Orthopoxvirus K3 orthologs show virus- and host-specific inhibition of the antiviral protein kinase PKR

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Abstract

The antiviral protein kinase R (PKR) is an important host restriction factor, which poxviruses must overcome to productively infect host cells. To inhibit PKR, many poxviruses encode a pseudosubstrate mimic of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2), designated K3 in vaccinia virus. Although the interaction between PKR and eIF2α is highly conserved, some K3 orthologs were previously shown to inhibit PKR in a species-specific manner. To better define this host range function, we compared the sensitivity of PKR from 17 mammals to inhibition by K3 orthologs from closely related orthopoxviruses. The K3 orthologs showed species-specific inhibition of PKR and exhibited three distinct inhibition profiles. In some cases, PKR from closely related species showed dramatic differences in their sensitivity to K3 orthologs. Vaccinia virus expressing the camelpox virus K3 ortholog replicated more than three orders of magnitude better in human and sheep cells than a virus expressing vaccinia virus K3, but both viruses replicated comparably well in cow cells. Strikingly, in site-directed mutagenesis experiments between the variola virus and camelpox virus K3 orthologs, we found that different amino acid combinations were necessary to mediate improved or diminished inhibition of PKR derived from different host species. Because there is likely a limited number of possible variations in PKR that affect K3-interactions but still maintain PKR/eIF2α interactions, by chance PKR from some potential new hosts may be susceptible to K3-mediated inhibition from a virus it has never previously encountered. We conclude that neither the sensitivity of host proteins to virus inhibition nor the effectiveness of viral immune antagonists can be inferred from their phylogenetic relatedness but must be experimentally determined.

Authors summary

Most virus families are composed of large numbers of virus species. However, in general, only a few prototypic viruses are experimentally studied in-depth, and it is often assumed that the obtained results are representative of other viruses in the same family. In order to test this assumption, we compared the sensitivity of the antiviral protein kinase PKR from various mammals to inhibition by multiple orthologs of K3, a PKR inhibitor expressed by several closely related orthopoxviruses. We found strong differences in PKR inhibition by the K3 orthologs, demonstrating that sensitivity to a specific inhibitor was not indicative of broad sensitivity to orthologs of these inhibitors from closely related viruses. We also show that PKR from even closely related species displayed markedly different sensitivities to these poxvirus inhibitors. Furthermore, we identified amino acid residues in these K3 orthologs that are critical for enhanced or decreased PKR inhibition and found that distinct amino acid combinations affected PKRs from various species differently. Our study shows that even closely related inhibitors of an antiviral protein can vary dramatically in their inhibitory potential, and cautions that results from host-virus interaction studies of a prototypic virus genus member cannot necessarily be extrapolated to other viruses in the same genus without experimental verification.

Introduction

Poxviruses are a large family of double-stranded (ds) DNA viruses encompassing two subfamilies and 14 currently recognized genera, which include many viruses of both medical and veterinary importance. Poxviruses, as a group, infect a large range of animals; however, the host range of individual virus species can vary dramatically [1]. For example, members of the well-studied orthopoxvirus genus have one of the broadest host ranges of all poxviruses with vaccinia viruses (VACV), cowpox viruses (CPXV) and monkeypox viruses (MPXV) infecting a wide range of mammals. Yet the genus also includes viruses that only infect a single species such as variola virus (VARV), the causative agent of smallpox in humans, and ectromelia virus (ECTV), which only infects mice. There are also orthopoxviruses with a narrow host range such as camelpox virus (CMLV), which primarily infects camels but can also cause infections in humans [2, 3]. This diversity in poxvirus host range is believed to be mediated, at least in part, by a set of host range genes, which have largely evolved to counteract the host antiviral response [1].

One important host antiviral protein that is targeted by poxvirus host range factors is protein kinase R (PKR), which exerts its antiviral effect against many virus families. PKR is ubiquitously expressed in host cells in an inactive monomeric form. In the presence of dsRNA, produced during the replication cycle of most viruses, PKR is activated through a process of dimerization and autophosphorylation. Activated PKR phosphorylates the alpha subunit of eukaryotic translation initiation factor 2 (eIF2), which ultimately prevents delivery of methionyl-tRNA to the ribosome and blocks cap-dependent translation [4-6]. In response to this potent antiviral activity, many viruses have evolved at least one PKR antagonist [7]. These conflicting host and viral proteins exert strong selective pressure on each other, driving rapid, successive changes to the coding sequence of each protein to either disrupt or re-engage the interaction. Signatures of positive selection resulting from this evolutionary “arms race” have been identified across the PKR coding sequence, and some of these positively selected residues have been shown to be determinants of differential susceptibility to both poxvirus and herpesvirus PKR antagonists [8-10].

Many poxviruses express two PKR antagonists, designated E3 and K3 in VACV. E3, the gene product of E3L, contains a N-terminal Z-nucleic acid binding domain and a C-terminal dsRNA binding domain. E3 inhibits PKR by binding to dsRNA and preventing PKR homodimerization [11, 12]. K3, the gene product of K3L, is a structural homolog of eIF2α and acts as a pseudosubstrate inhibitor by binding to activated PKR, thus precluding phosphorylation of eIF2α [13, 14]. Both E3 and K3 have been identified as host range factors, because deletion of either E3L or K3L led to impaired VACV replication in cell lines derived from different species [13, 15]. While the mere presence or absence of host range genes likely contributes to the host range of poxviruses, many orthopoxviruses share a similar profile of host range genes, despite having different host tropisms [16]. This indicates that variations in these host range genes may influence host range by altering interactions with host molecules.

Our recent work has supported this hypothesis in the leporipoxvirus and capripoxvirus genera. We have demonstrated that capripoxvirus K3 orthologs inhibit PKR in a species-specific manner, strongly inhibiting sheep, goat, and human PKR, while only

weakly inhibiting cow and mouse PKR [17]. Similarly, M156, the K3 ortholog from the rabbit- and hare-specific myxoma virus (MYXV), inhibited European rabbit PKR but not PKR derived from seven other mammals. Moreover, this work demonstrated that changes in viral antagonists can be rapidly fixed in the population, as demonstrated by the discovery that M156 evolved a loss of function mutation that might contribute to the attenuation of the virus in European rabbits [18].

Most research on orthopoxviruses has focused on the prototypic poxvirus VACV as a model for poxvirus virology and host-virus interactions. However, very few studies have systematically investigated the activity of VACV orthologs in other orthopoxviruses. It is therefore unclear whether the activity of VACV proteins is representative of their orthologs in other orthopoxvirus. Orthopoxviruses such as CPXV and VARV represent substantial public health or bioterror threats, and human infections with orthopoxviruses are predicted to become more common as immunity due to the cessation of the smallpox vaccination decreases in the population [19]. Therefore, it is critically important to identify orthopoxvirus determinants of host range and to identify viruses most likely to infect new species.

In this study, we compared inhibition of PKR from a panel of mammalian species by K3 orthologs from VACV, VARV, CMLV and CPXV-rat09 to determine whether the ability of VACV K3 to inhibit PKR from a given host was predictive of the ability for other orthopoxvirus K3 orthologs to inhibit this host antiviral protein. The results presented here show that the tested K3 orthologs exhibited distinct inhibition profiles and also that the phylogenetic relatedness of PKR was not a good predictor for how susceptible they were to inhibition by K3 orthologs. Mapping K3 residues that governed the species-specificity of these interactions revealed that multiple amino acid substitutions were necessary to convert a weak K3 ortholog into a better inhibitor of most tested PKRs. Furthermore, different amino acid combinations were needed to most effectively inhibit PKR from different species.

Results

Species-specific inhibition of mammalian PKR by vaccinia virus K3

Previous yeast-based and transfection assays indicated differential inhibition of PKR from several vertebrate species by VACV K3 [8, 9]. In order to get a more complete picture about the breadth of species-specific PKR inhibition, we analyzed the sensitivity of PKRs from 17 mammals to inhibition by VACV K3 in an established luciferase-based reporter (LBR) assay (Fig. 1A) [9]. In agreement with previous findings, human, and sheep PKRs were largely resistant, whereas mouse and cow PKRs were sensitive to K3 inhibition in this assay [17]. Among the primate PKRs tested, tamarin PKR was most strongly inhibited, consistent with previous yeast assays [8]. Because vaccinia virus is thought to have originated from a rodent virus, it was surprising that only Syrian hamster and mouse PKRs were sensitive, whereas PKRs derived from other rodents, rats and guinea pigs, were resistant. Among PKRs from ungulates, horse, cow and camel PKR were sensitive, whereas sheep PKR was only weakly sensitive and pig PKR was resistant. The observation of such extensive variability in sensitivity to VACV K3, even among related species, raised the question as to whether other orthopoxvirus K3 orthologs would follow this pattern of PKR inhibition.

