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Characterization of a ranavirus inhibitor of the antiviral protein kinase PKR

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Abstract

Background: Ranaviruses (family *Iridoviridae*) are important pathogens of lower vertebrates. However, little is known about how they circumvent the immune response of their hosts. Many ranaviruses contain a predicted protein, designated vIF2 α , which shows homology with the eukaryotic translation initiation factor 2 α . In analogy to distantly related proteins found in poxviruses vIF2 α might act as an inhibitor of the antiviral protein kinase PKR.

Results: We have characterized the function of vIF2 α from *Rana catesbeiana* virus Z (RCV-Z). Multiple sequence alignments and secondary structure prediction revealed homology of vIF2 α with eIF2 α throughout the S1-, helical- and C-terminal domains. Genetic and biochemical analyses showed that vIF2 α blocked the toxic effects of human and zebrafish PKR in a heterologous yeast system. Rather than complementing eIF2 α function, vIF2 α acted in a manner comparable to the vaccinia virus (VACV) K3L protein (K3), a pseudosubstrate inhibitor of PKR. Both vIF2 α and K3 inhibited human PKR-mediated eIF2 α phosphorylation, but not PKR autophosphorylation on Thr446. In contrast the E3L protein (E3), another poxvirus inhibitor of PKR, inhibited both Thr446 and eIF2 α Ser51 phosphorylation. Interestingly, phosphorylation of eIF2 α by zebrafish PKR was inhibited by vIF2 α and E3, but not by K3. Effective inhibition of PKR activity coincided with increased PKR expression levels, indicative of relieved autoinhibition of PKR expression. Experiments with vIF2 α deletion constructs, showed that both the N-terminal and helical domains were sufficient for inhibition of PKR, whereas the C-terminal domain was dispensable.

Conclusions: Our results show that RCV-Z vIF2 α is a functional inhibitor of human and zebrafish PKR, and probably functions in similar fashion as VACV K3. This constitutes an important step in understanding the interaction of ranaviruses and the host innate immune system.

Background

Infectious diseases have devastating ecological and economical impacts on fish, amphibian and reptile populations worldwide (reviewed in [1]). Despite those effects, the precise pathogenesis of infectious diseases of ectotherm vertebrates and the interaction with the immune system of their respective hosts are mostly poorly understood. Recently, marked progress has been made in the characterization of the immune system of lower vertebrates. This has been facilitated by concentrated focus on the cloning of pathogen-induced genes and by accumulating sequence data from genome and expressed sequence tag (EST) projects. Similarly, increased information about the genomes of pathogens

of lower vertebrates is becoming available. However, there are still large gaps in our knowledge, especially concerning the interaction of ectothermic pathogens with the host immune system.

Ranaviruses, which constitute a genus within the family *Iridoviridae*, are important pathogens of ectotherms and have been associated with massive die-offs of both wild and farmed populations of fish, frogs and salamanders in diverse areas of the world [2-5]. Ranaviruses are double-stranded DNA viruses with genomes ranging from 105 to 140 kb. Currently the genomes of seven ranaviruses have been sequenced: *Ambystoma tigrinum* virus (ATV, accession no. NC_005832[6]); Frog virus 3 (FV3, accession no. NC_005946[7]); Tiger frog virus (TFV, accession no. AF389451 [8]); Grouper iridovirus (GIV, accession no. AY666015 [9]); Singapore grouper iridovirus (SGIV, accession no. NC_006549[10]); Soft-shelled turtle iridovirus (STIV, accession no. EU627010 [11]); and Epizootic

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hematopoietic necrosis virus (EHNV, accession no. FJ433873 [12]). Phylogenetic analysis showed the existence of two major clades among ranaviruses, one that included GIV and SGIV, and another comprised of ATV, EHNV, FV3, STIV and TFV. Interestingly, the latter clade could be further subdivided with ATV and EHNV in one subclade, and FV3, STIV and TFV in the other. The diversity of organisms (amphibians, fish and reptiles) infected by viruses from this second clade, combined with short branch lengths within its two subclades may indicate recent host switches among ranaviruses [12]. However, it should be noted that the host range of ranaviruses is incompletely understood at this time.

The host immune system has evolved multiple ways to fight virus infection and replication. One important arm of the host immune response is the innate immune system, which recognizes molecular patterns present in many pathogens and initiates antimicrobial responses [13,14]. An important component of the host response is the antiviral protein kinase PKR, which contains double-stranded (ds) RNA binding domains (RBD) and a kinase domain. PKR is activated by dsRNA, which is formed during infection by many RNA and DNA viruses, and phosphorylates the α subunit of eukaryotic translation initiation factor 2 (reviewed in [15]). PKR is inactive in its latent monomeric form. However, upon binding dsRNA, two PKR molecules dimerize and undergo autophosphorylation on residue Thr446 (for human PKR) [16-18]. Activated PKR then phosphorylates eIF2 α on Ser51, which subsequently acts as an inhibitor of the guanine nucleotide exchange factor eIF2B. As eIF2B normally exchanges GDP for GTP on eIF2, a step necessary for successful translation initiation, eIF2 α phosphorylation leads to a general inhibition of translation initiation [19,20]. The function of mammalian PKR and its interaction with viruses has been extensively characterized (reviewed in [15]). However, PKR-like molecules in ectotherms eluded molecular characterization until recently. PKR-like activity was first described in fish cells [21,22]. This was followed by the cloning and functional characterization of crucian carp and zebrafish PKR-related genes, which contain Z-DNA binding ($Z\alpha$) domains instead of the dsRBDs and were hence named PKZ [23,24]. PKZ was subsequently described in Atlantic salmon and the rare minnow [25,26]. Recently, authentic PKR genes were described and characterized in many ectotherm species including zebrafish, pufferfish, Japanese flounder and two *Xenopus* species [27,28]. Like mammalian PKR, both PKZ and PKR are induced by immunostimulation [23,27,28]. Phylogenetic analyses indicate that a duplication of an ancestral PKR-like gene in the fish lineage probably led to the emergence of PKR and PKZ in a fish ancestor, and might have helped to extend the spectrum of viral

