imager.

162

163   **Cell fractionation.** For glycerol gradient fractionation of infected cells, 100 mm dishes

164   of NIH 3T3 cells were infected with MCMV MC.55. At 48 h post-infection the cells

165   were scraped into PBS, pelleted, then resuspended in 250 µl of cell fractionation buffer

166   (20 mM Hepes (pH 8.0), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1% NP-40, 1 mM

167   benzamidine, 10% glycerol). The samples were incubated for 30 min., 4°C on a rotator,

168   then clarified by centrifugation (16,000 x g). Supernatants were separated on 5 ml 15-

169   35% glycerol gradients at 237,000 x g, 17 h, 4°C. Fractions were collected from the

170   bottom of the gradients and separated on 10% polyacrylamide gels, transferred to PVDF,

171   probed with a mixture of m142 and m143 antisera, then stripped and re-probed with anti-

172   PKR antiserum. Molecular weight standards were centrifuged on parallel gradients, and

173   their distribution was determined by polyacrylamide gel electrophoresis and Coomassie

174   blue staining.

175   Similar glycerol gradient fractionation of transfected cells was performed after

176   transfection of plasmids expressing m142-B-H and m143-B-H into HeLa cells. Lysates

177   prepared at 24 h post-transfection were analyzed as described for infected cells, except

178   that the PVDF membrane was probed with avidin-AP for protein detection.

179   Nuclear- and cytoplasmic-enriched fractions were prepared from 100 mm dishes

180   of NIH 3T3 cells after mock-infection or infection with MCMV MC.55. At 24, 48, and

181 72 h post-infection, the cells were washed with PBS and lysed with 1 ml of cell

182 fractionation buffer (see above). The lysates were transferred to 1.5 ml microfuge tubes,

183 and placed on a rotator at 4°C for 30 min. The samples were then centrifuged at 1000 x

184 g, 5 min., 4°C, and the supernatant (cytoplasmic-enriched fraction) removed carefully

185 without disturbing the pellet. The pellet fraction was then gently resuspended in 0.5 ml

186 of fractionation buffer, microfuged as before, and the remaining pellet (nuclear-enriched

187 fraction) resuspended in 60 µl of 2% SDS and sonicated to shear DNA. Equal amounts

188 of protein were separated on 10% polyacrylamide gels, transferred to PVDF, and

189 immunoblotted with the following antisera according to the manufacturer's

190 recommendations: PKR (B-10), lamin A/C (#2032, Cell Signaling), calnexin (#610523,

191 BD Transduction Laboratories), ß-actin (#A2066, Sigma). and a mixture of m142 and

192 m143 antisera.

193

194 **Immunofluorescence.** NIH 3T3 cells or PKR null mouse fibroblasts grown on glass

195 coverslips were mock-infected or infected with MCMV K181. At 48 h post-infection the

196 cells were washed three times with PBS (4°C), fixed in 100% MeOH at -20°C for 5 min.,

197 then washed again with PBS. The coverslips were blocked with 5% normal goat serum

198 for 1 h, then incubated for 1 h with PKR (B-10) antibody or an anti-ß-Gal antibody

199 (Promega) that served as an IgG2a isotype control. The coverslips were washed three

200 times with PBS, then incubated with FITC-conjugated anti-mouse secondary antibody

201 (SIGMA) for 1 h in the dark. After washing the cells three times with PBS they were

202 incubated with Hoechst 33342 (5 µg/ml; Invitrogen) for 5 min., washed twice with PBS,

203 then mounted on slides using ProLong Gold anti-fade mounting medium (Invitrogen).

204   All incubations were performed at room temperature. The samples were analyzed using a

205   Deltavision RT Wide-field Deconvolution Microscope (Applied Precision, Inc.).

206

207   **RESULTS**

208   **pm142 and pm143 form a complex that binds to PKR in MCMV-infected cells.**

209   Several viral proteins that block PKR activation bind to both dsRNA and PKR (4, 5, 10,

210   11, 16, 20, 24). We have shown that MCMV pm142 and pm143 together bind to dsRNA

211   and block PKR activation (7). Therefore, as a next step in elucidating the mechanism by

212   which pm142 and pm143 function, we investigated whether they also physically

213   associate with PKR.

214   First, we infected NIH 3T3 cells with MCMV and at 48 h post-infection prepared

215   lysates. After immunoprecipitation with pre-immune serum or antisera specific for either

216   pm142 or pm143, we analyzed the precipitated proteins by immunoblot analyses using

217   either a mixture of rabbit anti-m142 and anti-m143 antisera or with a mouse monoclonal

218   antibody directed against PKR (Fig. 1). Although the immunoglobulin heavy chain

219   partially obscured the pm142 band following precipitation of MCMV-infected lysates

220   with anti-m142 or anti-m143 antiserum, these experiments confirmed that pm142 and

221   pm143 form a complex as previously reported (7). Also, as noted previously, anti-m143

222   appeared to precipitate more pm142 compared to the amount of pm143 precipitated by

223   anti-m142 (compare lanes 7 and 8 in Fig. 1A). Importantly, pm142 and pm143 antisera

224   each precipitated PKR while preimmune serum did not (Fig 1B). The amount of PKR

225   that co-immunoprecipitated with pm142 or pm143 was relatively small, suggesting the

226   possibility that only a minority of PKR in the cell is associated with these two proteins

227   (see below). As expected, pm142, pm143 and PKR were absent from precipitates from

228   mock-infected cell lysates after precipitation with the same antisera. Thus, PKR

229   associates with pm142 and pm143 in MCMV-infected cells.

230

231   **pm142 and pm143 each bind to PKR in transfected HeLa cells.** In order to determine

232   which of the MCMV proteins (pm142, pm143, or both) associates with PKR, we used an

233   AV-agarose pull-down system as described in Materials and Methods. This system takes

234   advantage of the high affinity of the biotin-avidin interaction and eliminates background

235   resulting from the immunoglobulin heavy chain. The plasmids used in these assays

236   expressed proteins that were either His-tagged (designated pm142-H, for example), or

237   contained a 14 amino acid biotinylation signal in addition to the His tag (e.g. pm142-B-

238   H). When co-transfected into cells along with a plasmid containing the *E. coli* biotin

239   ligase (BirA) gene and incubated in the presence of 25 µM biotin, biotin is efficiently

240   added to a lysine residue in the biotinylation signal of signal-tagged proteins (2).

