

Evolution of PKR combats viral mimicry

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The innate immune system provides the first line of host defense to pathogens. Part of this response is mediated by the cellular RNA-activated protein kinase, PKR. In a recent article published in the journal *Nature* [1], findings on the rate of evolution of PKR and how it relates to K3L, a pseudosubstrate viral mimic from poxviruses, are presented. The authors employ an impressive diversity of approaches, including phylogenetic analyses of PKR from 21 primates as well as functional yeast growth assays, both of which are interpreted at the molecular level using crystal structures. Phylogenetic analyses reveal positive selection throughout PKR and K3L, but little or no change in the sequence of PKR's true cellular substrate eIF2 α or the three other cellular kinases that phosphorylate it. Thus, certain genes in the primate genome are capable of evolving rapidly. Functional assays conducted on mutant PKRs in which a subset of the positively selected residues was changed confirm that certain amino acids confer species-specific resistance to K3L. Rapid evolution of genes involved in innate immunity may be a mechanism for keeping pace with viral challenges.

Introduction

The double-stranded RNA (dsRNA)-activated protein kinase PKR is comprised of three structural domains: a tandem N-terminal double-stranded RNA binding domain (dsRBD) consisting of two double-stranded RNA binding motifs (dsRBMs),

a C-terminal kinase domain, and a connecting linker that is unstructured (**Figure 1A**). The best characterized cellular substrate of PKR is eukaryotic initiation factor 2 α (eIF2 α). In the cell, PKR is present in a latent state, but in response to infection by dsRNA [2] or ssRNA with a 5'-triphosphate [3], PKR becomes activated by autophosphorylation and phosphorylates eIF2 α at serine 51. Phosphorylation of eIF2 α blocks translation and, in effect, obstructs viral replication in the cell (**Figure 1B**). In addition to PKR, eIF2 α can be activated by three other cellular stress-sensing kinases: HRI (activated under amino acid deprivation), PERK (senses misfolded proteins), and GCN2 (activated during heme deprivation). All four eIF2 α kinases have conserved kinase domains but distinct regulatory domains [4].