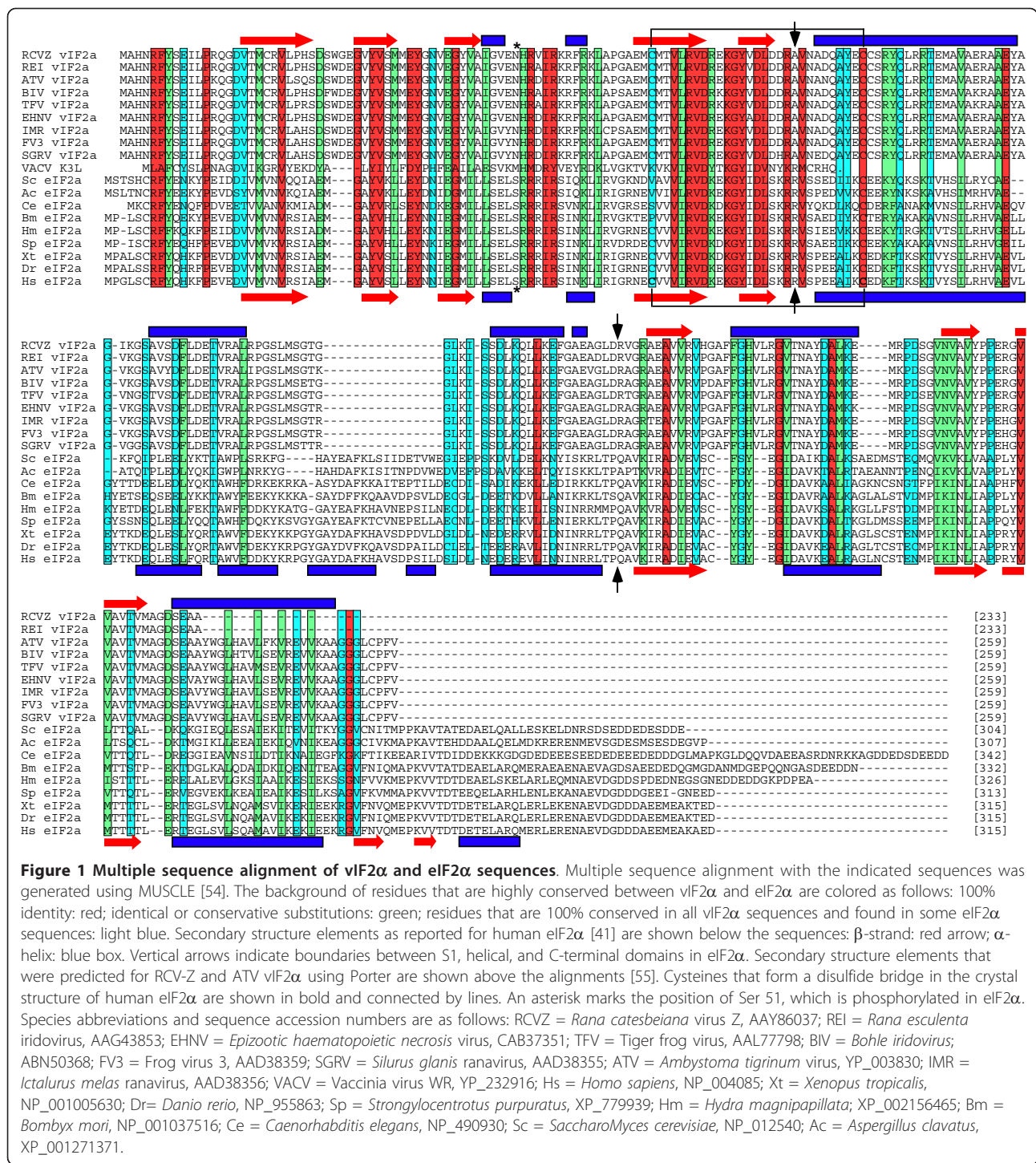
nucleic acids that can be recognized [27]. Although higher vertebrates lack PKZ genes, they contain a different $Z\alpha$ -containing protein, termed ZBP1, which binds Z-DNA and has been implicated in the recognition of viral DNA and the induction of an antiviral response [29-31].

In order to overcome the antiviral effects of PKR many mammalian viruses encode inhibitors of PKR, which block PKR activation or activity at different steps during or following the activation process (reviewed in [32]). Many poxviruses possess two PKR inhibitors, which are designated E3 and K3 in vaccinia virus (VACV) and are encoded by the E3L and K3L genes, respectively. E3 binds to dsRNA and prevents activation of PKR [33,34], whereas K3 encodes an S1 domain that is homologous to the N-terminus of eIF2 α and inhibits activated PKR by binding to the kinase domain and acting as a pseudosubstrate inhibitor of PKR [18,35,36].

Interestingly, most ranaviruses encode a protein with an S1 domain, which is related to the S1 domain of eIF2 α and K3 and is referred to as a viral homolog of eIF2 α or vIF2 α . In contrast to K3, which only possesses the S1 domain, vIF2 α proteins contain a C-terminal extension of between 165 to 190 amino acids, for which no sequence homology to any other proteins was described. It was previously speculated that vIF2 α in analogy to K3 might be an inhibitor of PKR and might therefore play an important role in the pathogenesis of ranaviruses [37-39]. Herein, using a heterologous yeast assay system, we describe the characterization of vIF2 α as an inhibitor of human and zebrafish PKR.

Results

We present three lines of evidence that the C-terminus of vIF2 α is actually homologous to the helical and parts of the C-terminal domains of eIF2 α . Firstly, we performed PSI-BLAST searches with vIF2 α from ATV and RCV-Z. During the first iteration, sequence similarity for regions spanning amino acids 5-118 of ATV-vIF2 α with the S1 and helical domains eIF2 α from multiple eukaryotes was noted. During the second iteration, this region of similarity to eIF2 α was extended to amino acid position 253 of vIF2 α . Secondly, multiple sequence alignments including vIF2 α from many ranaviruses and eIF2 α from a diverse set of eukaryotes showed conservation of amino acids outside the S1 domain: 8 amino acids are 100% conserved among the sequences (Figure 1, red background; Cys99, Glu118, Leu160, Ala177, Gly192, Ala199, Val220 and Gly253). Moreover, conservative amino acid differences are present at 22 positions outside the S1 domain (Figure 1, green background). At many other positions, amino acids that are identical to the ones found in vIF2 α are present in a subset of eIF2 α sequences (Figure 1, light blue background). While the multiple sequence



alignment reveals sequence homology between vIF2α and eIF2α throughout the reading frame, sequence similarity is highest within the S1 domain, with the highest levels of sequence identity surrounding strands β4 and β5 (Val74 - Leu88 in vIF2α) as previously described [38,39]. Interestingly, in VACV K3 this region was previously shown to be important for PKR inhibition [40]. Thirdly,

secondary structure prediction with ATV and RCV-Z vIF2α resulted in predicted β-sheets and α-helices that coincide very well with the solved structural features observed in the NMR structure of human eIF2α [41]. These observations indicate that the middle and C-terminal parts of vIF2α are homologous to the helical and C-terminal domains, respectively, of eIF2α.

Yeast-based assays were previously employed to characterize PKR and its interaction with viral inhibitors [34,40,42,43]. To test whether vIF2 α can inhibit PKR-mediated toxicity in yeast, we transformed a control strain and a strain expressing human PKR under the control of the galactose-regulated *GAL-CYC1* hybrid promoter with plasmids designed to express RCV-Z vIF2 α and VACV K3L also under control of the *GAL-CYC1* promoter. When grown under inducing conditions (galactose medium), comparable growth was seen in the control strain transformed with vector, K3L or vIF2 α , demonstrating that K3 and vIF2 α had no effect on yeast cell growth (Figure 2A). In contrast, induction of PKR expression was toxic in the vector-transformed yeast, whereas the toxicity was suppressed by co-expression of K3L or vIF2 α (Figure 2B).

Based on the homology of vIF2 α with eIF2 α throughout the entire ORF we tested whether suppression of PKR toxicity might be caused by the complementation of eIF2 α function by vIF2 α . To this end, we transformed a yeast strain that carries a temperature-sensitive mutant of eIF2 α (*sui2-1*) [44] with an empty vector, with a plasmid designed to express wild-type eIF2 α (*SUI2*) under

the control of its native promoter, or with the plasmids that express vIF2 α or K3L under the control of the galactose regulated *GAL-CYC1* promoter. Yeast transformants were streaked on synthetic complete medium containing galactose (SC-Gal) and incubated at different temperatures. At permissive temperatures (27°C and 30°C) all transformants grew well (Figure 3). However, when incubated at restrictive temperatures (33°C and 36°C), only wild type eIF2 α was able to rescue growth

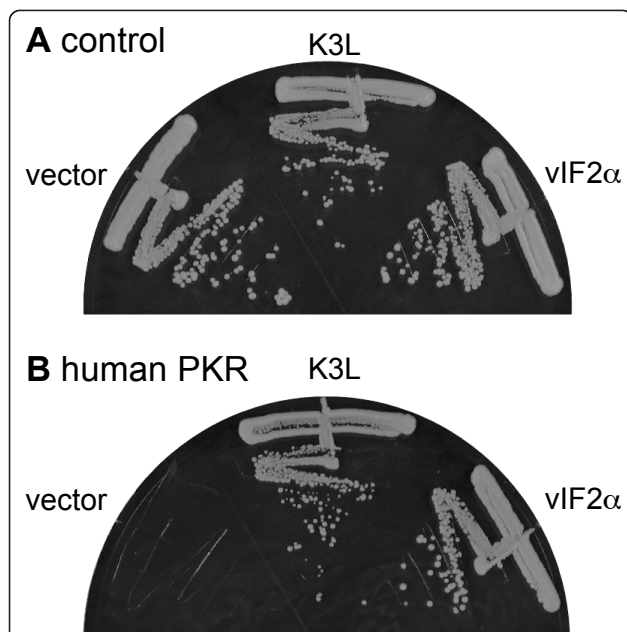


Figure 2 vIF2 α inhibits human PKR-mediated toxicity in yeast. Plasmids expressing VACV K3L (pC140) or RCV-Z vIF2 α (pC3853) under the control of a yeast *GAL-CYC1* hybrid promoter, or the vector pEMBLyex4 alone, were introduced into isogenic yeast strains having either an empty vector (A, control, J673) or a *GAL-CYC1*-human PKR construct (B, J983) integrated at the *LEU2* locus. The indicated transformants were streaked on SC-Gal medium where expression of both PKR and the viral proteins was induced, and incubated at 30°C for 4 days. Results shown are representative of 4 independent transformants for each plasmid.