241   For these analyses, we transfected HeLa cells with various combinations of

242   expression plasmids, prepared lysates at 48 h post-transfection, and detected proteins

243   present in the total lysates and in the fractions that bound to AV-agarose by assays using

244   anti-His or avidin-AP. When probed with anti-His antibody, all of the His-tagged proteins

245   were detected in the lysates (Fig. 2A, top panel). Consistent with the known interactions

246   of pm142 and pm143, both proteins were detected in the AV bead-bound fractions when

247   either one was biotinylated (Fig 2A, bottom panel, lanes 2 and 3), while neither protein

248   bound to EGFP-B-H (lanes 7 and 8), even on a longer exposure (data not shown).

249   Omitting the BirA plasmid from the transfection eliminated the binding of pm142 or

250 pm143 to the AV beads (lane 4). Note that the biotinylation signal caused a slight but

251 detectable increase in the size of the pm142 and pm143 proteins (compare lanes 2 and 3).

252   Immunoblot assays of the same samples using PKR antiserum revealed that at

253 least a small amount of PKR was precipitated by the pm142:pm143 complex and by

254 pm142 and pm143 individually (Fig 2B, lanes 2, 3, 5, and 6). pTRS1-B-H also

255 precipitated PKR (lane 1), consistent with our previous finding that pTRS1 binds to PKR

256 (11). As expected, the negative controls (samples lacking BirA or pulled down with

257 EGFP-B-H) did not precipitate PKR (lanes 4, 7 and 8). Treatment of the

258 immunoprecipitated material with dsRNA-specific RNase (RNaseIII) under conditions

259 that completely digested reovirus genomic dsRNA, did not disrupt the association of

260 pm143 with pm142 or with PKR (data not shown). However, dsRNA that is embedded in

261 the complex might not be accessible to the RNaseIII, so these results do not exclude the

262 possibility that dsRNA is a key component of the complex. However, these data do

263 reveal that pm142 and pm143 each appear to bind to PKR independently.

264

265 **Expression of pm142 stabilizes pm143.** We noticed that the abundance of pm143 in

266 lysates was consistently much lower when it was expressed by itself or with EGFP than

267 when expressed with pm142 (e.g. compare lanes 6 and 8 to lanes 2, 3, and 4 in Fig. 2A,

268 top panel). To evaluate the possibility that pm143 might be stabilized by its association

269 with pm142, we measured the half-life of pm143 in cells transfected with pm143-B-H in

270 the presence or absence of pm142-H (Fig. 3). At 24 h post-transfection, we pulse-labeled

271 cells with [^{35}S]-methionine for 1 h, then chased with excess cold methionine and

272 analyzed proteins bound to AV-agarose by electrophoresis and phosphorimager analysis.

273   As is evident in Fig. 3, even by two hours post-chase there was substantially less pm143

274   in the absence of pm142 than when the proteins were co-expressed. The calculated half-

275   life of pm143 was approximately 8 h in the absence of pm142 and 25 h when expressed

276   in combination with pm142. Thus, pm143 is stabilized by coexpression of pm142.

277

278   **The pm142:pm143 complex is larger than a heterodimer but lacks substantial**

279   **amounts of PKR.** The observation that pm142 and pm143 bind to each other and that

280   both also bind to PKR (Figs. 1 and 2) suggested that these three proteins might form a

281   heterotrimeric complex in infected cells. To test this hypothesis, we fractionated infected

282   cell lysates over glycerol gradients and analyzed the distribution of these three proteins

283   by SDS-PAGE and immunoblot assays (Fig. 4). In parallel gradients, we fractionated

284   molecular weight standards in an identical manner and determined their distribution by

285   polyacrylamide gel electrophoresis and Coomassie Blue staining.

286   Most of the pm143 migrated with a peak of pm142 at an apparent size of ~200

287   kDa (Fig. 4A, fractions 12 - 17), which is close to the calculated size (178 kDa) of a

288   heterotrimer consisting of one molecular each of pm142 (49 kDa), pm143 (61 kDa) and

289   PKR (68 kDa). Surprisingly, we detected little or no PKR in the fractions containing the

290   pm142:pm143 complex (Fig 4B). Instead, PKR migrated mostly in the range of ~70 kDa

291   to ~160 kDa (fractions 17 – 24). The smaller size within this range corresponds to that

292   of the PKR monomer (68 kDa), while the larger species could represent PKR dimers or

293   PKR bound to other factors. Although the small amount of overlap between pm142,

294   pm143, and PKR (fractions 16-18) might represent PKR:pm142:pm143 heterotrimers,

295   these results indicate that the majority of the pm142:pm143 complex in infected cells is

296   not bound to PKR. Conversely, most of the PKR in the cell appears not to be bound to

297   the pm142:pm143 complex, which may explain the low amount of PKR associating with

298   pm142 and pm143 in our immunoprecipitation experiments (Figs. 1 and 2). However,

299   these data do not exclude the possibility that there is a PKR:pm142:pm143 trimeric

300   complex in infected cells but that it dissociates under the conditions of these experiments.

301   In addition to its presence in the pm142:pm143 complexes, pm142 migrates as a

302   smaller species, possibly representing pm142 monomers and homodimers (see below), as

303   well as in larger complexes of unknown composition. Perhaps due to its labile nature in

304   the absence of pm142 (Fig. 3), pm143 is present only in fractions containing pm142.

305   These results, suggesting that there is a stable ~200 kDa complex consisting of

306   pm142 and pm143 that lacks substantial amounts of PKR, raise two questions. First,

307   what is the composition of the ~200 kDa complex?   Second, can we reconcile the

308   observations that pm142 and pm143 can each bind to PKR and prevent its activation yet

309   most of the PKR in the cells appears not to be stably associated with pm142 and pm143?

310   The subsequent studies were designed to evaluate these issues.

311

312   **The pm142:pm143 complex lacks other proteins.** Since the pm142:pm143 complex

313   migrated at a size of ~200 kDa (Fig. 4) and a heterodimer of the two proteins would be

314   expected to be only ~110 kDa, we investigated the possibility that the complex contains

315   additional proteins. First, we transfected cells with plasmids expressing pm142-B-H and

316   pm143-B-H, and at 24 h post-transfection prepared cytoplasmic lysates and analyzed the

317   distribution of the proteins by glycerol gradient centrifugation. Detection of the two

318   transfected proteins using avidin-AP demonstrated that, similar to the complex present in

319   infected cells, the pm142:pm143 multimer sedimented as a ~200 kDa complex in these

320   transfected, uninfected cells (Fig. 5). This result demonstrates that no other MCMV

321   proteins are required for formation of the major pm142:pm143 complex.