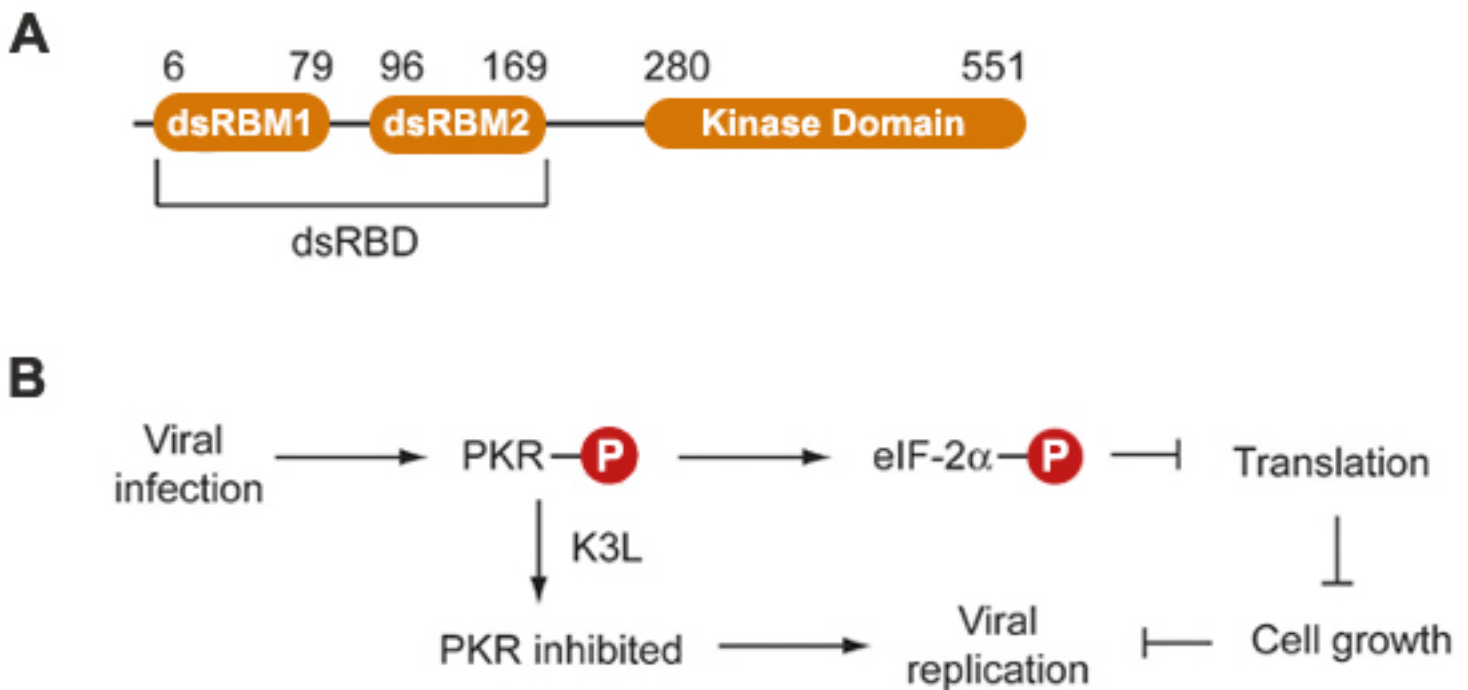


Figure 1. PKR function and its regulation by viral K3L. (A) PKR is comprised of tandem N-terminal dsRBMs (dsRBD) and a C-terminal kinase domain connected by a flexible linker. (B) PKR is activated (autophosphorylated) in the presence of viral RNA. Activated PKR then phosphorylates eIF2 α , which inhibits translation and ultimately prevents cell growth and viral replication. Viral eIF2 α -mimic K3L binds and inhibits PKR, thereby allowing viral replication to persist.

The structural biology of PKR has advanced in recent years. High-resolution structures of PKR include an NMR structure of the dsRBD [5] and a crystal structure of the kinase domain bound to eIF2 α [6]. The NMR structure reveals each dsRBM folds in a typical α - β - β - β - α topology characteristic of dsRBMs, and a flexible linker connecting the two dsRBMs. The crystal structure reveals a kinase domain dimerized in a back-to-back orientation, in complex with eIF2 α , an

orientation possibly conserved by other eIF2 α kinases. In addition, a low-resolution structural analysis of full-length PKR has been performed using SAXS [7]; flexibility in the linker regions is observed and thought to contribute to PKR dimerization on dsRNAs of diverse secondary structure. It should be noted that currently there are no available high-resolution structures of full-length PKR alone or bound to RNA, or of its dsRBD bound to RNA. Nonetheless, possible modes of non-specific sequence recognition of dsRNA by PKR can be inferred from a crystal structure of an unrelated dsRBM from *Xenopus laevis* bound to dsRNA [8].

Viruses have evolved various strategies to evade detection by PKR, including synthesis of PKR pseudosubstrates and non-activating RNA decoys [9]. This observation suggests that the evolution of PKR and of viral factors might be intimately linked. Recently, Malik and co-workers published a breakthrough study in the journal *Nature*, in which phylogenetic and functional analyses were employed to investigate PKR evolution and the sensitivity of various primate PKRs to a poxvirus pseudosubstrate protein, K3L [1].

Phylogentic Analyses

In the current study, Elde *et al.* cloned and sequenced PKR cDNA from 21 primates, representing 30 million years of evolutionary divergence. Total RNA was obtained from Coriell Cell Repositories (ccr.coriell.org). Established in 1972, these repositories provide scientists with thousands of cell lines, including those from healthy primates, as well as human disease states, chromosomal abnormalities, and distinct human populations. In the present study, the cell repository provided the authors with a diverse collection of primate DNA for sequencing PKR and analyzing its evolution.

Protein function is directly affected by nucleotide changes that code for a different amino acid (*i.e.* non-synonymous changes), but not by nucleotide changes that code for the same amino acid (*i.e.* synonymous changes). Positive selection occurs when the ratio of non-synonymous changes (dN) to synonymous changes (dS), is greater than 1 (*i.e.* dN/dS > 1, or dN > dS), which indicates that nucleotide substitutions frequently code for different amino acids. Conversely, purifying selection occurs when dN/dS < 1 (or dS > dN), which indicates that nucleotide substitutions more frequently code for the same amino acid. In this study, dN/dS was determined for all four eIF2 α kinases, for K3L, and for eIF2 α itself.

First, the authors aligned the 21 primate PKR cDNAs, where they observed strong positive selection (dN > dS) in all three structural domains of PKR (**Table 1**). For

instance, dN/dS calculated from human and rhesus monkey was 2.01. In one striking example, the authors found one branch of a PKR phylogenetic tree, along which Old World monkeys had 22 non-synonymous and no synonymous changes, thus illustrating an extraordinary period of PKR evolution.