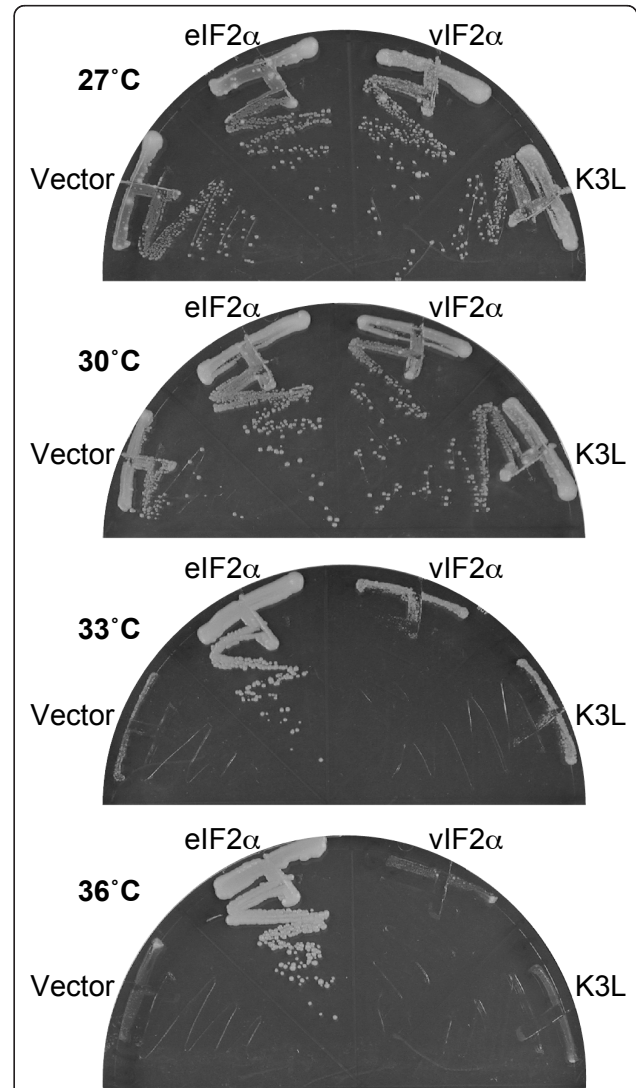


Figure 3 vIF2 α does not complement eIF2 α function in yeast. Plasmids expressing VACV K3L (pC140) or RCV-Z vIF2 α (pC3853) under the control of a yeast *GAL-CYC1* hybrid promoter, or yeast eIF2 α (p919) under the control of its native promoter, or the vector pEMBLyex4, were introduced into the temperature-sensitive eIF2 α (*sui2-1*, TD304-10B) mutant strain. The indicated transformants were streaked on SC-Gal medium, where eIF2 α expression was maintained and the viral protein expression was induced, and incubated at the indicated temperatures. Results shown are representative of 4 independent transformants for each plasmid.

(Figure 3). It is important to note that under these growth conditions vIF2 α and K3L were able to suppress PKR toxicity (data not shown), indicating that the viral proteins are functional under these conditions. As expression of neither vIF2 α nor K3L suppressed the growth defects of the *sui2-1* mutant strain, we conclude that vIF2 α does not functionally substitute for eIF2 α .

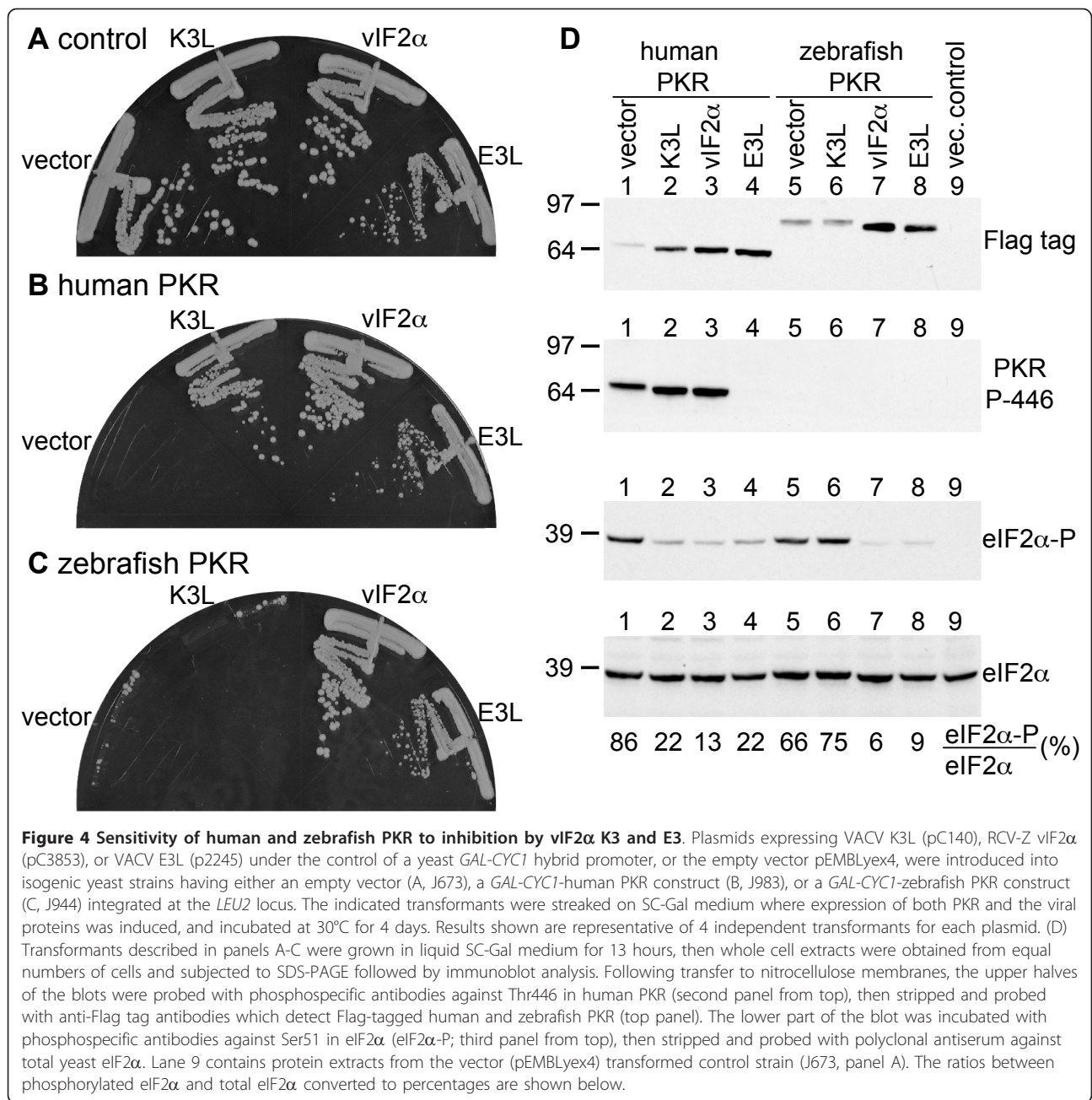
We next compared the effect of vIF2 α on human and zebrafish PKR with the effects of the two VACV PKR inhibitors K3 and E3. In the control strain not expressing PKR, expression of K3L or vIF2 α had no effect on yeast cell growth, whereas expression of E3L induced a slow growth phenotype as previously described [34] (Figure 4A). The toxicity associated with expression of human PKR was inhibited by co-expression of K3L, vIF2 α or E3L (Figure 4B). Interestingly, the toxicity associated with expression of zebrafish PKR in yeast was only inhibited by vIF2 α or E3L, but not by K3L (Figure 4C). Thus in accord with the virus host range vIF2 α , but not VACV K3L, may have evolved to inhibit fish PKR. To assess the effectiveness of K3, E3, and vIF2 α to inhibit human and zebrafish PKR, matching sets of strains expressing a particular inhibitor and either no PKR, human PKR, or zebrafish PKR were streaked on the same plate for comparison. Examining the colony sizes of the transformants in the streaks revealed that K3 did not fully inhibit human PKR (colonies of cells expressing human PKR plus K3L were smaller than colonies expressing K3L and no PKR, Additional file 1: Figure S1A). In contrast, vIF2 α and E3 appeared to fully inhibit both human and zebrafish PKR (Additional file 1: Figure S1B, C).