322   To evaluate whether any cellular proteins were present in the pm142:pm143

323   multimer, we transfected cells with pm142-H and pm143-B-H and pulled-down pm143-

324   B-H and any associated proteins by binding to AV-agarose. We then detected these

325   proteins by gel electrophoresis followed by silver staining. As a negative control we

326   analyzed lysates from cells transfected with pm142-H and pm143-H, both lacking the

327   biotinylation signal. For comparison, we also analyzed HCMV pTRS1-H-B. Consistent

328   with prior evaluations using immunoblot assays, pm142 was detectable in the pm143-

329   bound material (Fig. 6). Although there were several other minor protein bands, some of

330   which also were detected in the negative control sample, none approached the abundance

331   of the bands corresponding to pm142 and pm143.   Thus, the relatively large size of the

332   pm142:pm143 multimer cannot easily be explained by the presence of either additional

333   MCMV or cellular proteins.

334

335   **The pm142:pm143 complex may be a heterotetramer.** The absence of other proteins

336   in the pm142:pm143 complex might be explained by its being a higher order multimer of

337   just these two proteins To investigate this possibility, we again transfected various

338   plasmids expressing proteins tagged with either His or the biotinylation signal into HeLa

339   cells and assayed total and AV-bound proteins using either anti-His antibody or avidin-

340   AP.

341   When expressed by itself, pm142-B was detectable in lysates and the AV-bound

342   samples using avidin-AP, but not with anti-His (Fig. 7, panels A-D, lanes 1). On the

343   other hand, pm142-H was expressed in lysates (A, lane 2), but it lacks the biotin tag and

344   so, as expected, did not bind to AV-agarose (B, lane 2) nor was it detected by avidin-AP

345   (C and D, lanes 2). However, when pm142-B and pm142-H were co-transfected, pm142-

346   H was pulled down by AV beads (B, lane 3), demonstrating that pm142-H binds to

347   pm142-B, thus suggesting that pm142 can homodimerize.

348   Consistent with its relative instability in the absence of pm142 (Fig. 3), pm143-B

349   transfection by itself resulted in only a faint band in both the lysates and bound fractions

350   (C and D, lanes 4). On an exposure comparable to that shown in panels C and D, pm143-

351   H was almost undetectable (not shown) but with a longer exposure it was clearly present

352   (A, lane 5). In lysates prepared after transfection of both pm143-B and pm143-H, we

353   could detect both proteins in the lysates but only pm143-B in the bound fraction. The

354   observation that all of the other proteins shown Fig. 7 that bound directly or indirectly to

355   AV beads produced bands that appeared at least as intense in bound samples as in the

356   lysates, coupled with the detection of pm143-H in the lysates but not the bound samples,

357   suggests that pm143 does not self-associate.

358   Interestingly, when pm142-H was expressed along with pm143-B and pm143-H,

359   pm143-H was present in the bound fraction along with pm142-H (Fig. 7B, lane 7). This

360   result indicates that pm143-B and pm143-H were tethered together by one or more

361   molecules of pm142. The absence of detectable pm142-H or pm143-H binding to the

362   EGFP-B-H control (lane 8, B) supports the specificity of the binding reactions.

363   Collectively, the results of Figs. 3-6 suggest that the major form of the

364   pm142:pm143 complex is a heterotetramer in which two pm142 molecules bind to each

365   other in addition to each binding to separate pm143 monomers.

366

367   **MCMV causes relocalization of PKR.** We previously reported that pTRS1 and pIRS1

368   not only bind to PKR, but that they also cause accumulation of PKR in the nucleus (11).

369   Because of the similarities between HCMV and MCMV, we hypothesized that MCMV

370   infection might also cause PKR relocalization. To test this idea, we prepared nuclear-

371   and cytoplasmic-enriched fractions of mock- or MCMV-infected NIH 3T3 cells at 24, 48,

372   and 72 h post-infection and evaluated the distribution of PKR by immunoblot analysis

373   (Fig. 8). PKR remained relatively constant in abundance and remained predominantly

374   cytoplasmic during this time interval in mock-infected cells. In contrast, its abundance

375   increased and it redistributed to the nuclear fraction in MCMV-infected cells. We

376   performed control immunoblots on the same cytoplasmic and nuclear extracts to evaluate

377   the efficacy of the fractionation procedure. Antibodies directed against the nuclear

378   marker lamin A/C revealed its presence exclusively in the nuclear fraction. The

379   cytoplasmic marker calnexin was upregulated by MCMV infection, but remained

380   predominantly cytoplasmic in the infected cells. An anti-ß-actin immunoblot showed that

381   the abundance of ß-actin was similar in mock-infected and infected cells, although it

382   appeared to be slightly more concentrated in the nuclear than the cytoplasmic fractions in

383   both cases. The same extracts were also probed with a mixture of anti-m142 and anti-

384   m143 antisera, and as reported by Hanson et al. (12), pm142 and pm143 were present in

385   both the cytoplasmic and nuclear fractions throughout infection.

386   As a complementary method for assessing the effects of MCMV on PKR, we

387   examined the distribution of PKR in mock- and MCMV-infected cells by indirect

388   immunofluorescence using an Olympus DeltaVision microscope as described in

389   Materials and Methods. In mock-infected NIH 3T3 cells (Fig. 9, panel A), incubation

390   with anti-PKR antibody showed the predominantly cytoplasmic localization of PKR with

391   a small amount of nuclear PKR appearing to localize in the nucleoli, as has been reported

392   by others (14, 15) and consistent with our immunoblot data (Fig. 8). At 48 h post

393   infection, PKR expression was elevated in MCMV-infected cells and a substantially

394   higher amount was detectable in the nucleus. Surprisingly, contrary to the results based

395   on cell fractionation methods (Fig. 8), the majority of the PKR in the infected cells

396   appeared to remain localized within the cytoplasm. The PKR signal in infected cells also

397   seemed distributed in a coarser punctate pattern in the cytoplasm of infected cells

398   compared to mock-infected cells. Staining with an isotype-matched control antibody

399   showed minimal background staining in these cells. As an additional control, we

400   incubated mock- or MCMV-infected PKR-null MEFs with anti-PKR antiserum or the

401   isotype-matched control antibody (Fig. 9B). In these cells, both the anti-PKR and control

402   antibodies revealed only a low level of background fluorescence.

403   Thus, the results of the immunoblot assays and immunofluorescence assays both

404   revealed an increase in the amount of nuclear PKR after MCMV infection. Compared to

405   the immunoblot assays, the immunofluorescence studies indicate that there is more

406   abundant PKR in the cytoplasm at late times post infection. These results suggest that

407   MCMV infection results in relocalization of PKR to both the nucleus and to insoluble

408   cytoplasmic complexes.