157 **Inhibition profiles of K3 orthologs from other orthopoxviruses are distinct from**
158 **VACV K3**
159 To compare the inhibition profiles of VACV K3 orthologs from other
160 orthopoxviruses, we cloned VARV C3L, CMLV (CMS strain) 032, and CPXV 043 from the
161 CPXV-rat09 strain, the latter of which is identical to multiple CPXV isolates from the D
162 clade [20, 21], into the mammalian expression vector pSG5. CPXV-rat09 043 is also
163 identical to protein 037 from taterapox virus (TATV) and will henceforth be referred to as
164 CPXV-D 043/TATV 037. Relative to VACV K3, CPXV-D 043/TATV 037 has 11 amino acid
165 (aa) differences (87.5 % identity), CMLV 032 has 12 aa differences (86.4 % identity), and
166 VARV C3 has 17 aa differences (80.7 % identity) (Fig. 2A, B). Transient transfection of
167 HeLa-PKR^{kd} cells with the K3L orthologs revealed comparable expression by Western
168 blot (Fig. 2C). These three K3 orthologs showed different inhibition profiles by the LBR
169 assay, which were distinct from each other and from the VACV K3 inhibition pattern (Fig.
170 1 and Fig. 3). Compared to VACV K3, VARV C3 showed slightly better inhibition of human
171 and horse PKR, and considerably better inhibition (> 2-fold) of gibbon and sheep PKR. In
172 contrast, VARV C3 inhibited PKR from eight other species less well than VACV K3.
173 CPXV-D 043/TATV 037 and CMLV 032 showed overall similar inhibition profiles to one
174 another, inhibiting PKR from most species better than VACV K3. However, there was
175 essentially no difference in the ability of CPXV-D 043/TATV 037 or CMLV 032 to inhibit
176 rhesus, cat, horse or camel PKR, compared to VACV K3. Pig and guinea pig PKR were
177 largely resistant to inhibition by all K3 orthologs tested.
178 To visualize if the phylogenetic relatedness of a given PKR may be predictive of
179 susceptibility to these K3 orthologs, we generated a phylogenetic tree using PKR
180 sequences derived from 40 mammalian species. We included PKRs from several species
181 that were not tested in the LBR assays to achieve better statistical support for the
182 branches. We then mapped the relative sensitivities of the tested PKRs to each K3
183 ortholog obtained from multiple LBR assays using a susceptibility scale ranging from
184 weakly sensitive to highly sensitive on a scale from 1 to 6, respectively (Fig. 4). By this
185 analysis there was no discernible pattern to predict PKR sensitivity by phylogenetic
186 relatedness.
187
188 **Sensitivity of PKR to K3-mediated inhibition correlates with host range function**
189 **during infection**
190 In order to analyze whether the potency of PKR inhibition by VACV and CMLV K3
191 orthologs correlates with the ability of these proteins to support productive infection, we
192 cloned VACV K3L and CMLV 032 into the E3L locus of the highly attenuated VACV strain
193 VC-R4, which lacks both K3L and E3L and can only replicate in the absence of PKR or
194 in cells that express exogenous PKR inhibitors [22]. Using these viruses, we compared
195 plaque formation and virus replication relative to wild type vaccinia virus (VC-2,
196 Copenhagen strain) in cells of human (HeLa), gibbon (Tert-GF), sheep (OA1) or cow (BT)
197 origin. In HeLa cells that have PKR knocked out (HeLa-PKR^{ko}), all four viruses formed
198 comparably sized plaques (Fig. 5A). In contrast, infected wild type HeLa or Tert-GF cells
199 only developed plaques when they were infected with VC-2 or VC-R4+CMLV 032. These
200 viruses also caused large plaque formation in OA1 cells, whereas infection with VC-
201 R4+VACV K3L led to the formation of small plaques. In BT cells, all viruses, except for
202 VC-R4, formed comparably sized plaques.

203 Multiple cycle replication assays also correlated with the results of the plaque
204 assays, with VC-2, VC-R4+CMLV 032, and VC-R4+VACV K3L replicating to comparable
205 titers in HeLa PKR^{ko} cells and BT cells. In Contrast, VC-2 and VC-R4+CMLV 032 were
206 the only viruses that replicated well in HeLa, Tert-GF, and OA1 cells. Viruses containing
207 VACV-K3L replicated to slightly higher titers than input (~10-fold increase) in HeLa and
208 Tert-GF cells indicating that the weak inhibition of PKR allowed limited virus replication.
209

210 **Identification of amino acid residues that confer differential PKR inhibition between**
211 **VARV C3L and CMLV 032**

212 VARV C3L and CMLV 032 differ by seven aa residues (Fig. 2). In order to identify
213 which residues are responsible for the virus-specific differences in PKR inhibition, we first
214 exchanged single aa residues in VARV C3L with the ones found in CMLV 032 and
215 performed the LBR assay with human, tamarin, mouse, dog, cow and sheep PKR. None
216 of these mutants inhibited human PKR better, whereas some mutants showed better
217 inhibition of some other PKRs. Only one VARV C3L mutant (V25A) reached comparable
218 PKR inhibition as CMLV 032, and only for dog PKR. VARV C3L-V25A showed more than
219 2-fold increased PKR inhibition of tamarin and sheep PKR and more than 50% higher
220 inhibition of cow and mouse PKR. VARV C3L-I70V showed more than 2-fold increased
221 PKR inhibition of tamarin PKR and more than 50% higher inhibition of mouse and dog
222 PKR. VARV C3L-V3A displayed more than 50% higher inhibition of dog and sheep PKR,
223 whereas VARV C3L-T44V only showed higher inhibition of mouse PKR (more than 2-
224 fold).

225 These experiments confirmed that a single aa residue was not responsible for the
226 differential PKR inhibition found for most of the tested PKRs. Therefore, we generated
227 mutants containing multiple aa changes, designated as M2-M7 (Fig. 7A). The majority of
228 these residues localized to the suspected PKR-K3 interacting face when we mapped
229 these positions on the existing VACV K3 structure (blue and purple, Fig. 7B). We
230 expressed each mutant VARV C3L in transfected HeLa-PKR^{ko} cells and confirmed
231 expression by Western blot analysis. VARV C3L and M1 were detected slightly less well,
232 which might be explained by the presence of isoleucine at position 70, potentially altering
233 the epitope detected by the primary antibodies. Mutation of this residue to valine (M2 and
234 up), which is included in the native epitope, resulted in detection comparable to CMLV
235 032. Remarkably, all tested PKRs showed different sensitivity patterns (Fig. 7D). For
236 human PKR, a combination of four mutations (M4) resulted in inhibition comparable to
237 CMLV 032, while mutations in M2 and M3 had no effect. Inhibition of tamarin PKR
238 increased more gradually, with M4 being as effective as CMLV 032. Inhibition of mouse
239 PKR increased sharply with the inclusion of T44V in M6. Inhibition of cow PKR showed
240 the most gradual, stepwise increase from M1 to M7.
241

242 **Alanine 25 and isoleucine 70 in CMLV 032 are important for strong PKR inhibition**

243 Because swapping residues 25 and 70 in VARV C3L with the ones in CMLV 032
244 resulted in better inhibition of PKRs from many species, we replaced the corresponding
245 residues in CMLV 032 with the VARV C3L residues either alone or in combination. Single
246 mutations led to strongly decreased inhibition of human, mouse, cow and sheep PKR.
247 For these species' PKR, the double mutant CMLV 032-A25V/V70I, showed PKR inhibition
248 comparable to VARV C3L, indicating additive effects of both mutations. Single CMLV 032

249 mutants changed the inhibition of gibbon PKR only weakly, and replacement of both A25V
250 and V70I resulted in inhibition comparable to VARV C3L. Tamarin PKR was the only PKR
251 tested for which the double-mutant displayed substantially stronger PKR inhibition than
252 VARV C3L. We next compared replication of VACV containing CMLV 032-A25V/I70L with
253 a virus expressing either CMLV 032 or VACV K3L. Correlating with the LBR assays,
254 VACV-CMLV 032-A25V/I70L showed only modestly lower (3.4-fold) replication than
255 VACV-CMLV 032 in tert-GF, whereas 117-fold lower replication was observed in HeLa
256 cells. Notably, VACV-CMLV 032-A25V/I70L showed higher replication than VACV-K3L in
257 HeLa cells.
258