Suppression of PKR toxicity in yeast could be due to impaired PKR expression or due to inhibition of eIF2 α phosphorylation. In order to examine eIF2 α phosphorylation, yeast whole cell extracts were prepared by the TCA method to prevent protein degradation and dephosphorylation, and Western blot analyses were performed using phospho-specific antibodies directed against phospho-Ser51 in eIF2 α . To normalize for protein loading, the blot was then stripped and probed with anti-yeast eIF2 α antiserum. As shown in Figure 4D (next to bottom panel), induction of either human or zebrafish PKR expression in the absence of a viral inhibitor led to high levels of eIF2 α phosphorylation. Co-expression of K3L, vIF2 α , or E3L greatly reduced eIF2 α phosphorylation in cells expressing human PKR (Figure 4D and Additional file 2: Figure S2). Consistent with the growth assays, vIF2 α and E3, but not K3, inhibited eIF2 α phosphorylation in yeast expressing zebrafish PKR. Next, PKR expression levels were monitored using an anti-Flag tag antibody. Expression levels of PKR were higher in the presence of effective eIF2 α phosphorylation inhibitors. As observed previously PKR autoinhibits its own

expression in yeast [34,40,45]. Presumably PKR phosphorylation of eIF2 α leads to suppression of total protein synthesis including PKR expression. Accordingly, inhibition of PKR by the viral inhibitors restores protein synthesis and leads to higher PKR levels. Taken together, the results of the PKR expression and eIF2 α phosphorylation studies demonstrate that vIF2 α can effectively inhibit eIF2 α phosphorylation by human and zebrafish PKR.

In the presence of effective eIF2 α phosphorylation inhibitors, PKR migrated faster on SDS-PAGE than in the controls (Figure 4D, top panel, lanes 2-4 versus 1 and lanes 7-8 versus 5). This might have been caused by inhibition of PKR autophosphorylation. To examine PKR autophosphorylation, we probed the Western blots with a phospho-specific antibody that recognizes human PKR phosphorylated on Thr446. High levels of Thr446 phosphorylation were detected in the absence of inhibitors and when either K3 or vIF2 α were present. Thr446 phosphorylation was effectively inhibited in the presence of E3 (Figure 4D, second panel, lanes 1-4). These results indicate that K3 and vIF2 α are unable to block Thr446 phosphorylation and are consistent with previous findings that K3 binding to PKR is dependent on Thr446 phosphorylation [18]. Presumably vIF2 α , like K3, binds to PKR following autophosphorylation on Thr446 and blocks subsequent autophosphorylation events that lead to altered mobility of PKR on SDS-PAGE. Zebrafish PKR was not detected with the antibody directed against Thr446-phosphorylated human PKR (Figure 4D, second panel, lanes 5-8). This was expected because of the strong sequence divergence between human and zebrafish PKR surrounding the phosphorylation site [27]. Finally, using yeast growth rate assays as described above, vIF2 α was found to inhibit, at least partially, both *Xenopus laevis* PKR1 and zebrafish PKZ (data not shown). However, precise determination of PKR1 and PKZ sensitivity to vIF2 α inhibition will depend on the ability to obtain yeast strains expressing the appropriate level of each kinase.

In order to test which domains of vIF2 α are important for PKR inhibition we tested various vIF2 α deletion mutants for their ability to inhibit PKR activity. Additionally, the C-terminus of RCV-Z vIF2 α was extended to match the length of ATV vIF2 α (see Figure 1). For the latter constructs, the 26 C-terminal amino acids found in ATV vIF2 α that are not in RCV-Z vIF2 α due to an early termination codon were appended to the C-terminus of RCV-Z vIF2 α (vIF2 α +26C, Figure 5A). None of the vIF2 α constructs led to a growth defect in the control strain not expressing PKR (Figure 5B). In a zebrafish PKR-expressing strain, wild-type vIF2 α , vIF2 α +26C, and vIF2 α Δ 59C (lacking the C-terminal 59 amino acids) led to comparable inhibition of PKR toxicity



(Figure 5C, sectors 2-4 versus 1). In contrast, no PKR suppression was observed when the helical domain was partly (vIF2 α Δ 94C) or completely (vIF2 α Δ 138C) deleted or when the N-terminus was deleted (vIF2 α Δ 94N and vIF2 α Δ 94N+26C) (Figure 5C, sectors 5-8). Western analyses of eIF2 α phosphorylation in the strains expressing zebrafish PKR and the various vIF2 α mutants revealed that vIF2 α , vIF2 α +26C, vIF2 α 59C led to strong and comparable inhibition of eIF2 α phosphorylation (Figure 5D, next to bottom panel, lanes 2-4). Consistent with their inability to inhibit PKR toxicity in

yeast, high levels of eIF2 α phosphorylation were observed in strains expressing the other vIF2 α mutants (Figure 5D). As seen earlier, PKR was expressed at higher levels and migrated faster on SDS-PAGE when PKR toxicity and eIF2 α phosphorylation were suppressed (Figure 5D, top panel). Western blot analyses using antibodies against a C-terminal Myc-epitope tag in the vIF2 α constructs revealed detectable expression for only vIF2 α , vIF2 α +26C, and vIF2 α 59C. Comparable results were obtained in Western blot analyses of protein extracts from the control (-PKR) strain expressing