<u>Protein</u>	<u>dN/dS</u>
eIF-2α kinases¹	
PKR	2.01
HRI	0.27
PERK	0.21
GCN2	0.11
eIF-2α¹	0.00
K3L²	2.80
¹ dN/dS calculated from human and rhesus monkey or	
² vaccinia and smallpox virus	

Table 1.

dN/dS calculations for eIF-2 α kinases, eIF-2 α , & K3L

Next, to see if rapid evolution is unique to PKR, the authors performed phylogenetic analyses on the other known eIF2 α kinases in primates: HRI, PERK, and GCN2. In contrast to the positive selection observed in PKR, these eIF2 α kinases exhibit purifying selection (dS>dN). For instance, dN/dS values calculated from human and rhesus monkey were 0.27, 0.21, and 0.11 for HRI, PERK, and GCN2.

A study of the evolution of poxvirus K3L from vaccinia virus to variola major virus (smallpox) was also carried out. This analysis revealed that K3L, like PKR, is undergoing strong positive selection. In particular, dN/dS calculated from vaccinia and smallpox virus was 2.80 (**Table 1**). Unfortunately, even though this dN/dS values is similar to that for PKR, the co-evolution of PKR and K3L could not be tested due the young age of poxviruses.

Thus, PKR and K3L are undergoing rapid positive selection, while the other three

eIF2 α kinases are not. Why is this? PKR activation is vital to combating viral infection. As a key component of the innate immune system, its positive selection appears to be a ‘tug-of-war’ with rapidly evolving viral inhibitors. The other eIF2 α kinases, on the other hand, are sensors of non-evolving (or slowly evolving) stressors: amino acid deprivation, misfolded proteins, and heme deprivation.

In addition to examining rates of evolution in eIF2 α kinases and K3L, the authors analyzed the evolution of eIF2 α itself. In stark contrast to PKR, no amino acid changes were found in eIF2 α (dN/dS =0.00, **Table 1**). The absence of change in eIF2 α appears consistent with translation being a highly demanding and conserved process. Moreover, because eIF2 α is the substrate of four kinases, intense evolutionary pressure may be placed on eIF2 α to remain conserved. In summary, rapid evolution of PKR is unique among the eIF2 α kinases, presumably because it must discriminate against ever-changing viral mimics in such a way that it recognizes an unchanging and possibly unchangeable substrate.

Growth Assays

To evaluate the effects of positive selection, Elde *et al.* employed a yeast growth assay in which human PKR and variola and vaccinia virus K3L were co-expressed in yeast under a galactose-inducible promoter. When over-expressed in yeast cells, PKR has been shown to inhibit cell growth through phosphorylation of yeast eIF2 α [10]. This likely occurs due to the presence of endogenous activating RNAs in yeast [11], or in the absence of activating RNA wherein high levels of PKR promote self-dimerization and activation [12]. In the absence of K3L expression, Elde *et al.* observed PKR-induced absence of growth across a range of evolutionarily divergent primate PKR orthologues. Moreover, when the primate PKRs were expressed in a yeast strain containing a nonphosphorylatable mutant of eIF2 α (eIF2 α -S51A), growth was restored, indicating that PKR toxicity is mediated through phosphorylation of eIF2 α for all primates tested, as expected.

Once K3L was co-expressed with PKR, however, the authors observed a significant rescue of growth for Old and New World monkeys, whereas the hominoids tested, with the exception of gibbon, continued to exhibit growth arrest. Thus, while PKR from all primates studied has retained the ability to recognize eIF2 α , rapid evolution of hominoid PKR has led to greater resistance to K3L.

Functional Analysis

Elde *et al.* utilized the co-crystal structure of the dimerized human PKR kinase domain bound to eIF2 α [6] in order to establish the molecular basis for the observed divergence in PKR response to K3L among primates. Mapping of residues subject to positive selection revealed a clustering around the PKR/eIF2 α interface, particularly in the α G helix, which is involved in a hydrogen-bonding network with eIF2 α (**Figure 2**). Additionally, several of the positively selected PKR residues cluster in the α O helix and the β 4- β 5 loop, all of which do not appear to be involved in the specific contacts with eIF2 α .