409

DISCUSSION

411   The importance of PKR as a mediator of intracellular innate defenses against viral

412   infections is highlighted by the remarkable number and diversity of viral factors and

413   mechanisms that have evolved to counteract its inhibitory effects on host cell translation

414   (22). In several viral systems, mutants lacking anti-PKR factors are severely attenuated

415   for growth in cell culture and have extremely reduced virulence in animal models.

416   Moreover, the replication defect of some of these viruses has been shown to be reversed

417   at least in part in PKR-deficient cells and, in the case of herpes simplex type-1, even in

418   animals, thus providing genetic support for the functional importance of the interactions

419   between PKR and viral antagonists (19, 30, 31).

420   Many viruses encode a dsRNA-binding protein that can inhibit the PKR pathway.

421   Some viruses, including vaccinia virus, herpes simplex type 1 and HCMV, have been

422   shown to contain a second gene that also inhibits the PKR pathway (4, 6, 16, 22, 26). In

423   these examples, the two genes act independently and, in the vaccinia virus and herpes

424   simplex type 1 cases, at different steps in the PKR activation pathway. The two HCMV

425   genes are very closely related and likely act at the same step. MCMV also has two anti-

426   PKR genes but is unusual in that the two genes act together as a single functional unit.

427   Both m142 and m143 are required for effective dsRNA binding and for rescue of

428   VVΔE3L replication (7). Furthermore, deletion of either gene results in phosphorylation

429   of PKR and eIF2α, repression of protein synthesis in infected cells, and eliminates

430   MCMV replication (27). The genomic proximity of m142 and m143 combined with their

431   functional similarities suggest that evolution of MCMV may have resulted in separation

432   of the functions needed for blocking PKR into two proteins, while in other viruses these

433   functions are combined in a single protein.

434   Many well-studied viral dsRNA-binding proteins that prevent PKR activation

435   bind to PKR (4, 11, 20, 24). We found that the pm142:pm143 complex does so as well

436   (Fig. 1). The observation that pm142 and pm143 can each bind to PKR in transfection

437   assays (Fig. 2) argues against the possibility that the separation of dsRNA-binding

438   protein functions in MCMV led to one protein becoming specialized in binding to PKR.

439   We do not know whether the interactions between pm142:pm143 and PKR are direct

440   ones. Data showing that pm142 and pm143 are each able to bind to PKR, yet the

441   combination of both is required for strong binding to dsRNA (7), suggest that a dsRNA

442   tether is not required for PKR binding to pm142 or pm143, but it remains possible that

443   there are other proteins or nucleic acids contributing to the interaction.

444   We also do not yet know whether binding to PKR is required for pm142:pm143

445   function, although based on studies of other viral dsRNA-binding proteins we suspect

446   that it is. HCMV pTRS1 (and pIRS1) and influenza NS1 bind to PKR, and these

447   interactions appear to be essential for its inhibition (12, 20). In the case of vaccinia virus

448   E3L, the C-terminal dsRNA binding domain, in the absence of the N-terminal PKR-

449   binding domain, is sufficient for viral replication in many cell types but it is required for

450   preventing PKR activation late in infection (18), for blocking the inhibitory effects of

451   PKR in a yeast growth assay (24), and for virulence in animal models (3). Evaluating

452   whether E3L function requires it to bind to PKR is complicated by the presence of other

453   functions, including an N-terminal Z-DNA binding domain that may be responsible for

454   some of the observed phenotypes (17). Adding to the complexity, binding to PKR does

455   not always inhibit PKR function, as is clearly illustrated by the activation of PKR

456   resulting from its interaction with the cellular regulator PACT (21). Nonetheless, the fact

457   that both pm142 and pm143 associate with and inhibit PKR does suggest that this is a

458   functionally important physical interaction.

459   The observed binding of pm142, pm143 and PKR to each other in each binary

460   combination suggested that these three proteins might form a heterotrimeric complex.

461   Although we found clear evidence for a complex of pm142 and pm143 in infected or

462   transfected cells, little if any PKR co-migrated with the pm142:pm143 complex. One

463   possible explanation for the observed interaction between PKR and pm142:pm143 by

464   coimmunoprecipitation but not by glycerol gradient fractionation is that the interaction is

465   unstable and therefore the complex dissociated under the conditions used in the glycerol

466   gradient experiments. Alternatively, the interaction might be a transient one, possibly

467   resulting in inactivation of PKR by a mechanism that does not require continued

468   association with the MCMV proteins.

469   We also discovered that MCMV alters the distribution of PKR in infected cells.

470   Our analyses of uninfected cells are consistent with reports indicating that approximately

471   20% of the cellular PKR is present in the nucleus, usually in association with the

472   nucleolus (14, 15). Fractionating cells with detergent revealed accumulation of PKR in

473   the nuclear-enriched pellet fraction by 24 h post infection (Fig.8). Since such pellet

474   fractions may also contain insoluble cytoplasmic material, complementary methods are

475   important for evaluating protein distribution. Using immunofluorescence assays, we

476   found that PKR did indeed appear to accumulate in the nucleus during infection, but a

477   large fraction of it remained in the cytoplasm. PKR accumulates in the nucleus after

478   HCMV infection but not after vaccinia infection (11).   Using recombinant vaccinia

479   viruses, we showed that pTRS1 or pIRS1 could mediate this relocalization, suggesting

480   that pm142 and pm143 are plausible candidates for instigating the redistribution of PKR

481   during MCMV infection.

482   Altered distribution of PKR has been reported in other settings. ER stress can

483   cause PKR to accumulate in the nucleus (23). Activated PKR was recently shown to be

484   enriched in the nucleus of high risk myelodysplastic syndrome lymphoblasts (9) and in

485   neurons expressing the human immunodeficiency virus type I gp120 where it may

486   contribute to neurodegeneration (1). Human papilloma virus causes PKR to relocalize

487   both to the nucleus and to cytoplasmic P bodies (13). At present, the role of nuclear

488   accumulation of PKR in infected cells is unknown, but it might serve to keep PKR away

489   from its cytoplasmic targets, or alternatively, to enable interaction with a nuclear target

490   and enhance viral replication. The relocalization of PKR into insoluble cytoplasmic

491   compartments might represent another mechanism for ensuring PKR is sequestered away

492   from translational machinery. Note that our glycerol gradient experiments would not

493   have detected nuclear or insoluble cytoplasmic complexes since these were removed

494   during processing, so it remains possible that pm142 and pm143 are associated with PKR

495   in these sites. Consistent with this possibility, Hanson et al. found that pm142 and

496   pm143 appear to be primarily cytoplasmic by immunofluorescence, but were present in

497   the "nuclear" fractions as well when analyzed by cell fractionation and immunoblot assay

498   (12).