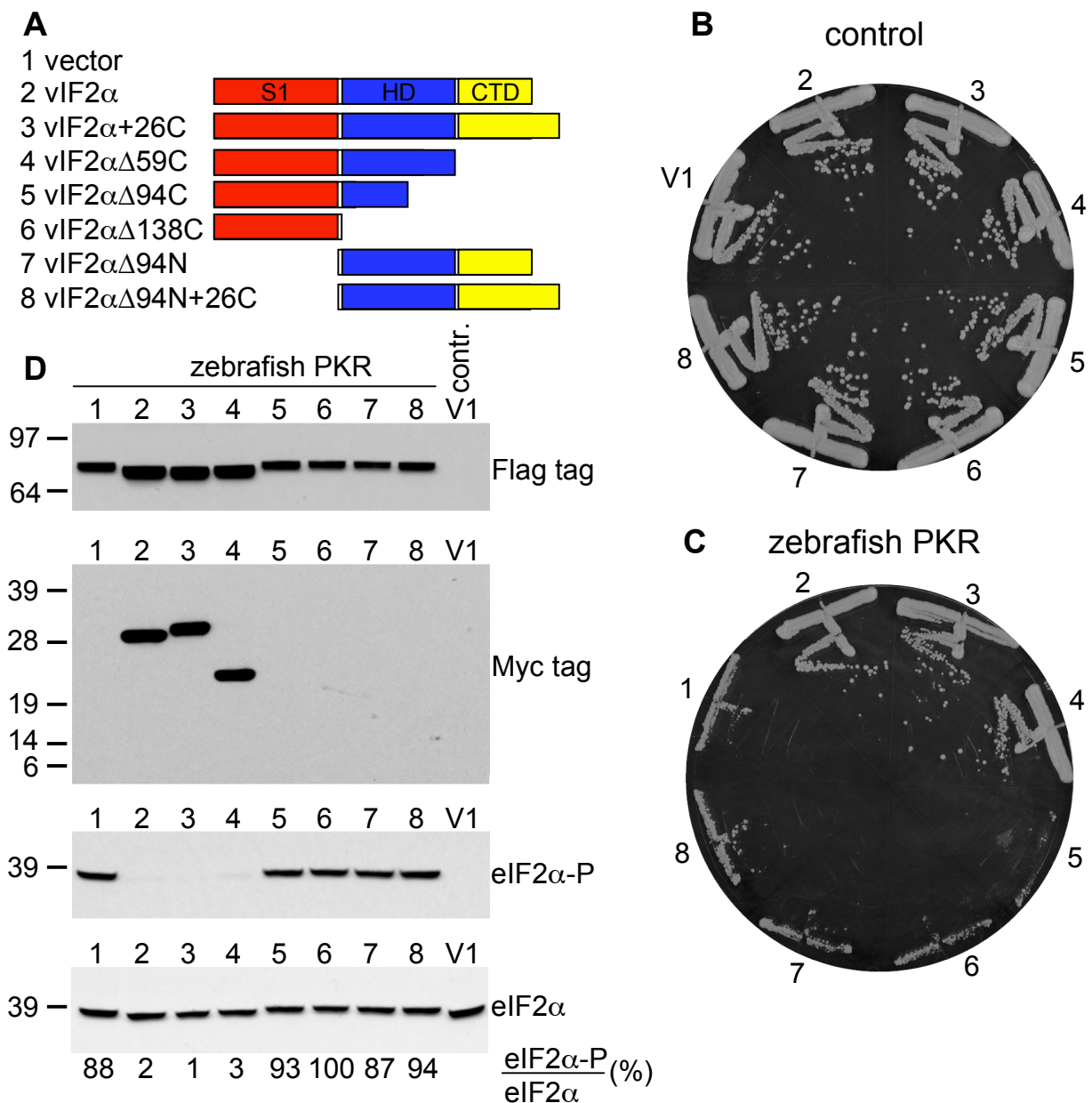


Figure 5 Both S1 and helical domains in vIF2 α are required for PKR inhibition. (A) Schematic representation of RCV-Z vIF2 α constructs tested in yeast growth assays and Western blots analyses. S1 domain (red), helical domain (HD; blue) and C-terminal domain (CTD, yellow) are represented by boxes. Numbers that follow deltas (Δ) indicate the (number of residues that were deleted from the C- or N-terminus, respectively). The extended C-terminus (26 amino acids) from ATV vIF2 α was added to the C-terminus of RCV-Z vIF2 α in the constructs with the +26C label. The indicated constructs were introduced into isogenic yeast strains having either an empty vector (B, J673) or a GAL-CYC1-zebrafish PKR construct (C, J944) integrated at the LEU2 locus. The indicated transformants were streaked on SC-Gal medium where expression of both PKR and the viral proteins was induced, and incubated at 30°C for 4 days. Results shown are representative of 4 independent transformants for each plasmid. (D) Transformants described in panels B-C were grown in liquid SC-Gal medium for 13 hours, then whole cell extracts were obtained from equal numbers of cells and subjected to SDS-PAGE followed by immunoblot analysis. Following transfer to nitrocellulose membranes, the upper half of the blot was probed with anti-Flag tag antibodies, which detect Flag-tagged zebrafish PKR (top panel). The lower part of the blot was incubated with anti-Myc tag antibodies to detect Myc-tagged vIF2 α (second panel from top), then stripped and probed with phosphospecific antibodies against Ser51 in eIF2 α (eIF2 α -P; third panel from top), and finally stripped again and probed with polyclonal antiserum against total yeast eIF2 α (bottom panel). The ratios between phosphorylated eIF2 α and total eIF2 α converted to percentages are shown below.

these same vIF2 α mutants (data not shown), indicating that both the S1 domain and the helical domain are essential for vIF2 α expression and/or stability.

Discussion

Ranaviruses are important pathogens of fish, amphibians and reptiles (reviewed in [2]). However, little is known about how they interact with the immune system of their hosts. Herein we show that RCV-Z vIF2 α , a homolog of eIF2 α , is an effective inhibitor of PKR in a heterologous yeast assay system. PKR is an important antiviral protein kinase that has been primarily studied in mammals (reviewed in [15]). PKR-related genes have recently been identified in a variety of fish and amphibian species. Fish PKR genes are expressed at low levels constitutively, but they are highly induced after viral infection and stimulation with the dsRNA analog poly(I:C), which mimics viral infection [27,28]. It was recently shown that PKR of the Japanese flounder (*Paralichthys olivaceus*) was able to inhibit replication of *Scophthalmus maximus rhabdovirus* [28]. To date, only PKR inhibitors from mammalian viruses have been functionally characterized (reviewed in [32]). Moreover, the only well-characterized viral PKR inhibitors that directly target the PKR kinase domain are the pseudosubstrates found in many poxviruses and represented by VACV K3L, which is homologous to the S1 domain of the PKR target eIF2 α [33,40,46,47]. It was speculated that the ranavirus vIF2 α protein, another eIF2 α homolog, might inhibit PKR of infected hosts [38,39]. A notable difference between K3 and eIF2 α is the presence of an extended C-terminal domain in eIF2 α . In addition to the C-terminal α/β domain, eIF2 α consists of an N-terminal S1 domain and a central α -helical domain. The K3 protein is homologous to the N-terminal domain in eIF2 α . Like K3, vIF2 α shows moderate sequence identity to eIF2 α in the S1 domain. In this study we used PSI-BLAST analyses, multiple sequence alignment and secondary structure prediction to show that the C-terminal parts of vIF2 α are likewise homologous to the helical and C-terminal domains of eIF2 α .

Functional analyses using deletion constructs of vIF2 α revealed that both the S1 and helical domains are sufficient for inhibition of PKR in yeast (Figure 5). Since the presence of both domains was necessary for detectable vIF2 α expression, it appears possible that the domains are important to stabilize each other. The crystal structure of human eIF2 α showed that the S1 and helical domains are connected by an intramolecular disulfide bridge formed by cysteine residues 69 and 97 [48]. Interestingly, a cysteine corresponding to position 69 is found in many Metazoa, including Chordata, Echinodermata, Cnidaria and Mollusca, but is missing in most Arthropoda (except *Ioxedes scapularis*), in all fungi and

plants sequences currently found in Genbank, and in all poxviral K3L orthologs (Figure 1 and data not shown). As cysteines corresponding to residues 69 and 97 in human eIF2 α are found in all vIF2 α sequences (Figure 1), we speculate that formation of a disulfide bridge between the two domains might be important for the stability of vIF2 α . Future *in vitro* kinase assays with vIF2 α constructs that are produced in a cell-free translation system might be suited to further investigate the importance of the individual domains.

It is striking that eIF2 α sequences and all known vIF2 α sequences display a high level of sequence identity within their respective groups. The sequence identity for eIF2 α is between 92% and 100% among vertebrates, while the sequence identity for vIF2 α is between 95% and 98% among ranaviruses. In contrast, K3L orthologs are very diverse, some of which display only around 30-40% sequence identity to each other [49]. The high sequence conservation in eIF2 α and vIF2 α indicates that eIF2 α might be under purifying (negative) selective pressure in order to maintain its primary sequence or, alternatively, that current ranaviruses might have experienced bottlenecks in their recent evolution. Overall the S1 domains of vIF2 α and K3 are comparably distantly related to eIF2 α .

Interestingly, some Ranaviruses do not encode functional vIF2 α orthologs. GIV and SGIV do not contain vIF2 α orthologs, and truncated vIF2 α genes lacking regions of the N-terminal and the helical domains are found in the completely sequenced FV3 strain and in STIV [7,11]. As our studies indicate that the N-terminus of vIF2 α is essential for PKR inhibition, these complete or partial deletions might lead to the attenuation of the viruses. In accord with this notion FV3, which lacks most of vIF2 α , is much less pathogenic than RCV-Z in North American bullfrog (*Rana catesbeiana*) tadpoles [39]. Alternatively the absence of predicted functional vIF2 α proteins in some ranaviruses suggests that, as in vaccinia virus, a second PKR inhibitor may be present in ranaviruses.