259 ## Discussion

260

261 Most research on poxviruses has focused on VACV, which is often called the
262 prototypic poxvirus. VACV K3 has been shown to inhibit PKR from several mammals in
263 a species-specific manner, as defined in both LBR assays and yeast-based studies [8, 9,
264 17]. The data presented here support and extend this species-specific sensitivity of PKR
265 to VACV K3L and add to a growing body of evidence that virus-specific differences play
266 an important role in viral host range, as do species-specific differences in host genes [23,
267 24]. Consistent with this concept, in this study we show that the VACV K3 orthologs from
268 four different orthopoxviruses displayed dramatic differences in their ability to inhibit PKR
269 from different species, including different sensitivities in closely related species such as
270 mice and rats. Furthermore, VACV, VARV and CMLV/CPXV-D 043/TATV 037 orthologs
271 showed three distinct inhibition profiles when tested against 17 mammalian PKR
272 orthologs. The extent of this phenomenon across orthopoxvirus K3L orthologs uncovered
273 here was unanticipated.
274 The three distinct patterns of PKR inhibition mediated by K3 orthologs that we
275 identified in this study do not appear to be predictable by either phylogenetic distance
276 between different PKRs, or by whether the PKR is derived from a natural or alternative
277 host species. For example, among the primate PKRs tested, VACV K3L only inhibited
278 tamarin PKR efficiently, although VACV can infect many primates [25]. However, VARV
279 K3 ortholog C3L only inhibited gibbon PKR efficiently. Surprisingly, despite VARV being
280 a human-specific virus, C3L did not efficiently inhibit human PKR. In contrast, although
281 CMLV does not efficiently infect primate species, CMLV 032 inhibited all primate PKRs
282 except rhesus PKR. The inhibition of PKRs by VACV K3 and CMLV 032 in the LBR
283 assays correlated with the ability of those inhibitors to support virus replication *in vitro*. A
284 VACV expressing only K3L replicated three to four orders of magnitude better in bovine
285 cells than in human, gibbon, or sheep cells. In contrast, the same virus expressing the
286 CMLV K3 ortholog 032 replicated as efficiently as wild type VACV in all of these cell types.
287 This also demonstrates that one efficient K3 ortholog can substitute for both VACV E3
288 and K3, in at least some cell lines.
289 The strong activity of CMLV K3 against PKR from its natural host (camel) is in line
290 with previous studies that showed that myxoma virus, sheepox virus and goatpox virus
291 inhibited PKR from their naturals hosts with high efficiency [17, 18]. In contrast, we have
292 shown here and in a previous study that K3 orthologs from two other poxviruses, lumpy
293 skin disease virus and VARV, only weakly inhibited PKR from their natural hosts, cattle
294 and humans, respectively [17]. Because CPXVs possess very broad host ranges it is

295 difficult to make conclusions about their natural hosts [25]. As of now, 26 CPXV isolates
296 possess K3 orthologs that are identical to CPXV-D 043/TATV 037 on the protein level.
297 These viruses were mostly isolated from humans (38%), rats (31%) and cats (23%) [20,
298 26, 27], and PKR derived from these hosts were intermediately sensitive to this K3
299 ortholog. The host range of TATV, which was isolated from an healthy gerbil (*Tatera*
300 *kempi*), is unknown [28].
301 It is not clear, why some poxvirus K3 orthologs are efficient inhibitors of their host's
302 PKRs, whereas others are not. A possible explanation is that different poxviruses may
303 produce different amounts of dsRNA during infection. The formation of dsRNA is an
304 important danger signal detected by multiple innate immune system sensors, and
305 perhaps as a consequence, the amount of dsRNA produced during infection varies even
306 between closely related orthopoxviruses. For example, vaccinia virus produces high
307 amounts of dsRNA during infection, while monkeypox virus (MPXV) and ectromelia virus
308 (ECTV) produce lower amounts of dsRNA [29, 30]. In agreement with this observation,
309 total RNA produced during MPXV infection was a less potent activator of human PKR
310 than total RNA produced during VACV infection [29]. Strikingly, MPXV and ECTV contain
311 inactivating mutations in their K3L orthologs [16]. Thus, while K3 may be a necessary
312 PKR antagonist in viruses that produce large amounts of dsRNA, possibly releasing some
313 E3 to antagonize other dsRNA-activated pathways, poxviruses that naturally produce less
314 dsRNA might not require redundant PKR inhibitors. It will be interesting to determine
315 whether this correlation between reduced dsRNA production and K3 activity holds across
316 multiple poxviruses.
317 In many cases, K3 orthologs inhibited PKR from species that are not the natural
318 hosts of the respective poxvirus, e.g. CMLV 032 strongly inhibited gibbon, dog and cow
319 PKR, even though these species are likely never infected by CMLV. There is evidence of
320 substantial positive selection in PKR throughout the mammalian phylogeny, likely driven
321 by a diverse set of viral PKR inhibitors [8, 9]. These positively selected residues cluster
322 around the eIF2α binding region of PKR. Because the PKR/eIF2α interaction must be
323 preserved, there are likely a limited number of evolutionary options available to disrupt
324 this interface between PKR and pseudosubstrate inhibitors like K3. Thus, even though
325 CMLV 032 likely evolved to inhibit PKR in the camelid lineage, the evolutionary
326 constraints on PKR increase the likelihood that PKR from other species might by chance
327 be susceptible to this inhibitor. In line with this is a recent report that shows that VACV
328 strains that contain different poxvirus K3 orthologs as their only PKR inhibitors exhibited
329 different replication profiles in cell lines from different species, with some strains showing
330 broader host ranges, whereas others showed narrower host ranges [31]. In a broader
331 context, these chance susceptibilities of host antiviral factors to viral inhibitors that a host
332 has otherwise never been exposed to, might play an important role in cross-species
333 transmission of viruses [24].
334 Although VARV is a human-specific pathogen, this study and previous yeast-
335 based assays demonstrated that the VARV K3L ortholog does not inhibit human PKR
336 efficiently [8, 9]. However, mutation of some of the positively selected residues in human
337 PKR to residues found in other species differentially affected the sensitivity to VACV K3
338 and VARV C3, further illustrating that these differences are mediated by only a few
339 residues in either the host or viral protein [9]. The close relatedness between VARV C3
340 and CMLV 032 allowed the identification of aa residues that are responsible for the

differential inhibition of PKR. In 5 of the 6 tested PKR mutants, single aa substitutions in VARV C3 led to little or no change in activity. Distinct combinations of mutations were necessary to make VARV C3 as efficient as CMLV 032 depending on the particular PKR ortholog being tested. For example, only V25A was necessary to make VARV C3 inhibit dog PKR as efficiently as CMLV 032; however, four aa changes were necessary to phenocopy CMLV 032 inhibition of human PKR by VARV C3, and only three aa changes were necessary for this effect against sheep PKR. It is noteworthy that the loss of PKR inhibition by CMLV 032 was more easily achieved by single aa substitutions than an increase in PKR inhibition. The differences in VARV C3L were all acquired after the split from a CMLV/TATV ancestor, and all of these C3L mutations are non-synonymous, which is indicative of positive selection [8]. This might indicate that there was some selective advantage for VARV C3 to become a less efficient inhibitor of human PKR. Alternatively, these mutations might have occurred in an ancestor of VARV as an adaption to a different host, before it became human-specific.

In addition to the work on K3 orthologs, there is evidence that other poxvirus immune antagonists can have varying effects on the host. For example, while the E3 orthologs derived from swinepox virus and myxoma virus rescued E3L-deficient VACV in HeLa cells, the sheeppox virus E3 ortholog was not able to rescue this virus [32]. Along this line, sheeppox virus E3L was not effective in inhibiting sheep PKR in the LBR assay [17]. These findings indicate that not all E3L orthologs are as broadly functional as currently believed. Thus, it is possible that poxviruses whose E3 orthologs inhibit PKR less efficiently might depend more on their K3 orthologs for PKR inhibition than is currently recognized. Similarly, orthologs of the complement-binding factor VCP from VACV, VARV and MPXV were shown to inhibit complement factors in a species-specific manner [33-37]. Another example of variations in poxviral immune antagonist activity is found in the poxvirus C7L family, which confers host range function in different poxviruses [38-40]. C7 orthologs were shown to bind and inhibit host restriction factors SAMD9 and SAMD9L in a species-specific manner [41-43]. Interestingly, SAMD9/SAMD9L inhibition can be mediated by different, unrelated poxvirus proteins such as CPXV CP77 or K1 in some poxviruses. CPXV CP77 was recently shown to bind and inhibit Chinese hamster SAMD9L, which was not inhibited by VACV C7 or K1 [44]. In combination with the data we present in this study, these results emphasize the fact that immunomodulation is an important component of species-specificity. Changes in a small number of residues in viral immunomodulators may confer unexpected sensitivities to antiviral factors from species that have not encountered the virus. This chance sensitivity may simply be due to the relatively small number of residues that appear to govern these host-virus interactions. In some cases, exemplified by SAMD9/SAMD9L inhibition, virus-specific differences in strategies to inhibit a particular host immune response adds a further layer of complexity to the task of predicting virus host range *a priori*. Ultimately, it is likely that a combination of some or all of these interactions is necessary to permit a virus to replicate in a given host [24].