499   Our data suggest that pm142 and pm143 form a stable complex, likely a

500   heterotetramer, consisting of two molecules of pm142 associated with each other and

501   each one associating with a molecule of pm143 (Fig 10). Data supporting this proposed

502   structure include the observations that in infected cells, pm142 and pm143 (i) colocalize

503   by immunofluorescence (12), (ii) coimmunoprecipitate (Fig. 1 and (7)), and (iii) migrate

504   through glycerol gradients as a complex having a size consistent with a heterotetramer

505   (Fig. 4). Further evidence comes from transfection assays showing that (iv) pm143

506   stability requires pm142, (v) the molarity of the two proteins in the complex is similar

507   (based on experiments in which their relative amounts can be compared, as in Figs. 2A,

508   Fig. 5, and Fig. 6) and (vi) pm142 can self-associate while pm143 appears not to do so

509   (Fig. 7).

510   Based on our results, we propose a model in which pm142 and pm143 form a

511   stable soluble heterotetrameric cytoplasmic complex during infection (Fig. 10). This

512   complex serves as a reservoir, poised to interact with PKR and dsRNA. As has been

513   proposed for the mechanism by which E3L functions, the pm142:pm143 complex may

514   associate with PKR and prevent its homodimerization (24). The absence of a stable

515   soluble pm142:pm143:PKR complex may result from its relocalization to the nucleus and

516   insoluble cytoplasmic fractions where PKR would be unable to shut off translation,

517   although it is also possible that a transient interaction with pm142 and pm143 is sufficient

518   to cause PKR to relocalize. Although we do not yet understand the significance of the

519   separation of PKR inhibitory function into two proteins in MCMV, this organization is

520   unique among viruses studied thus far, and we expect that further exploration of this

521   system will help uncover fundamentals of the mechanisms by which dsRNA-binding

522   proteins interfere with PKR function and enable viral replication.

523

531

532

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630

631   **FIGURE LEGENDS**

632

633   **Figure 1.** The pm142 and pm143 proteins bind PKR in MCMV-infected cells. NIH 3T3

634   cells were mock-infected (lanes 1, 3, 4, and 5) or infected with MCMV (lanes 2, 6, 7 and

635   8; MOI = 3), and at 48 h post-infection lysates were prepared and analyzed by

636   immunoblot assay directly (lanes 1 and 2) or after immunoprecipitation with the

637   preimmune serum (lanes 3 and 6), pm142 antiserum (lanes 4 and 7) or pm143 antiserum

638   (lanes 5 and 8). (A) Unbound mock and infected cell lysates (lanes 1 and 2; 3% of the

639   amount immunoprecipitated) and 20% of each bound sample (lanes 3 – 8) were probed

640   using a mixture of m142 and m143 antisera. The migration of pm142, pm143, and the

641   IgG heavy chain (HC) are indicated on the right. (B) Lysates and the remaining 80% of

642   each bound sample were probed with the anti-PKR antibody.

643

644   **Figure 2.** Both pm142 and pm143 bind to PKR in transfected cells. HeLa cells were

645   transfected with the indicated plasmids, all of which express proteins containing His tags

646   alone (-H) or with a biotinylation signal (-B-H). All transfections, except that in lane 4,

647   also included the plasmid encoding biotin ligase (pSC2+BirA). At 48 h post-transfection

648   cell lysates were prepared and incubated with AV-agarose and analyzed by immunoblot

649   assay as described in Materials and Methods. (A) Membranes with unbound lysates (top

650   panel) or 20% of the bound samples (bottom panel) were probed with the anti-His

651   antibody.   (B) Membranes with unbound lysate (top panel) and 80% of the bound

652   samples (lower panel) were subjected to immunoblot analysis with the PKR antibody.

653

654   **Figure 3.** The pm143 protein is stabilized by co-expression of pm142. HeLa cells were

655   transiently transfected with m143-B-H alone or in combination with m142-H. At 24 h

656   post-transfection the cells were pulse labeled with [^{35}S]methionine for 1 h then re-fed

657   with medium containing excess cold methionine. Cell lysates were prepared at the

658   indicated times post-chase and proteins binding to AV-agarose were analyzed by

659   electrophoresis and phosphorimager analysis.

660

661   **Figure 4.** Size fractionation of pm142:pm143 complexes and PKR on glycerol density

662   gradients. NIH 3T3 cells were infected with MCMV (MOI = 3), and at 48 h post-

663   infection cell lysates were prepared and centrifuged through 15-35% glycerol density

664   gradients as described in Materials and Methods. Following centrifugation, fractions

665   were collected from the bottom of the gradients, and the fractions were separated on

666   polyacrylamide gels, transferred to PVDF, and probed with (A) a mixture of m142 and

667   m143 antisera, after which the blot was stripped and re-probed with (B) an anti-PKR

668   antibody. Molecular weight standards were size fractionated on a parallel gradient, then

669   analyzed by polyacrylamide gel electrophoresis and Coomassie blue staining. The

670   migration of the molecular weight standards is indicated below panel B. Note that

671   fractions 5 and 7 were lost during processing and that aliquots of the lysates (lys) prior to

672   fractionation were included in the first and last lanes.

673

674   **Figure 5.** The pm142:pm143 complex does not contain additional MCMV proteins.

675   HeLa cells were transfected with plasmids expressing BirA, m142-B-H and m143-B-H,

676   and at 24 h post-transfection lysates were prepared and fractionated on 15-35% glycerol

677   density gradients as described in Materials and Methods. The indicated subset of

678   fractions were analyzed by gel electrophoresis, transfer to PVDF, and detection of the

679   biotin-tagged pm142 and pm143 proteins using avidin-AP. The migration of molecular

680   weight standards analyzed on a parallel gradient is indicated below.

681

682   **Figure 6.** The pm142:pm143 complex does not include cellular proteins. HeLa cells

683   were transfected with plasmids expressing m143-B-H and m142-H, TRS1-B-H, or m143-

684   H plus m142-H. All samples also included pSC2+BirA. At 48 h post-transfection

685 lysates were prepared and incubated with AV-agarose as described in Materials and

686 Methods, and bound proteins were analyzed by gel electrophoresis and silver staining.

687

688 **Figure 7.** The pm142 and pm143 proteins appear to form a heterotetrameric complex.