Western blot analyses showed that human PKR was expressed at higher levels in yeast expressing the PKR inhibitors vIF2 α , K3L, or E3L, consistent with the notion that the viral inhibitors suppress autoinhibition of PKR expression. Moreover, PKR from cells expressing viral inhibitors migrated faster on SDS-PAGE, suggesting that the inhibitors might block PKR autophosphorylation. Thr446 is the only site in the human PKR kinase domain that is stoichiometrically phosphorylated and visible in the PKR crystal structure, where it is thought to stabilize the active PKR conformation [18,50]. Once activated, PKR can phosphorylate eIF2 α as well as autophosphorylate other residues in the kinase [17,34]; however the significance of the latter is not fully

understood. Interestingly, only E3L was able to prevent Thr446 phosphorylation. In cells expressing K3L or vIF2 α , Thr446 was phosphorylated to the level observed in the absence of an inhibitor, whereas PKR mobility was comparable to that in E3L transformed cells. A likely explanation is that K3 and vIF2 α bind after the initial Thr446 autophosphorylation and block subsequent phosphorylation events. This is in agreement with the finding that activated WT PKR, but not the PKR-T446A mutant, was able to bind K3 [18].

Like human PKR, zebrafish PKR was inhibited by E3 and vIF2 α . Moreover, as was seen for human PKR, zebrafish PKR from cells expressing the inhibitors migrated faster on SDS-PAGE, indicative of blocked secondary phosphorylation events. An interesting difference between human and zebrafish PKR is that zebrafish PKR was resistant to K3 inhibition in both the growth and eIF2 α phosphorylation assays. In accord with our previous studies on PKR inhibition by K3 [49], we propose that K3L might have evolved to suppress PKR of the natural poxvirus hosts and that zebrafish PKR is too different to be targeted with high efficiency. It is not clear why vIF2 α , which is found in amphibian and fish viruses, can inhibit both human and zebrafish PKR, but it is possible that vIF2 α targets more conserved residues in the PKR kinase domain than does K3. Previously we showed that K3 exhibits species specificity for inhibition of PKR. Whereas human PKR was only moderately inhibited by VACV K3, mouse PKR was much more sensitive [49]. This difference in sensitivity was attributed to residues that were subject to positive selection during evolution. Interestingly, positive selection was also observed in the kinase domains of fish and amphibian PKR and fish PKZ [49]. It will be interesting to determine whether vIF2 α also shows altered specificity for PKR or the related PKZ of the species that are naturally infected with vIF2 α -containing ranaviruses.

Conclusions

Overall, it appears that vIF2 α and K3 inhibit PKR in a similar fashion, by acting as pseudosubstrates and inhibiting PKR following kinase activation. As vIF2 α does not act as an eIF2 α substitute, but instead inhibits PKR function, the renaming of vIF2 α might be considered. We suggest changing the name from vIF2 α to RIPR, the acronym for Ranavirus Inhibitor of Protein kinase R.

Methods

Yeast strains and plasmids

Human (hs) and zebrafish (dr) PKR cDNAs containing both N-terminal His6- and Flag tags were first cloned into the yeast expression vector pYX113 (R&D systems) under the control of a *GAL-CYC1* hybrid promoter [27]. Next, the two DNA fragments containing the *GAL-CYC1*

promoter and a PKR cDNA were subcloned into the *LEU2* integrating vector pRS305, which was then directed to integrate into the *leu2* locus of the strain H2557 (*MAT α ura3-52 leu2-3 leu2-112 trp1 Δ 63 gcn2 Δ*) generating the strains J983 (*MAT α ura3-52 leu2-3 leu2-112 trp1 Δ 63 gcn2 Δ <GAL-CYC1-hsPKR, LEU2 >*) and J944 (*MAT α ura3-52 leu2-3 leu2-112 trp1 Δ 63 gcn2 Δ <GAL-CYC1-drPKR, LEU2 >*). Construction of the control strain J673 (*MAT α ura3-52 leu2-3 leu2-112 trp1 Δ 63 gcn2 Δ <LEU2 >*) was described previously [51]. The temperature-sensitive eIF2 α strain TD304-10B (*MAT α his4-303 ura3-52 leu2-3 leu2-112 sui2-1*) is a derivative of the previously described *sui2-1* strain 117-8AR20 [44].

A DNA fragment encoding RCV-Z vIF2 α [39] was amplified by PCR using viral DNA as a template and primers C27 (5'-TAGGATCCAAAATGGCACACAA-CAGGTTTTAC-3') and C28 (5'-TAAAGTCGACCGCCGCCTCAGAGTCGCCGG-3'). The PCR product was then subcloned between the *Bam*H I and *Sal* I restrictions sites of a modified version of the yeast expression vector pEMBLyex4 that contains two Myc tags at the C-terminal end of the multiple cloning site (pC3852) generating the plasmid pC3853. The following primer combinations were used for cloning of vIF2 α mutant constructs: vIF2 α Δ 59C: C27 plus C29 (5'-TAAAGTCGACCCGACCGACTCTGTCGAGGC-3'); vIF2 α Δ 94C: C27 plus C30 (5'-TAAAGTCGACTCTCAGGGCCCTCACGGTCTC-3'); vIF2 α Δ 138C: C27 plus C31 (5'-TAAAGTCGACCTGATCGG-CATTCACGGC-3'); vIF2 α +26C: C27 plus C32 (5'-TAAAGTCGACCACAAAGGGGCACAGTCCTC-3'); vIF2 α Δ 94N: C33 (5'-TAGGATCCAAAATGGCCGATCAGGCGTACGAGTG-3') plus C28; and vIF2 α Δ 94N+26C: C33 plus C32. The plasmid template for vIF2 α +26C and vIF2 α Δ 94N+26C was generated by fusion PCR using vector primer C23 (5'-CATATGGCATGCATGTGCTCTG-3') plus primer C21 (5'-GCCTTTACGACCTCTCGCACCTCAGACAGCACGGCGTGCAGTCCCCAGTACGCCGCTCAGAGTCGCCGG-3') for the first PCR and primer C22 (5'-GTGCGAGAGGTCGTAAAGGCTGCGGGGAGGACTGTGCCCTTTGTGTAAGTCGACCTGCAGGCATGC-3') plus vector primer C24 (5'-CGCTTCCGAAAATGCAACGC-3') for the second PCR. Following PCR purification, the two PCR products were mixed and used as a template for PCR along with the vector primers A46F (5'-ATTCTTTCTTATACAT-TAGGTCC-3') and A20R (5'-TGCTGCCACTCCTCAATTGG-3'). Finally, the PCR products were cloned into the *Bam*HI and *Sal*I sites of pEMBLyex4. All PCRs were carried out using Pfu Polymerase (Stratagene) and all plasmids were sequenced to verify correct sequences. Derivatives of pEMBLyex4 expressing VACV K3L (pC140) and VACV E3L (p2245), as well as the low copy-number *SUI2*, *URA3* plasmid p919 were described previously [34,40,52].

Yeast strains were transformed using the LiAcetate/PEG transformation method. For each transformation, four independent colonies were analyzed by streaking on inducing medium, SC-Gal minus uracil (synthetic complete medium containing 2% galactose and all amino acids, but lacking uracil) and grown at 30°C if not otherwise indicated.

Protein expression and Western Blot analyses

Yeast transformants were grown to saturation in 2 ml of SD medium. This starter culture was diluted 1:50 in 25 ml SD medium and grown to OD₆₀₀ = 0.6 and then shifted to SC-Gal medium to induce expression. After 13 hours, ODs of the cultures were measured and carefully adjusted by dilution in water to obtain comparable ODs and thus to lyse equivalent amounts of cells for each sample. Whole-cell extracts (WCEs) were prepared using the trichloroacetic acid (TCA) method as described previously [53] and then suspended in 200 µl 1.5 × loading buffer with reducing agent (both Invitrogen) and neutralized by the addition of 100 µl 1 M Tris base. Samples (5 µl) were fractionated on 10% Bis-Tris gels (Invitrogen), run in MOPS buffer (Invitrogen), and then transferred to nitrocellulose membranes. Upper parts of the membranes were incubated with rabbit phosphospecific anti-human PKR Thr446 antibodies (21st Century Biochemicals) and then stripped and probed with anti-D (Flag) tag antibody (Applied Biological Materials). Lower halves of the membranes were incubated with an anti-Myc tag antibody (Applied Biological Materials), rabbit phosphospecific antibodies directed against phosphorylated Ser51 of eIF2α (BioSource International), or rabbit polyclonal antiserum against total yeast eIF2α. Immune complexes were detected using enhanced chemiluminescence. Band intensities were quantified by densitometry using ImageJ (<http://rsbweb.nih.gov/ij/>) and ratios between phosphorylated eIF2α and total eIF2α were calculated.