Species-specific interactions of PKR with viral inhibitors have also been observed with cytomegalovirus (CMV) proteins. Human CMV TRS1 only inhibited human PKR but not PKR from old world monkeys, while the TRS1 ortholog from African green monkey (AGM) CMV inhibited AGM but not human PKR. Intriguingly, mutation of a single amino acid in human PKR (position 489), which determines susceptibility to human or AGM

387 TRS1, is also involved in the weak sensitivity of human PKR to VACV K3 inhibition. Taken
388 together, these data highlight that inhibitors from different pathogens can be affected by
389 mutations in individual antiviral proteins [8, 10].
390 Large DNA viruses commit about half of their genes to counteract and redirect the
391 host immune response. Because many viruses evolve with specific hosts, it can be
392 assumed that a considerable portion of their encoded proteins will show some level of
393 host-specificity, which ultimately contributes to their host range and virulence. Our study
394 shows that orthologs of even closely related viruses can show dramatic differences in
395 their interaction with host innate immune system. Furthermore, the phylogenetic
396 relatedness of PKR was not a good predictor for how well they were inhibited by K3
397 orthologs. These findings imply that research obtained with one specific virus and one
398 specific host species cannot necessarily be generalized, instead these interactions likely
399 require experimental validation for other viruses and species.
400
401
402 ## Materials and methods
403
404 **Cell lines**
405 HeLa (human, ATCC #CCL-2), HeLa PKR-knockdown(^{kd}) (kindly provided by Dr.
406 Charles Samuel [45]), HeLa PKR-knockout (kindly provided by Dr. Adam Geballe [10]),
407 RK13+E3L+K3L cells (rabbit) [46], tert immortalized gibbon fibroblasts (tert-GF, kindly
408 provided by Dr. Stephanie Child and Dr. Adam Geballe), OA1 (sheep, ATCC #CRL-6538)
409 and BT (cow, ATCC #CRL-1390) were maintained in Dulbecco's Modified Eagle's
410 Medium (DMEM) supplemented with 5% fetal bovine serum or 10% horse serum (BT
411 cells) and 100 IU/ml penicillin/streptomycin (Gibco). HeLa PKR^{kd} cells were maintained
412 in medium containing 1 µg/ml puromycin (Sigma). RK13+E3L+K3L cell culture medium
413 contained 500 µg/ml geneticin and 300 µg/ml zeocin (Life Technologies).
414
415 **Plasmids**
416 All PKR and viral genes were cloned into the pSG5 expression vector (Stratagene)
417 for transient transfection assay. Cloning of cow and sheep PKR, European rabbit PKR,
418 mouse PKR, rat PKR, Syrian hamster PKR and guinea pig PKR was described previously
419 [18]. Orangutan PKR, gibbon PKR, macaque PKR, and tamarin PKR were kindly provided
420 by Dr. Nels Elde [8]. Synonymous mutations were introduced in the target site for the
421 shRNA in orangutan PKR, gibbon PKR, macaque PKR and tamarin PKR to generate
422 knock-down resistant PKRs. Horse PKR was cloned from efc cells (kindly provided by Dr.
423 Udeni Balasuriva and Dr. Ying Fang). Pig PKR was cloned from PK15 cells (ATCC-CCL-
424 33). Camel PKR was cloned from Dubca cells (ATCC-CRL2276). Cloning of VACV_K3L
425 (YP_232916.1) was previously described [9]. VARV_C3L (DQ441439.1) and CPXV-D
426 043/TATV 037 (AGY97204.1/YP_717344.1) were cloned into pSG5 from synthesized
427 DNA. CMLV_032 (NP_570422.1) was generated from pSG5-CPXV-D_043/TATV_037 by
428 site-directed mutagenesis. VACV K3L and CMLV 032 constructs were cloned into P837-
429 GOI-mCherry-E3L for recombinant VACV generation using primers containing *Sal*I
430 (forward primer) and *Nhe*I (reverse primer) sites, as described [22]. VARV C3L and CMLV
431 032 single and overlapping mutations were generated by site-directed mutagenesis using
432 Phusion High-Fidelity DNA polymerase (NEB). All ORFs were validated by Sanger

433 sequencing (UC Davis sequencing facility). Plasmids containing variola virus DNA were
434 handled and stored in the absence of replicating poxviruses.
435
436 **Luciferase-based reporter (LBR) assays**
437 Assays were performed as described [9] with the following modifications: 5×10^4
438 Hela PKR^{kd} cells per well 24 well plates were seeded 16 hours prior to the experiment.
439 Cells were transfected with 200 ng of the indicated PKR expression vector, the indicated
440 amount of each K3L expression vector, and 50 ng of pGL3 firefly luciferase expression
441 vector (Promega) using GenJet (Signagen) at a DNA to GenJet ratio of 1:2 following the
442 manufacturer's protocol. Empty pSG5 vector was used to maintain the DNA concentration
443 at 200 or 100 ng where appropriate. 48 hours post-transfection cells were lysed with
444 mammalian lysis buffer (GE Healthcare), then luciferin (Promega) was added following
445 the manufacturer's recommendations. Luciferase activity was measured using a GloMax
446 luminometer (Promega). Experiments were conducted in triplicate in three independent
447 experiments.
448
449 **Viruses and infection assays**
450 VACV Copenhagen strain VC2 and vP872 [13] were kindly provided by Dr.
451 Bertram Jacobs. Generation of VC-R4, a derivative of vP872, was described [22]. VC-
452 R4:VACV_K3L, VC-R4:CMLV_032 and VC-R4:CMLV_032A25V/V70I were generated by
453 the scarless integration of the open reading frames of the K3L orthologs into the E3L
454 locus in VC-R4 by the same method. The resulting viruses were plaque-purified three
455 times and the integrations were confirmed by Sanger sequencing (UC Davis sequencing
456 facility).
457 Plaque assays were performed with confluent six-well plates of the indicated cell
458 lines, which were infected with 30 plaque forming units (pfu) of each indicated virus, as
459 determined on RK13+E3L+K3L cells. One hour post-infection, the medium was replaced
460 with DMEM containing 1% carboxymethylcellulose (CMC). After 48 hours, cells were
461 washed with PBS and stained with 0.1% crystal violet. The plates were imaged using an
462 iBright Imaging System (Invitrogen).
463 Multiple-cycle virus replication assays were carried out in confluent six-well
464 plates of the indicated cells, which were infected with each indicated virus (MOI = 0.01).
465 After 48 hours, cells and supernatants were collected and subjected to three rounds of
466 freezing at -80 ˚C and thawing at 37 ˚C. Lysates were sonicated for 15s, 50% amplitude
467 (Qsonica Q500). Viruses were titered by 10-fold serial dilutions on RK13+E3L+K3L cells.
468 Infections and viral titer were performed in duplicate.
469
470 **Western blot analyses**
471 To detect expression of K3 orthologs in transfected cells, 4×10^5 Hela PKR-knock-
472 down cells were seeded in six-well plates and transfected with 3 µg of the indicated K3-
473 expressing plasmids following the GenJet protocol described above. After 48 h, cells were
474 lysed with 1% sodium dodecyl sulfate (SDS) in PBS (VWR) and sonicated at 50%
475 amplitude for 10 seconds twice. All protein lysates were separated on 12% SDS-
476 polyacrylamide gels and transferred to polyvinyl difluoride (PVDF, GE Healthcare)
477 membranes. Membranes were blocked with 5% non-fat milk dissolved in TBST (20M Tris,
478 150mM NaCl, 0.1% Tween 20, pH 7.4) for 1 hour. Blots were probed with a primary

antibody (1:3000) directed against VACV K3 [17], or anti-β-actin (1:10,000, Sigma-Aldrich, A5441). All primary antibodies were diluted in TBST containing 5% BSA and 0.02% (w/v) sodium azide. Membranes were incubated overnight at 4˚C in the primary antibody, washed with TBS-T three times for 5 mins, and then incubated for 1 hour at room temperature with donkey anti-rabbit or goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (Invitrogen, A16110, 62–6520) at 1:10,000 in TBST containing 5% (w/v) nonfat milk. The membranes were then washed five times for 5 min and proteins were detected with Amersham ECL (GE Healthcare). Images were taken using the iBright Imaging System (Invitrogen).

PKR phylogeny

The amino acid sequences of PKR from 40 different species were aligned using MUSCLE [47] and the ambiguously aligned positions were eliminated using Gblocks [48]. The phylogenetic analysis was performed using the maximum likelihood approach, as implemented in PhyML v3.0 [49]. Branch support was estimated using the aLRT statistics, and the phylogenetic tree was constructed using the default substitution model, as implemented in the automatic model selection in PhyML [50]. One hundred bootstrap replicates were performed to estimate the nodal support at major branch points. The resulting phylogenetic tree was visualized using FigTree.