689 HeLa cells were transfected with pSC2+BirA in addition to the indicated plasmids that

690 express either His- or biotin-tagged forms of pm142 and pm143.   At 48 h post-

691 transfection lysates were collected, incubated with avidin-agarose, and subjected to

692 immunoblot analysis as previously described. Membranes containing (A) unbound

693 lysates (3% of total) and (B) 50% of the bound samples (bottom panel) were probed with

694 anti-His antibody. Membranes with (C) unbound (3% of total) and (D) bound lysates

695 (50% of total) were probed with avidin-AP to detect biotin-tagged proteins.

696

697 **Figure 8.** MCMV infection causes nuclear relocalization of PKR. NIH 3T3 cells were

698 mock-infected or infected with MCMV (MOI = 3). Cell lysates were prepared at 24, 48,

699 and 72 h post-infection and separated into cytoplasmic- (cyt) and nuclear-enriched (nuc)

700 fractions as described in Materials and Methods. Equal amounts of each fraction were

701 subjected to gel electrophoresis, transferred to PVDF membranes, and probed with the

702 indicated antisera including the nuclear marker lamin A/C and the cytoplasmic marker

703 calnexin.

704

705 **Figure 9.** MCMV infection appears to cause PKR relocalization to both the nucleus and

706 insoluble cytoplasmic complexes. NIH 3T3 and PKR null cells grown on coverslips were

707 mock-infected or infected with MCMV (MOI = 3). At 48 h post-infection the cells were

708 fixed, incubated with anti-PKR or an isotype control antibody, and prepared for

709 immunofluorescence as described in Materials and Methods. Samples were analyzed by

710 deconvolution microscopy.

711

712 **Figure 10.** Model depicting putative mechanisms of PKR inactivation and re-

713 localization by pm142 and pm143. In the absence of viral antagonists (1), PKR is

714 activated by dsRNA, which leads to dimerization, auto-phosphorylation, and subsequent

715 phosphorylation of the alpha subunit of the translation initiation factor eIF2, thereby

716 blocking translation and inhibiting viral replication. During MCMV infection, a stable

717 heterotetrameric pm142:pm143 complex forms which can interact with PKR and dsRNA.

718 (2), Whether or not pm142 and pm143 remain associated with PKR is not yet clear but

719 we hypothesize that these viral proteins cause sequestration of PKR in the nucleus and in

720 insoluble cytoplasmic complexes (gray area) where it is unlikely to be able to shut off

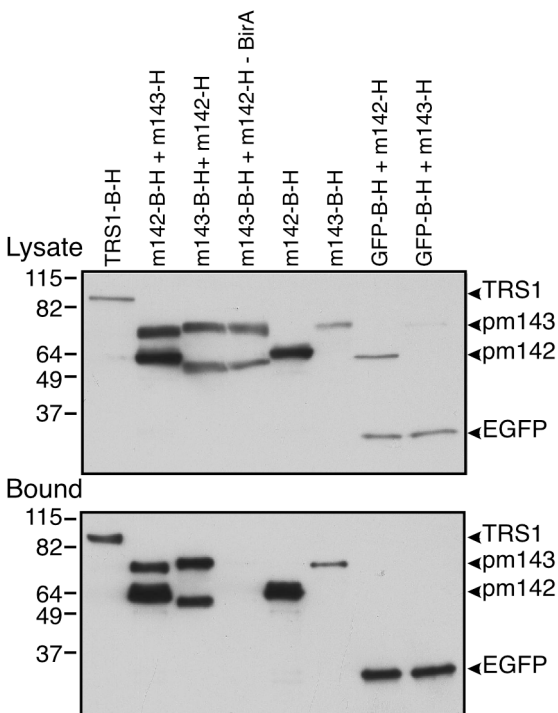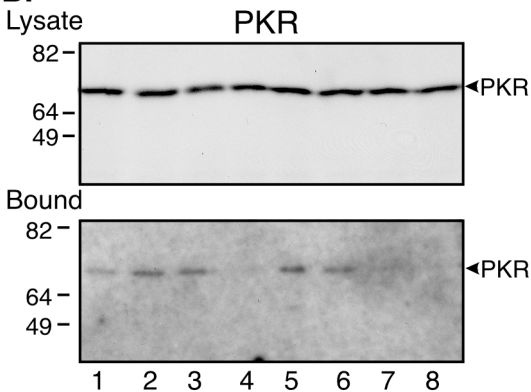721 translation and block viral replication.