Multiple sequence alignment and secondary structure prediction

Multiple sequence alignments of all sequences shown in Figure 1 plus all poxvirus K3L orthologs listed in [49] were performed using MUSCLE [54]. Secondary structure predictions for RCV-Z and ATV vIF2α sequences were performed using Porter [55].

Additional material

Additional file 1: Figure S1 Comparison of colony sizes of PKR-expressing and control stains expressing K3L, vIF2α or E3L. Plasmids expressing VACV K3L (A, pC140), RCV-Z vIF2α (B, pC3853), or VACV E3L (C, p2245) under the control of a yeast *GAL-CYC1* hybrid promoter were introduced into isogenic yeast strains having either an empty vector (J673), a *GAL-CYC1*-human PKR construct (hsPKR, J983), or a *GAL-CYC1*-

zebrafish PKR construct (drPKR, J944) integrated at the *LEU2* locus. The indicated transformants were streaked on SC-Gal medium where expression of both PKR and the viral proteins was induced, and incubated at 30°C for 4 days. Results shown are representative of 4 independent transformants for each plasmid.

Additional file 2: Figure S2 Relative PKR-induced eIF2α

phosphorylation levels after expression of vIF2α, K3L or E3L. Using data from Figure 4D and an independent experiment, the band intensities of phosphorylated and total eIF2α obtained from Western blots of TCA extracts of yeast cells expressing either human or zebrafish PKR and transformed with an empty vector or plasmids expressing K3L, vIF2α or E3L, as indicated, were measured using ImageJ. The ratios of phosphorylated and total eIF2α bands were calculated. Standard deviations from the two independent experiments are shown, and significant differences, as calculated using a t-test and as compared to the vector controls ($p < 0.05$), are shown. n. s. = non significant.

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Authors' contributions

SR and TED devised this study with important input from VGC. All experiments were performed by SR. The manuscript was drafted by SR with essential contributions from TED and VGC. All authors read and approved the final manuscript.

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References

1. Essbauer S, Ahne W: **Viruses of lower vertebrates.** *J Vet Med B Infect Dis Vet Public Health* 2001, **48**:403-475.
2. Williams T, Barbosa-Solomieu V, Chinchar VG: **A decade of advances in iridovirus research.** *Adv Virus Res* 2005, **65**:173-248.
3. Whittington RJ, Becker JA, Dennis MM: **Iridovirus infections in finfish - critical review with emphasis on ranaviruses.** *J Fish Dis* 2010, **33**:95-122.
4. Chinchar VG, Hyatt A, Miyazaki T, Williams T: **Family Iridoviridae: poor viral relations no longer.** *Curr Top Microbiol Immunol* 2009, **328**:123-170.
5. Chinchar VG, Storfer A: **Ecology of viruses infecting ectothermic animals - The impact of ranavirus infections on amphibians.** In *Viral Ecology*. 2 edition. Edited by: Hurst C. Wiley-Blackwell Publishing; 2011, Viral Ecology.
6. Jancovich JK, Mao J, Chinchar VG, Wyatt C, Case ST, Kumar S, Valente G, Subramanian S, Davidson EW, Collins JP, Jacobs BL: **Genomic sequence of a ranavirus (family Iridoviridae) associated with salamander mortalities in North America.** *Virology* 2003, **316**:90-103.
7. Tan WG, Barkman TJ, Gregory Chinchar V, Essani K: **Comparative genomic analyses of frog virus 3, type species of the genus Ranavirus (family Iridoviridae).** *Virology* 2004, **323**:70-84.
8. He JG, Lu L, Deng M, He HH, Weng SP, Wang XH, Zhou SY, Long QX, Wang XZ, Chan SM: **Sequence analysis of the complete genome of an iridovirus isolated from the tiger frog.** *Virology* 2002, **292**:185-197.
9. Tsai CT, Ting JW, Wu MH, Wu MF, Guo IC, Chang CY: **Complete genome sequence of the grouper iridovirus and comparison of genomic organization with those of other iridoviruses.** *J Virol* 2005, **79**:2010-2023.
10. Song WJ, Qin QW, Qiu J, Huang CH, Wang F, Hew CL: **Functional genomic analysis of Singapore grouper iridovirus: complete sequence determination and proteomic analysis.** *J Virol* 2004, **78**:12576-12590.
11. Huang Y, Huang X, Liu H, Gong J, Ouyang Z, Cui H, Cao J, Zhao Y, Wang X, Jiang Y, Qin Q: **Complete sequence determination of a novel reptile**