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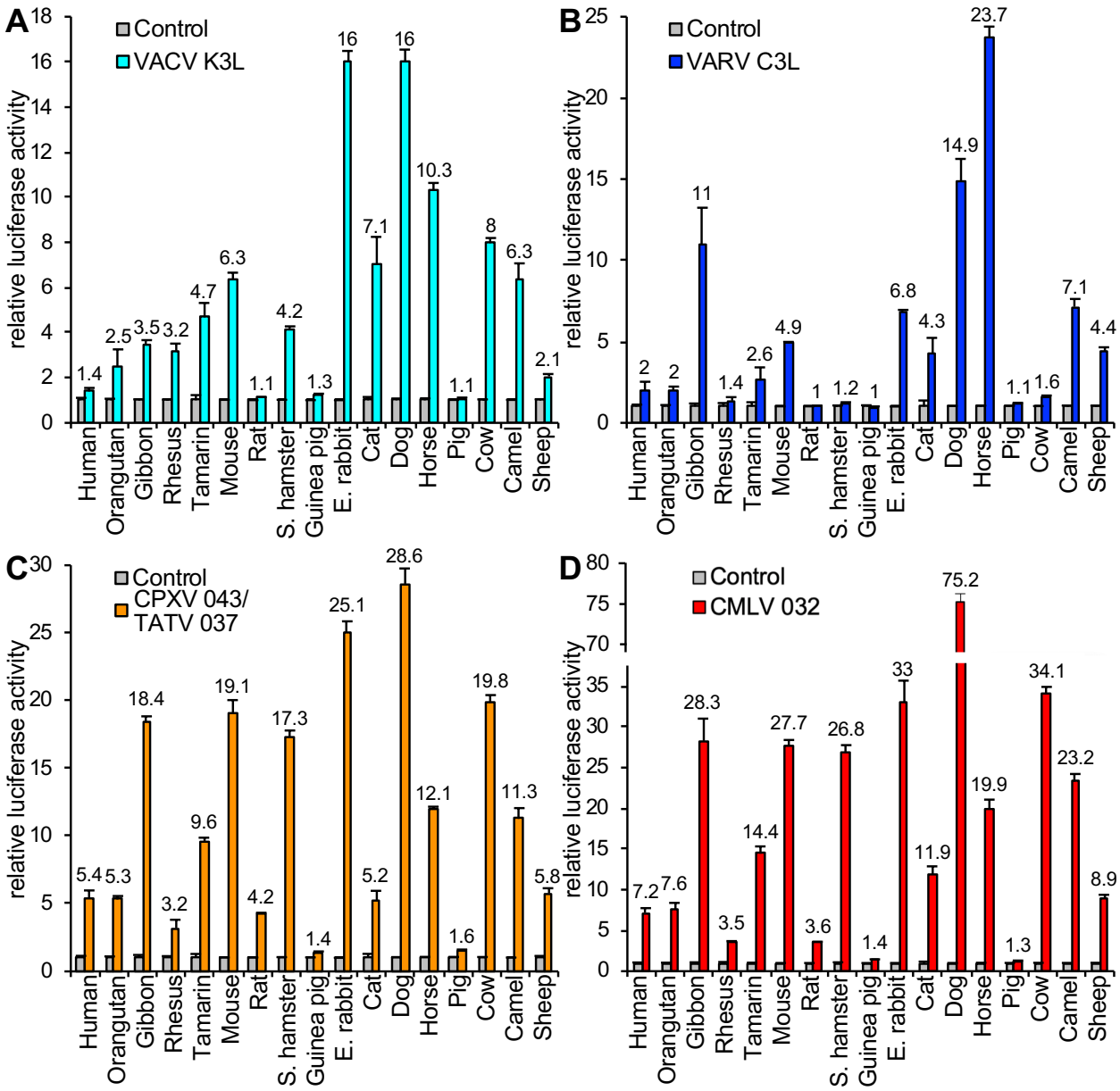
701 **Figures**

702



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705 **Figure 1. Species-specific inhibition of PKR by orthopoxvirus K3 orthologs.** HeLa-
706 PKR^{kd} cells were transfected with expression vectors encoding firefly luciferase (0.05 µg),
707 PKR from the indicated species (0.2 µg) and either 0.2 µg VACV K3L (**A**) or VARV C3L
708 (**B**), CPXV-D 043/TATV 037 (**C**), or CMLV 032 (**D**). Luciferase activities were measured
709 48 hours after transfection and normalized to PKR-only transfected cells to obtain relative
710 luciferase activities. Error bars represent the standard deviations from three independent
711 transfections. Results shown are representative of at least three independent
712 experiments.

713

A

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                    10         20         30         40         50
                     |          |  **    *   |        *  *|*        |
VACV K3    MLAFCYSLPNAGDVIKGRVYEKDYALYIYLFDYPHFEAILAESVKMHMDR
VARV C3    MLVFCYSLPNVGDVLKGKVYENGYVLYVDLFDYPHSEAILVESTQMHMNR
CPXV 042   MLAFCYSLPNVGDVLKGKVYENGYALYIDLFDYPHSEAILAESVQMHMNR
CMLV 032   MLAFCYSLPNVGDVLNGKVYENGYALYIDLFDYPHSEAILAESVQMHMNR