Figure 1

Figure 2

Figure 3

A. m142 + m143

lys 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 lys

116 —
84 —
64 —
51 —   ◄ pm143
37 —   ◄ pm142
25 —
20 —

B. PKR

lys 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 lys

116 —
84 —
64 —   ◄ PKR
51 —
37 —
25 —
20 —

443 kDa   200 kDA   150 kDa   66 kDa
                                   29 kDa
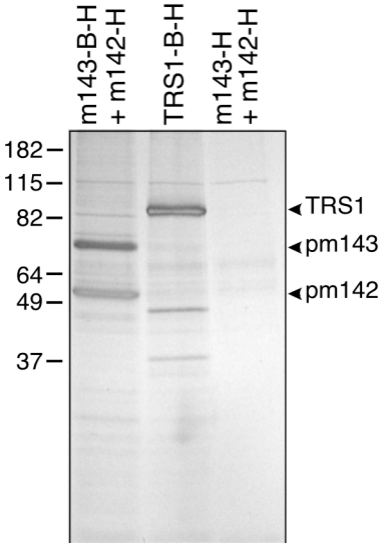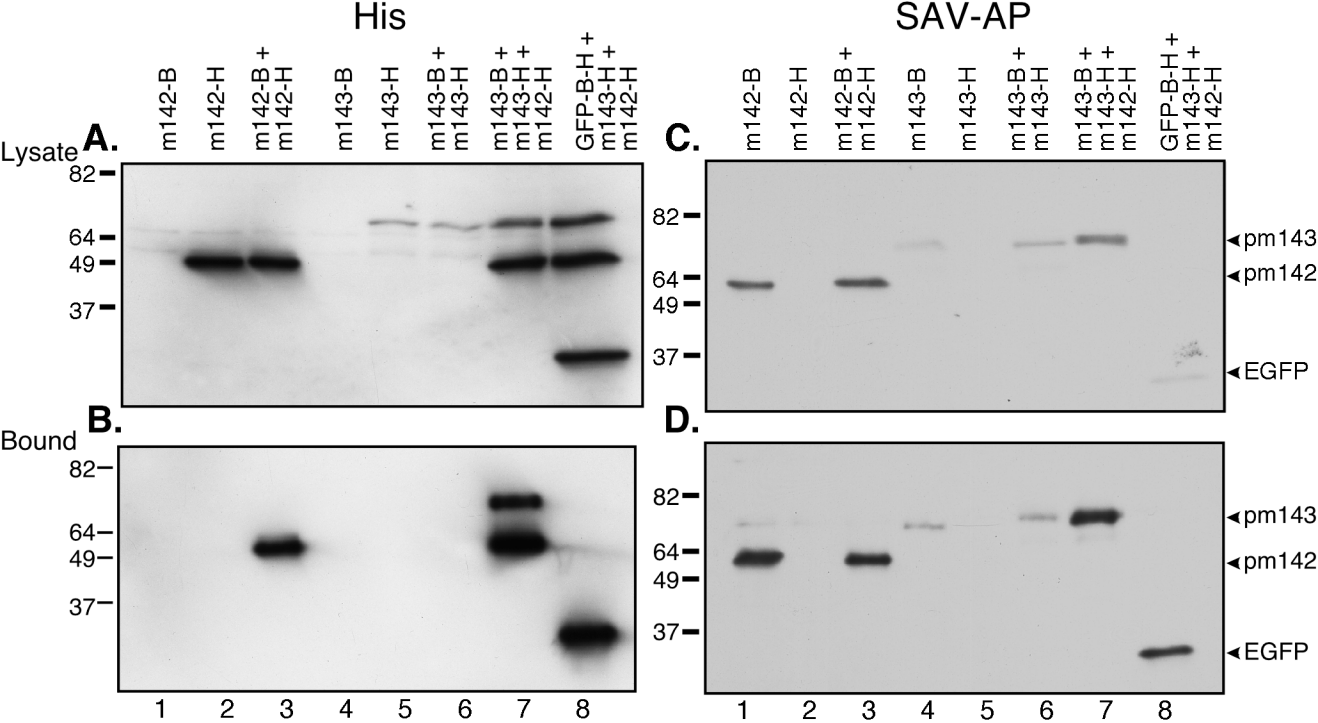   12.4 kDa

molecular weight standards
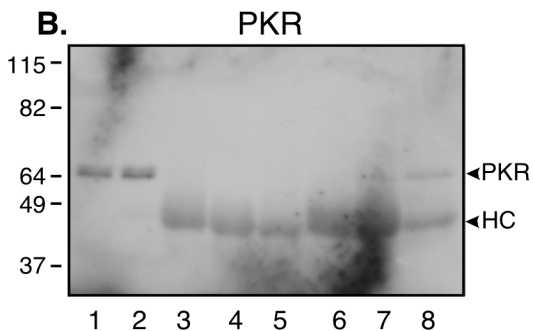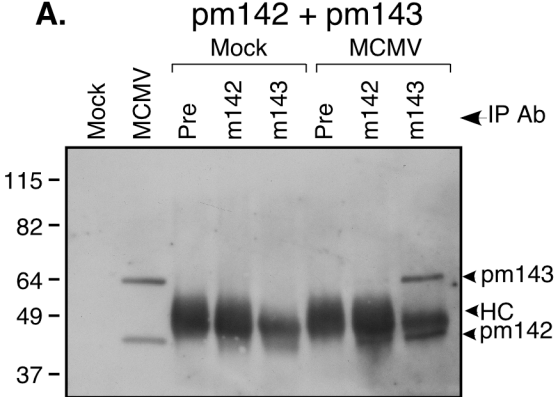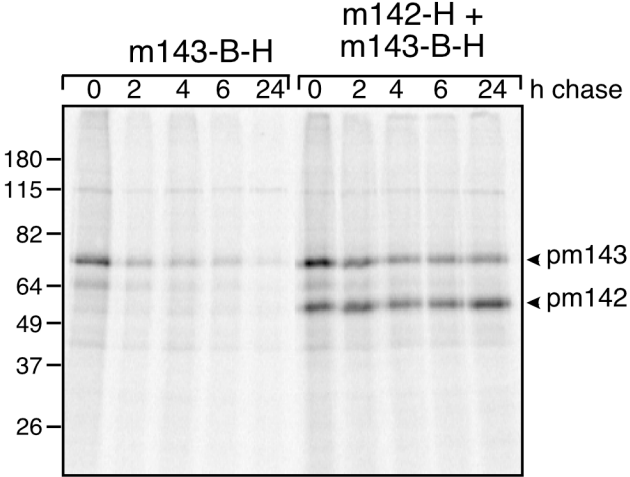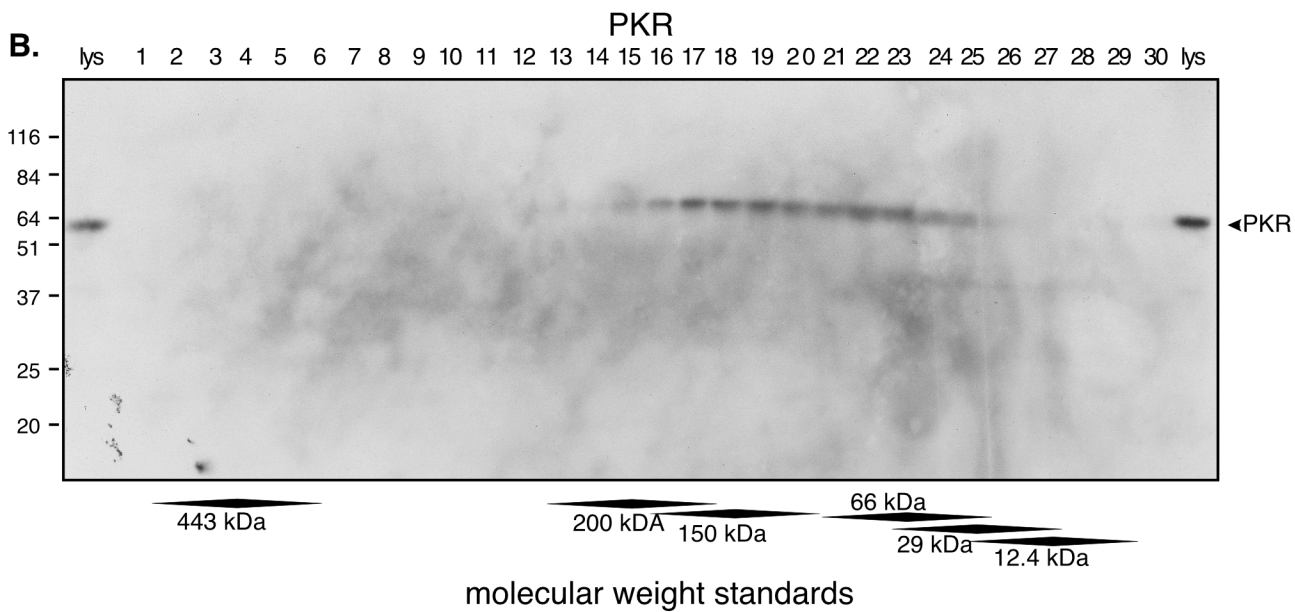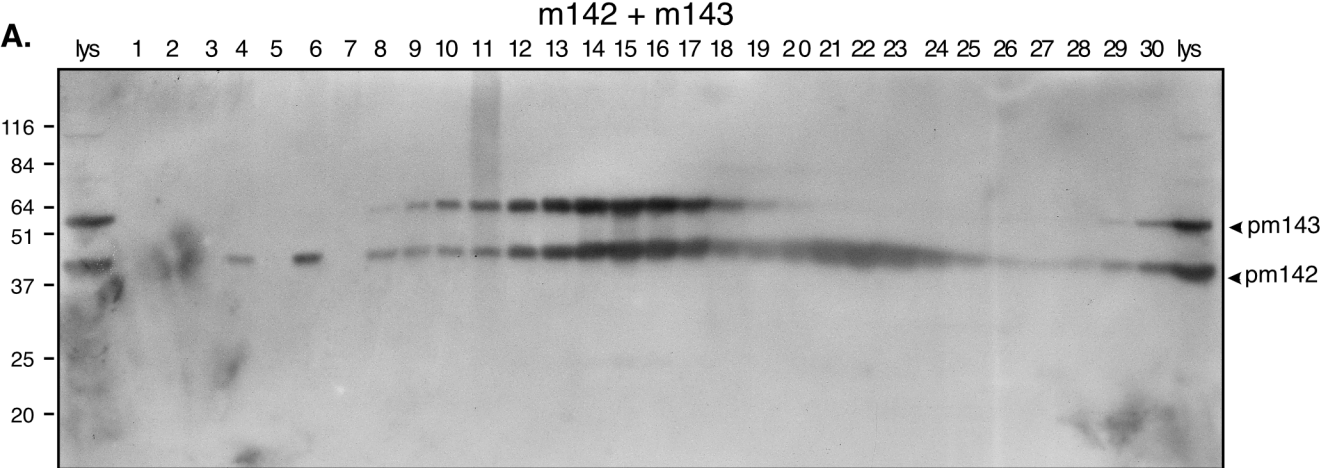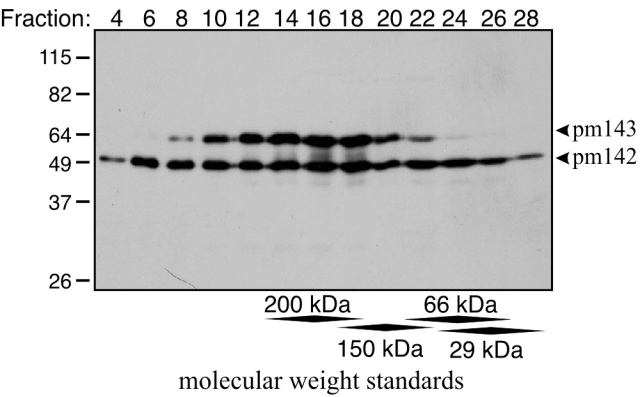
Figure 4