- iridovirus isolated from soft-shelled turtle and evolutionary analysis of Iridoviridae. *BMC Genomics* 2009, **10**:224.
12. Jancovich JK, Bremont M, Touchman JW, Jacobs BL: Evidence for multiple recent host species shifts among the Ranaviruses (family Iridoviridae). *J Virol* 2010, **84**:2636-2647.
 13. Kumagai Y, Takeuchi O, Akira S: Pathogen recognition by innate receptors. *J Infect Chemother* 2008, **14**:86-92.
 14. Ranjan P, Bowzard JB, Schwerzmann JW, Jeisy-Scott V, Fujita T, Sambhara S: Cytoplasmic nucleic acid sensors in antiviral immunity. *Trends Mol Med* 2009, **15**:359-368.
 15. Toth AM, Zhang P, Das S, George CX, Samuel CE: Interferon action and the double-stranded RNA-dependent enzymes ADAR1 adenosine deaminase and PKR protein kinase. *Prog Nucleic Acid Res Mol Biol* 2006, **81**:369-434.
 16. Nanduri S, Rahman F, Williams BR, Qin J: A dynamically tuned double-stranded RNA binding mechanism for the activation of antiviral kinase PKR. *Embo J* 2000, **19**:5567-5574.
 17. Zhang F, Romano PR, Nagamura-Inoue T, Tian B, Dever TE, Mathews MB, Ozato K, Hinnebusch AG: Binding of double-stranded RNA to protein kinase PKR is required for dimerization and promotes critical autophosphorylation events in the activation loop. *J Biol Chem* 2001, **276**:24946-24958.
 18. Dey M, Cao C, Dar AC, Tamura T, Ozato K, Sicheri F, Dever TE: Mechanistic link between PKR dimerization, autophosphorylation, and eIF2alpha substrate recognition. *Cell* 2005, **122**:901-913.
 19. Rowlands AG, Panniers R, Henshaw EC: The catalytic mechanism of guanine nucleotide exchange factor action and competitive inhibition by phosphorylated eukaryotic initiation factor 2. *J Biol Chem* 1988, **263**:5526-5533.
 20. Dever TE, Yang W, Astrom S, Bystrom AS, Hinnebusch AG: Modulation of tRNA(iMet), eIF-2, and eIF-2B expression shows that GCN4 translation is inversely coupled to the level of eIF-2.GTP.Met-tRNA(iMet) ternary complexes. *Mol Cell Biol* 1995, **15**:6351-6363.
 21. Chinchar VG, Dholakia JN: Frog virus 3-induced translational shut-off: activation of an eIF-2 kinase in virus-infected cells. *Virus Res* 1989, **14**:207-223.
 22. Garner JN, Joshi B, Jagus R: Characterization of rainbow trout and zebrafish eukaryotic initiation factor 2alpha and its response to endoplasmic reticulum stress and IPNV infection. *Dev Comp Immunol* 2003, **27**:217-231.
 23. Hu CY, Zhang YB, Huang GP, Zhang QY, Gui JF: Molecular cloning and characterisation of a fish PKR-like gene from cultured CAB cells induced by UV-inactivated virus. *Fish Shellfish Immunol* 2004, **17**:353-366.
 24. Rothenburg S, Deigendesch N, Dittmar K, Koch-Nolte F, Haag F, Lowenhaupt K, Rich A: A PKR-like eukaryotic initiation factor 2alpha kinase from zebrafish contains Z-DNA binding domains instead of dsRNA binding domains. *Proc Natl Acad Sci USA* 2005, **102**:1602-1607.
 25. Bergan V, Jagus R, Lauksund S, Kileng O, Robertsen B: The Atlantic salmon Z-DNA binding protein kinase phosphorylates translation initiation factor 2 alpha and constitutes a unique orthologue to the mammalian dsRNA-activated protein kinase R. *Febs J* 2008, **275**:184-197.
 26. Su J, Zhu Z, Wang Y: Molecular cloning, characterization and expression analysis of the PKZ gene in rare minnow *Gobiocypris rarus*. *Fish Shellfish Immunol* 2008, **25**:106-113.
 27. Rothenburg S, Deigendesch N, Dey M, Dever TE, Tazi L: Double-stranded RNA-activated protein kinase PKR of fishes and amphibians: varying number of double-stranded RNA binding domains and lineage-specific duplications. *BMC Biol* 2008, **6**:12.
 28. Zhu R, Zhang YB, Zhang QY, Gui JF: Functional domains and the antiviral effect of the double-stranded RNA-dependent protein kinase PKR from *Paralichthys olivaceus*. *J Virol* 2008, **82**:6889-6901.
 29. Deigendesch N, Koch-Nolte F, Rothenburg S: ZBP1 subcellular localization and association with stress granules is controlled by its Z-DNA binding domains. *Nucleic Acids Res* 2006, **34**:5007-5020.
 30. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T, Lu Y, Miyagishi M, Kodama T, Honda K, et al: DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* 2007, **448**:501-505.
 31. DeFilippis VR, Alvarado D, Sali T, Rothenburg S, Fruh K: Human cytomegalovirus induces the interferon response via the DNA sensor ZBP1. *J Virol* 2010, **84**:585-598.
 32. Langland JO, Cameron JM, Heck MC, Jancovich JK, Jacobs BL: Inhibition of PKR by RNA and DNA viruses. *Virus Res* 2006, **119**:100-110.
 33. Chang HW, Watson JC, Jacobs BL: The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase. *Proc Natl Acad Sci USA* 1992, **89**:4825-4829.
 34. Romano PR, Zhang F, Tan SL, Garcia-Barrio MT, Katze MG, Dever TE, Hinnebusch AG: Inhibition of double-stranded RNA-dependent protein kinase PKR by vaccinia virus E3: role of complex formation and the E3 N-terminal domain. *Mol Cell Biol* 1998, **18**:7304-7316, 29.
 35. Beattie E, Tartaglia J, Paoletti E: Vaccinia virus-encoded eIF-2alpha homolog the antiviral effect of interferon. *Virology* 1991, **183**:419-422.
 36. Dar AC, Sicheri F: X-ray crystal structure and functional analysis of vaccinia virus K3L reveals molecular determinants for PKR subversion and substrate recognition. *Mol Cell* 2002, **10**:295-305.
 37. Yu YX, Bearzotti M, Vende P, Ahne W, Bremont M: Partial mapping and sequencing of a fish iridovirus genome reveals genes homologous to the frog virus 3 p31, p40 and human eIF2alpha. *Virus Res* 1999, **63**:53-63.
 38. Essbauer S, Bremont M, Ahne W: Comparison of the eIF-2alpha homologous proteins of seven ranaviruses (Iridoviridae). *Virus Genes* 2001, **23**:347-359.
 39. Majji S, LaPatra S, Long SM, Sample R, Bryan L, Sinning A, Chinchar VG: Rana catesbeiana virus Z (RCV-Z): a novel pathogenic ranavirus. *Dis Aquat Organ* 2006, **73**:1-11.
 40. Kawagishi-Kobayashi M, Silverman JB, Ung TL, Dever TE: Regulation of the protein kinase PKR by the vaccinia virus pseudosubstrate inhibitor K3L is dependent on residues conserved between the K3L protein and the PKR substrate eIF2alpha. *Mol Cell Biol* 1997, **17**:4146-4158.
 41. Ito T, Marintchev A, Wagner G: Solution structure of human initiation factor eIF2alpha reveals homology to the elongation factor eEF1B. *Structure* 2004, **12**:1693-1704.
 42. Dever TE, Sripriya R, McLachlin JR, Lu J, Fabian JR, Kimball SR, Miller LK: Disruption of cellular translational control by a viral truncated eukaryotic translation initiation factor 2alpha kinase homolog. *Proc Natl Acad Sci USA* 1998, **95**:4164-4169.
 43. Kawagishi-Kobayashi M, Cao C, Lu J, Ozato K, Dever TE: Pseudosubstrate inhibition of protein kinase PKR by swine pox virus C8L gene product. *Virology* 2000, **276**:424-434.
 44. Cigan AM, Pabich EK, Feng L, Donahue TF: Yeast translation initiation suppressor sui2 encodes the alpha subunit of eukaryotic initiation factor 2 and shares sequence identity with the human alpha subunit. *Proc Natl Acad Sci USA* 1989, **86**:2784-2788.
 45. Dever TE, Chen JJ, Barber GN, Cigan AM, Feng L, Donahue TF, London IM, Katze MG, Hinnebusch AG: Mammalian eukaryotic initiation factor 2 alpha kinases functionally substitute for GCN2 protein kinase in the GCN4 translational control mechanism of yeast. *Proc Natl Acad Sci USA* 1993, **90**:4616-4620.
 46. Carroll K, Elroy-Stein O, Moss B, Jagus R: Recombinant vaccinia virus K3L gene product prevents activation of double-stranded RNA-dependent, initiation factor 2 alpha-specific protein kinase. *J Biol Chem* 1993, **268**:12837-12842.
 47. Davies MV, Chang HW, Jacobs BL, Kaufman RJ: The E3L and K3L vaccinia virus gene products stimulate translation through inhibition of the double-stranded RNA-dependent protein kinase by different mechanisms. *J Virol* 1993, **67**:1688-1692.
 48. Nonato MC, Widom J, Clardy J: Crystal structure of the N-terminal segment of human eukaryotic translation initiation factor 2alpha. *J Biol Chem* 2002, **277**:17057-17061.
 49. Rothenburg S, Seo EJ, Gibbs JS, Dever TE, Dittmar K: Rapid evolution of protein kinase PKR alters sensitivity to viral inhibitors. *Nat Struct Mol Biol* 2009, **16**:63-70.
 50. Dar AC, Dever TE, Sicheri F: Higher-order substrate recognition of eIF2alpha by the RNA-dependent protein kinase PKR. *Cell* 2005, **122**:887-900.
 51. Seo EJ, Liu F, Kawagishi-Kobayashi M, Ung TL, Cao C, Dar AC, Sicheri F, Dever TE: Protein kinase PKR mutants resistant to the poxvirus pseudosubstrate K3L protein. *Proc Natl Acad Sci USA* 2008, **105**:16894-16899.
 52. Dever TE, Feng L, Wek RC, Cigan AM, Donahue TF, Hinnebusch AG: Phosphorylation of initiation factor 2 alpha by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast. *Cell* 1992, **68**:585-596.

53. Reid GA, Schatz G: **Import of proteins into mitochondria. Extramitochondrial pools and post-translational import of mitochondrial protein precursors in vivo.** *J Biol Chem* 1982, **257**:13062-13067.
54. Edgar RC: **MUSCLE: multiple sequence alignment with high accuracy and high throughput.** *Nucleic Acids Res* 2004, **32**:1792-1797.
55. Pollastri G, McLysaght A: **Porter: a new, accurate server for protein secondary structure prediction.** *Bioinformatics* 2005, **21**:1719-1720.

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