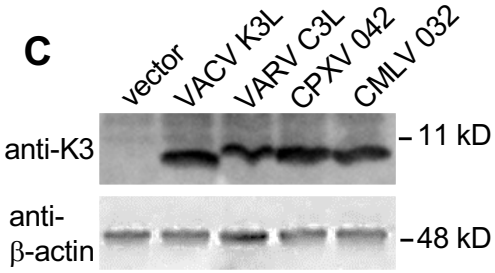
                    60         70         80
                     |         *|*     *  *  |
VACV K3    YVEYRDKLVGKTVKVKVIRVDYTKGYIDVNYKRMCRHQ 88
VARV C3    YFKYRDKLVGKTVKVKVIRIDYTKGYIDVNYKRMCRHQ 88
CPXV 042   YFKYRDKLVGKTVKVKVIRVDYTKGYIDVNYKRMCRHQ 88
CMLV 032   YFKYRDKLVGKTVKVKVIRVDYTKGYIDVNYKRMCRHQ 88
```

B

percent identity

	VACV	VARV	CPXV	CMLV
VACV	----	80.7	87.5	86.4
VARV	80.7	----	93.2	92.0
CPXV	87.5	93.2	----	98.9
CMLV	86.4	92.0	98.9	----

C



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716 **Figure 2. Amino acid differences between orthopoxvirus K3 orthologs.** (**A**) Multiple
717 sequence alignment of VACV K3, VARV C3, CPXV-D 043/TATV 037, and CMLV 032.
718 Amino acids that are unique to VACV K3 are shown in red. Residues that differ between
719 VARV C3 and CMLV 032 are highlighted in blue. The residue that is unique to CMLV 032
720 is shown in orange. Asterisks denote residues of K3 that correspond to the contacts of
721 eIF2α with PKR in a co-crystal structure [51]. The peptide sequence that was used to
722 generate anti-VACV K3 antibodies is underlined. (**B**) Amino acid identities between tested
723 K3 orthologs used in this study. Percent identities were calculated from a multiple
724 sequence alignment using MegAlign. (**C**) Expression of K3 orthologs in transfected cells.
725 HeLa-PKR^{kd} cells were transfected with the indicated K3L orthologs, and cell lysates were
726 collected 48 hours later. Total proteins from these samples were separated on 12% SDS-
727 PAGE gels and analyzed by immunoblot analysis with the indicated anti-K3 and anti-β-
728 actin antibodies.
729

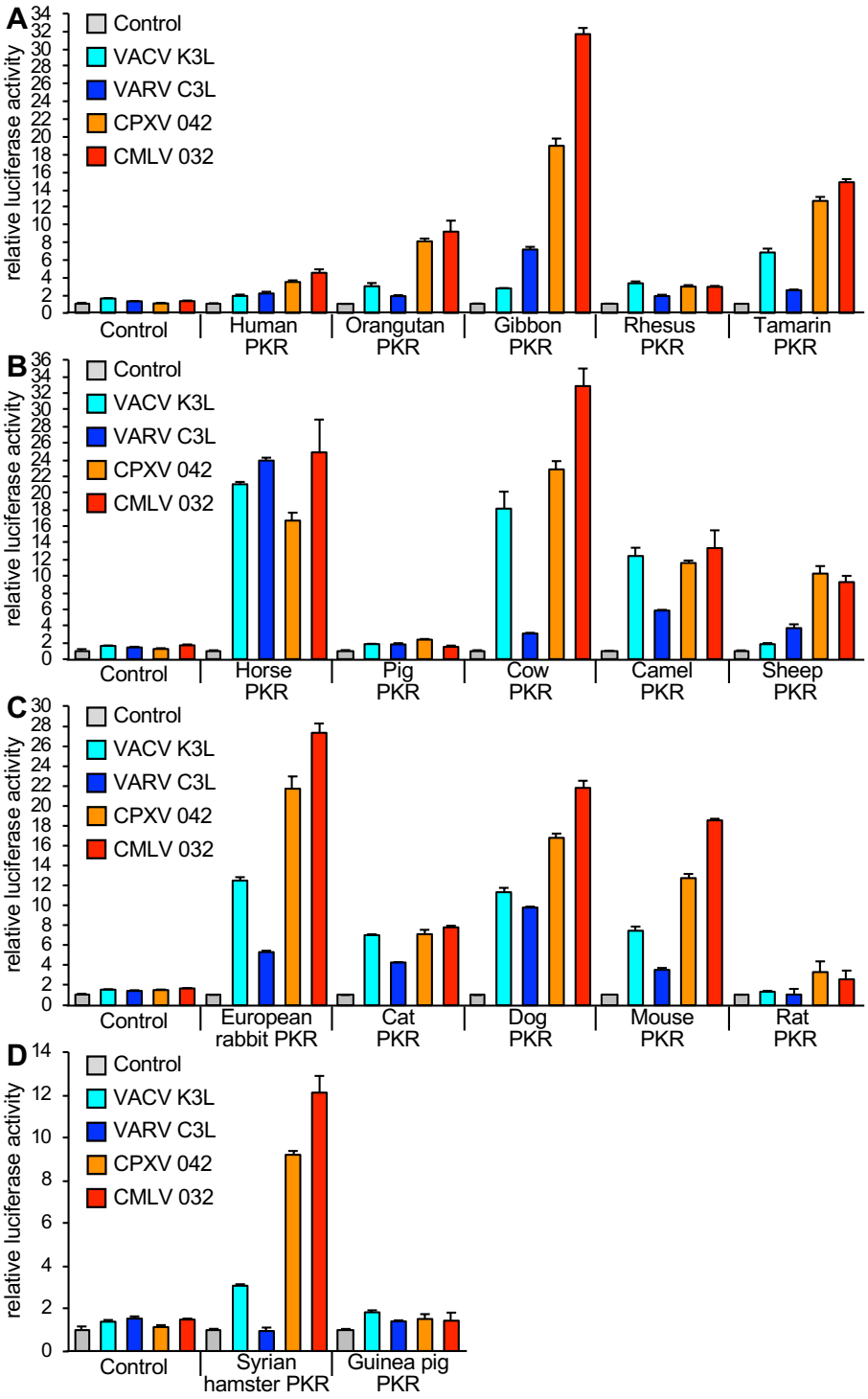


Figure 3. Orthopoxvirus K3 orthologs show differential PKR inhibition. HeLa-PKR^{kd} cells were transfected with expression vectors encoding firefly luciferase (0.05 µg), with either VACV K3L, VARV C3L, CPXV-D 043/TATV 037, or CMLV 032 (0.4 µg), and PKR (0.2 µg) from the indicated species in four groups (**A-D**). Luciferase activities were measured as described in Fig. 1. Error bars represent the standard deviations from three independent transfections. Results shown are representative of at least three independent experiments.

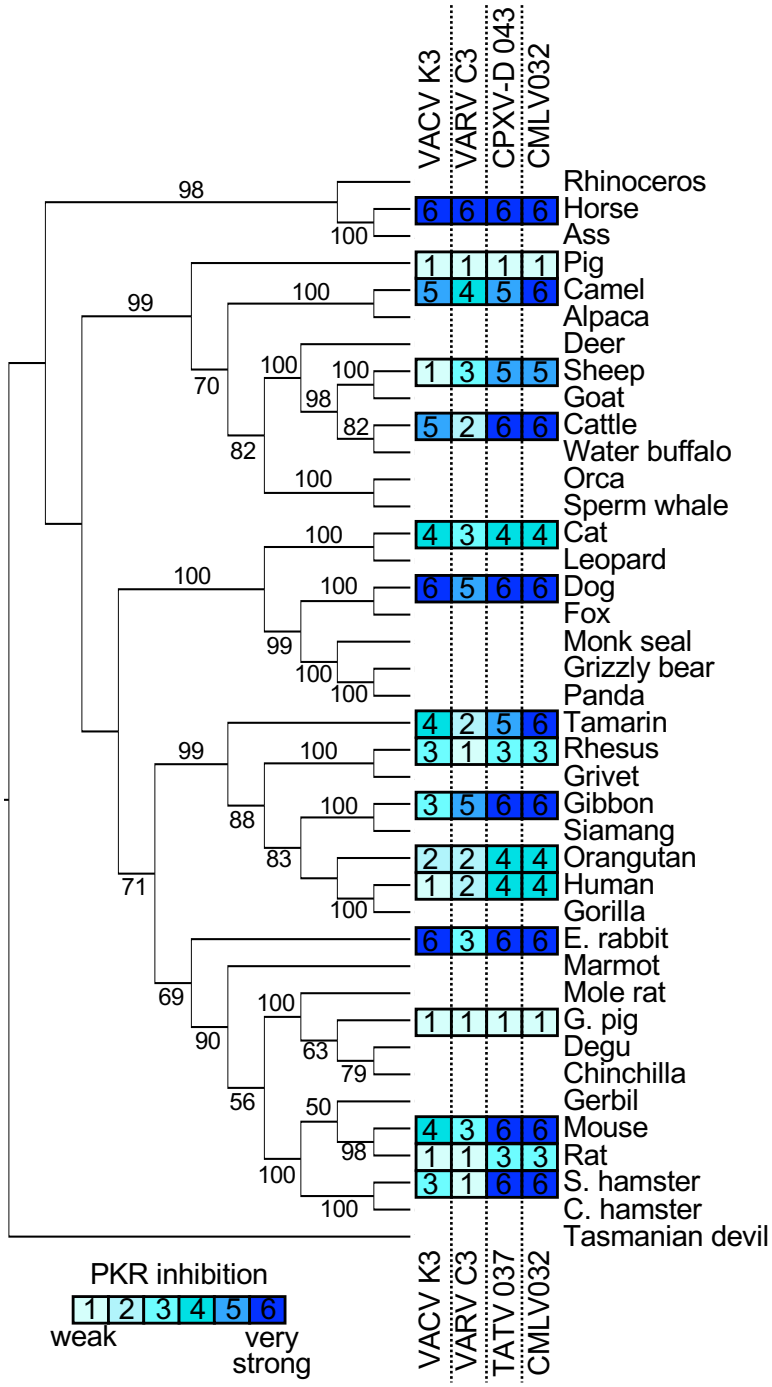