Figure 5
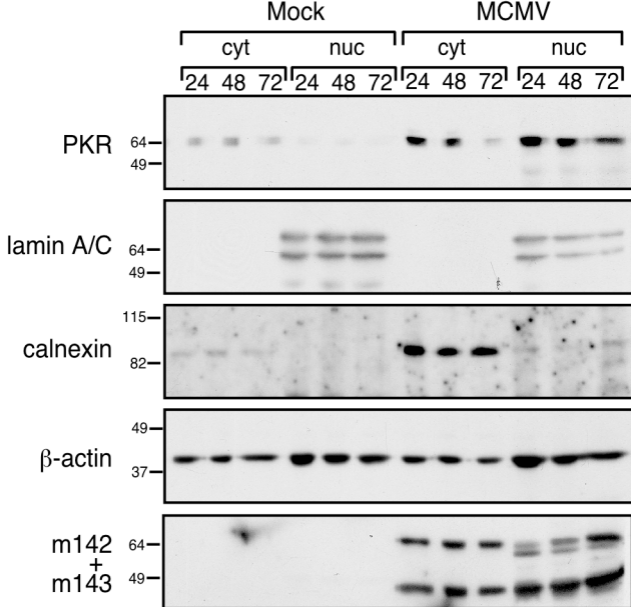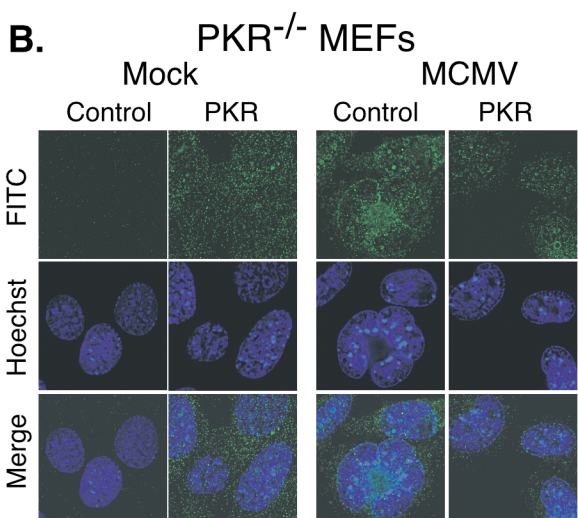
Figure 6

Figure 7

Figure 8

NIH 3T3 Cells

A.

	Mock		MCMV	
	Control	PKR	Control	PKR

FITC

Hoechst

Merge

B.

PKR$^{-/-}$ MEFs

	Mock		MCMV	
	Control	PKR	Control	PKR

FITC

Hoechst

Merge

Figure 9

heterotetramer

pm143

pm142

dsRNA

Sequestered PKR
possibly bound to
pm142:pm143

PKR monomer
(inactive)

2

1

eIF2α

eIF2α

PKR dimer
(active)

Figure 10