Figure 4. Sensitivities of tested PKRs projected on a PKR phylogenetic tree. A maximum-likelihood phylogenetic tree of PKR from selected mammalian species including the ones tested in this study was generated. The tree was rooted to Tasmanian devil PKR. Bootstrap values ≥ 50 are indicated on the branches. Relative sensitivities of PKRs to VACV K3, VARV C3, CPXV-D 043/TATV 037, or CMLV 032 obtained from multiple experiments with PKR:K3 ortholog ratios of 1:1 and 1:2 are shown on a scale from 1 to 6: 1 = no or very weak inhibition (≤ 2 fold); 2 = weak inhibition (2 to 3-fold); 3 = intermediate low inhibition (3 to 5-fold); 4 = intermediate high inhibition (5 to 7 fold); 5 = high inhibition (7 to 10-fold); 6 = very high inhibition (≥ 10-fold).

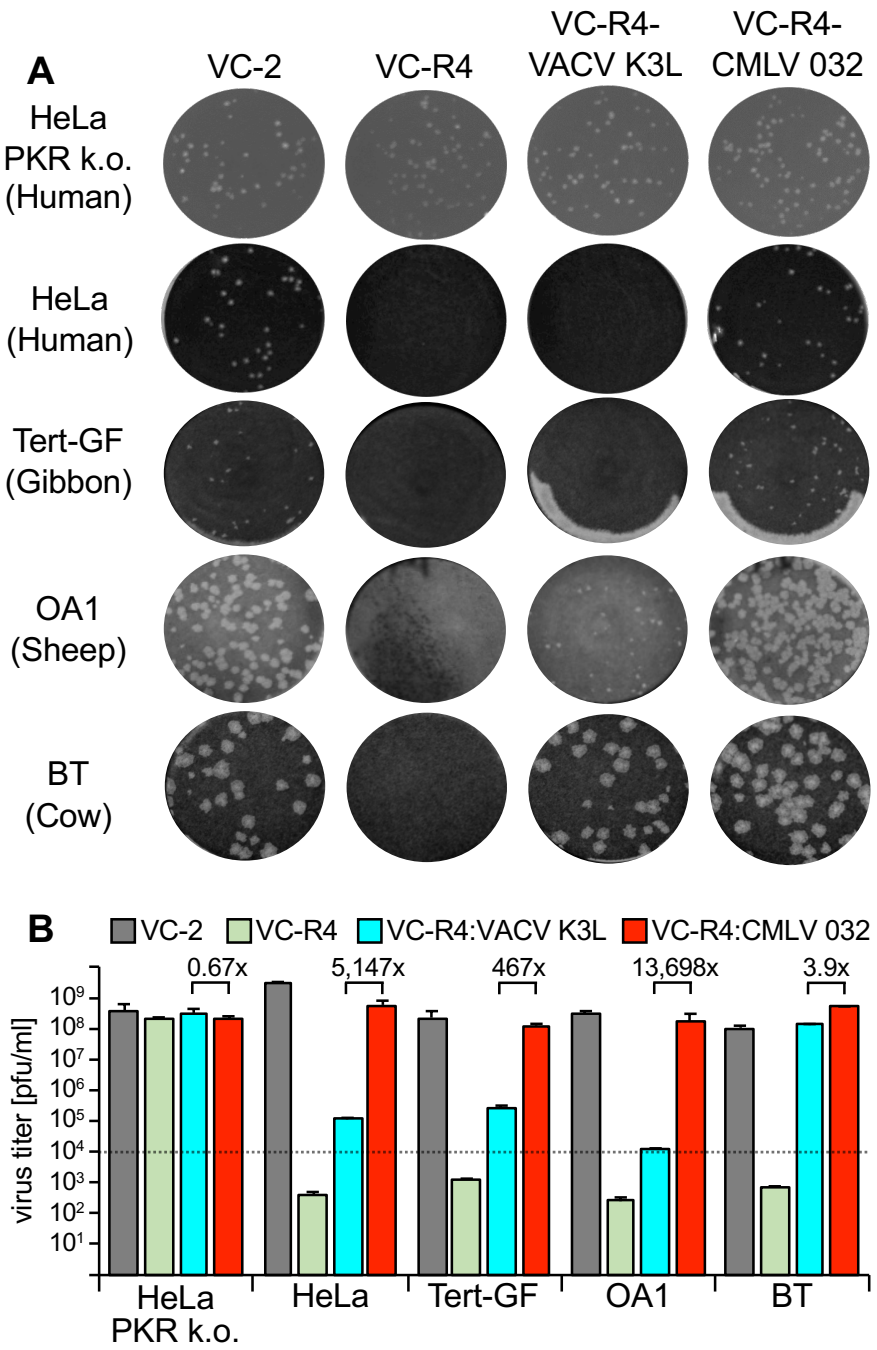
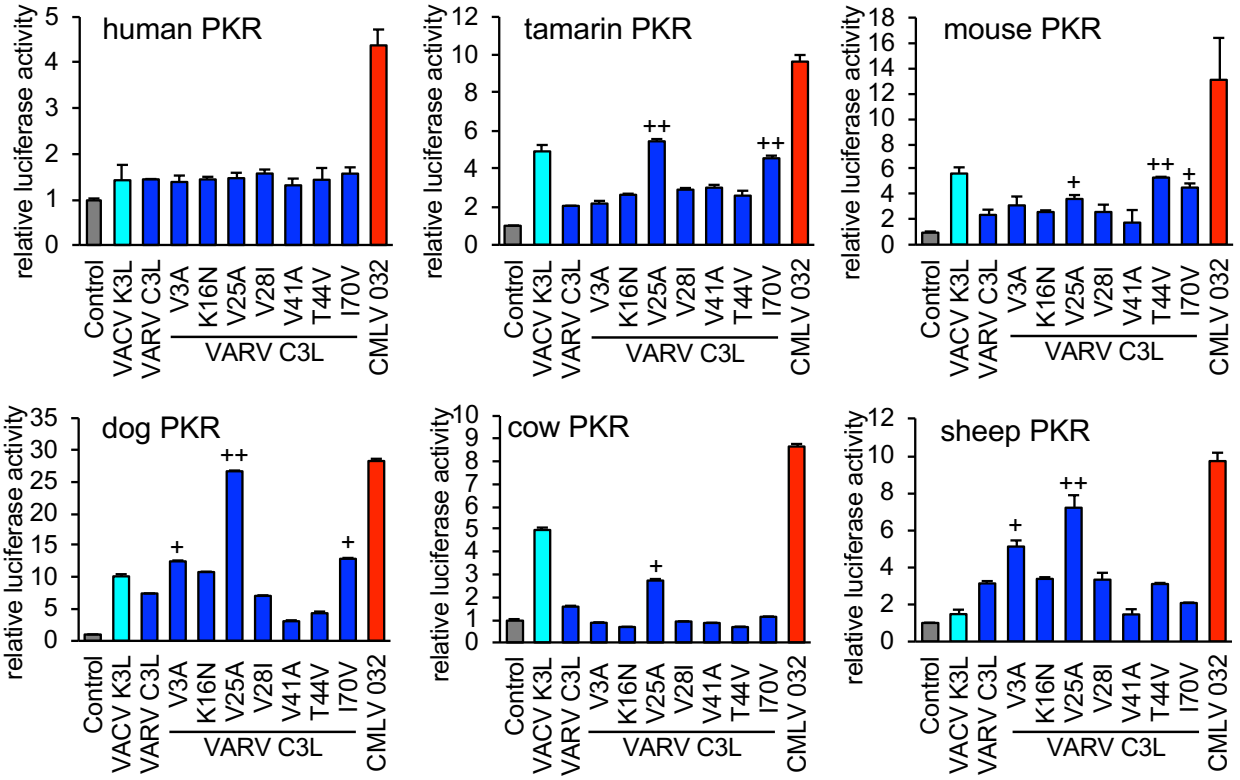


Figure 5. CMLV 032 facilitates strong VACV replication. HeLa-PKR^{ko}, HeLa, Tert-GF, OA1 and BT cells were infected with VC-2 (containing E3L and K3L), VC-R4 (missing E3L and K3L), VC-R4:VACV_K3L, and VC-R4:CMLV_032. (**A**) Cells were infected with each virus for 1 hour and the overlayed with DMEM + 2% carboxymethylcellulose to allow plaque formation. Cells were stained with 0.1% w/v crystal violet to visualize plaque size two days post-infection. (**B**) Cells were infected with the indicated viruses at MOI = 0.01. 48 hours after infection, viruses in cell lysates were tittered on RK13+E3L+K3L cells. Error bars represent the standard deviations from two independent infections. Fold differences in virus titers obtained with VC-R4:VACV_K3L, and VC-R4:CMLV_032 are shown.



759
760
761 **Figure 6. Identification of amino acid residues in VARV C3 that confer differential**
762 **PKR inhibition.** HeLa-PKR^{kd} cells were transfected with expression vectors encoding
763 firefly luciferase (0.05 μg), with either VACV K3L, VARV C3L, the indicated mutants of
764 VARV C3L, or CMLV 032 (0.4 μg), and PKR from the indicated species (0.2 μg).
765 Luciferase activities were measured 48 hours after transfection and normalized to PKR-
766 only transfected cells to obtain relative luciferase activities. Error bars represent the
767 standard deviations from three independent transfections. Results shown are
768 representative of three independent experiments. 1.5-fold and 2-fold (+) and ≥2 fold (++)
769 higher inhibition of the mutant than VARV C3L wild type are indicated.
770

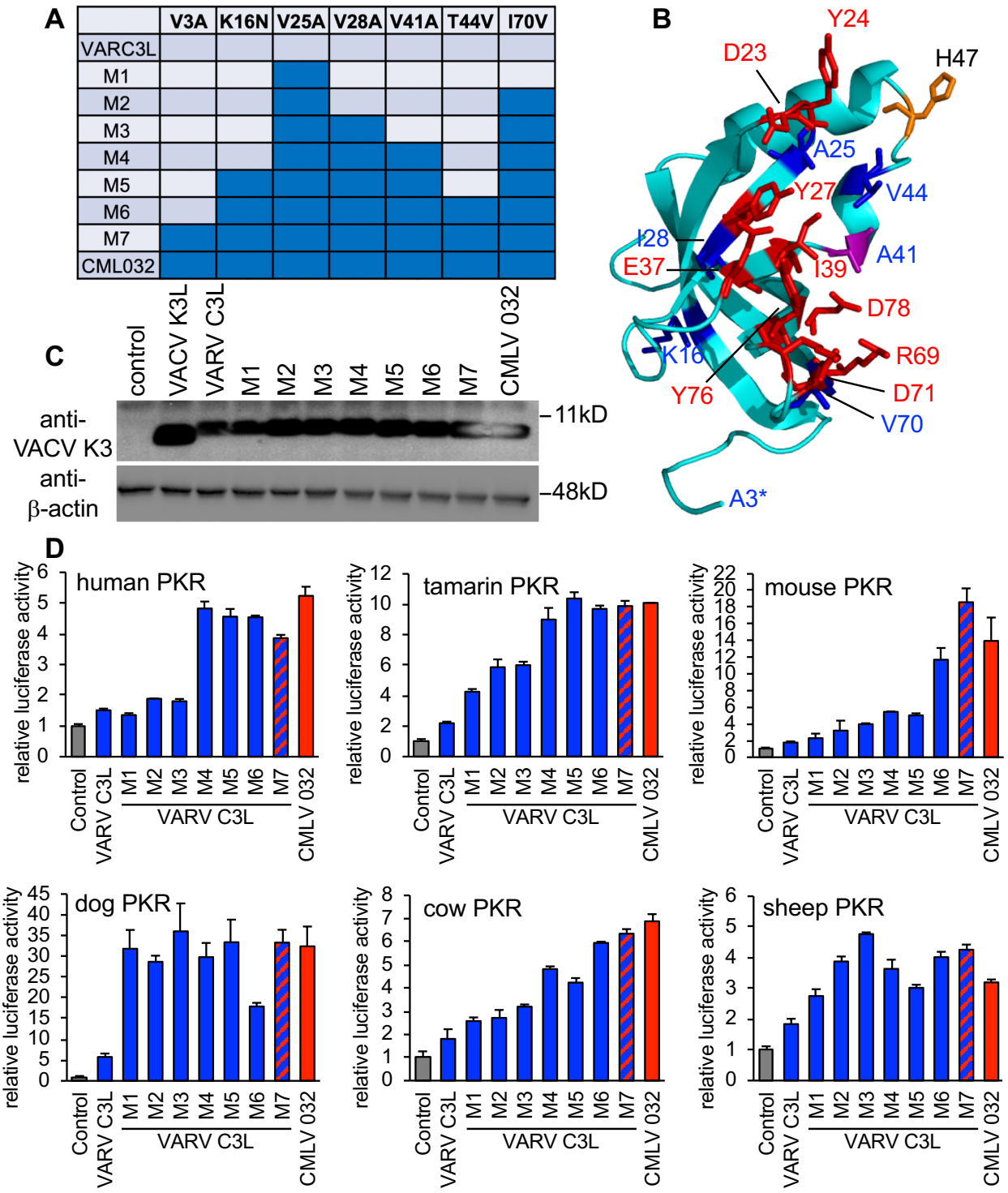


Figure 7. Distinct amino acids combinations are responsible for the higher inhibition of CMLV 032 for PKRs from different species.

(**A**) Seven different mutants of VARV C3L were generated that contain successive combinations of amino acid substitutions that are found in CMLV 032. (**B**) Crystal

structure of VACV K3 [14]. Residues that correspond to amino acids of eIF2α that contact human PKR in a co-crystal structure [51] are shown in purple (residue A41) or red (other residues). Residues that differ between VARV C3 and CMLV 032 are shown in purple (residue A41) or blue (other residues). Residue H47, which has been previously implicated in PKR binding [52], is shown in orange. Residues in eIF2α corresponding to residues 1-3 (asterisk) 44-52 in K3 were not resolved in the co-crystal structure. (**C**) Expression of VARV C3 mutants, CMLV 032 and VACV K3 in transfected HeLa-PKR^{kd} cells. Cell lysates from transfected cells were separated on 12% SDS-PAGE gels and analyzed by immunoblot analysis with anti-K3 and anti-β-actin antibodies. (**D**) HeLa-PKR^{kd} cells were transfected with expression vectors encoding firefly luciferase (0.05 µg), with VARV C3L, the indicated mutants of VARV C3L, or CMLV 032 (0.4 µg), and PKR (0.2 µg) from the indicated species. Luciferase activities were measured 48 hours after transfection and normalized to PKR-only transfected cells to obtain relative luciferase activities. Error bars represent the standard deviations from three independent transfections. Results shown are representative of two independent experiments.

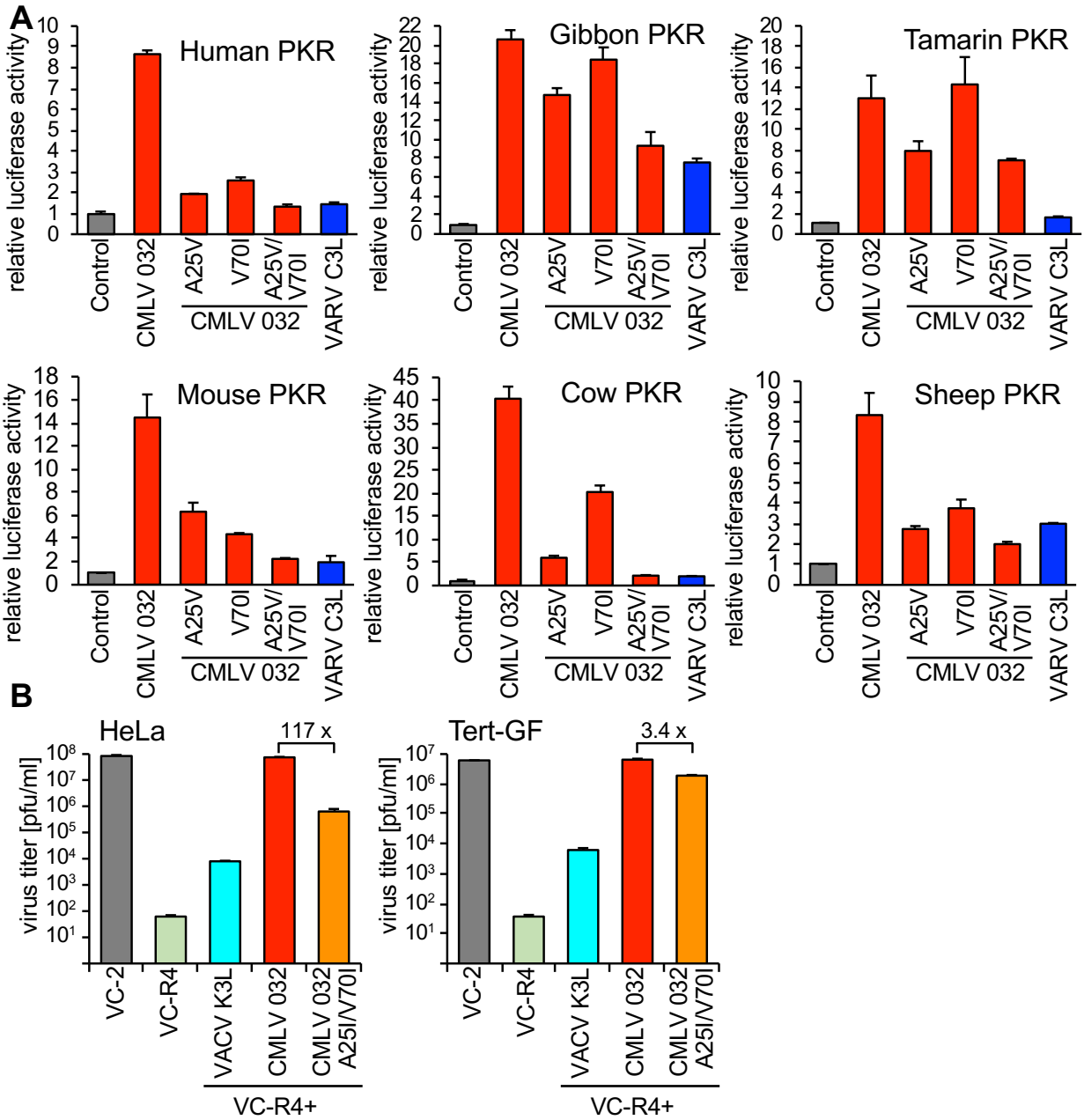


Figure 8. Residues A25 and V70 in CMLV 032 are critical for strong PKR inhibition. (**A**) HeLa-PKR^{kd} cells were transfected with expression vectors encoding firefly luciferase (0.05 µg), with CMLV 032, the indicated mutants of CMLV 032, or VARV C3L (0.4 µg), and PKR from the indicated species (0.2 µg). Luciferase activities were measured 48 hours after transfection and normalized to cells transfected only with PKR to obtain relative luciferase activities. Error bars represent the standard deviations from three independent transfections. Results shown are representative of two independent experiments. (**B**) HeLa or gibbon (Tert-GF) cells were infected with VC-2, VC-R4 or its derivatives containing either VACV K3, CMLV 032 or CMLV 032A25I/V70I at a MOI of 0.01. After 48 hours viruses were harvested and tittered on RK13+E3L+K3L cells. Error bars represent the standard deviations of two independent experiments. Fold differences in titer between VC-R4+CMLV 032 and VC-R4+CMLV 032-A25I/V70I are shown.