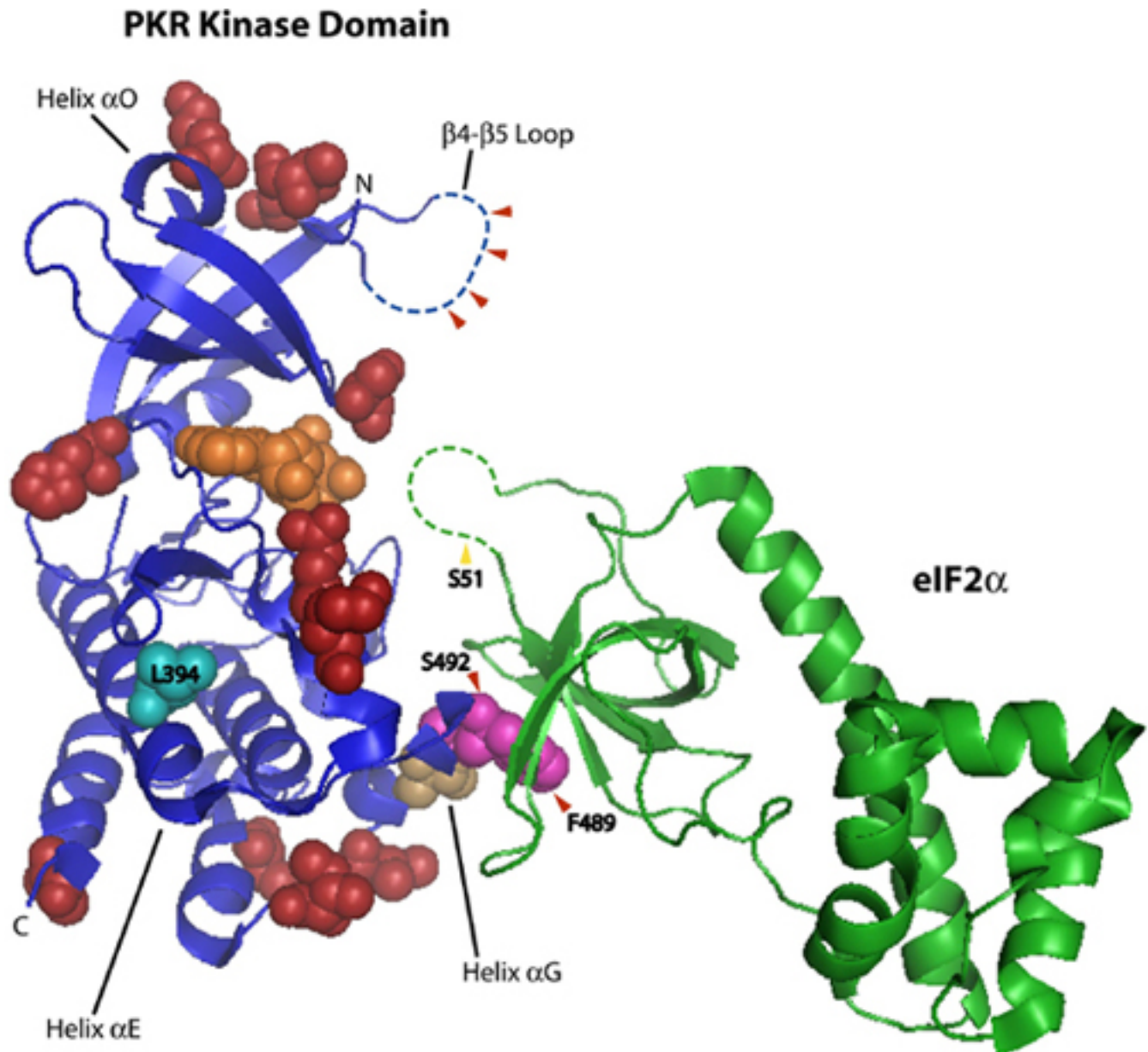


Figure 2. Mapping of positively selected PKR residues to PKR-eIF2 α co-crystal. This figure is based on Figure 1b by Elde *et al.* [1] but here the crystal structure (PDB 2A19) contains the ATP analog, AMPPNP (orange), bound in the active site [6], instead of the apo-structure pictured in the *Nature* article. Sites under positive selection are in red, space filling. Residues in

magenta (S492 and F489) and tan (T496) in the α G helix also evolved under positive selection and are known to interact directly with eIF2 α [6]. S492, F489, and L394 (teal) all confer increased K3L resistance to human PKR as compared to gibbon PKR. This was prepared using MacPyMol.

Employing the yeast growth assays described above, the authors devised a series of mutants in the K3L-susceptible gibbon PKR wherein three residues in the α G helix were exchanged for K3L-resistant human PKR residues in various combinations. Remarkably, swapping of these residues conferred K3L resistance to gibbon PKR, and in one case required as little as one substitution. Moreover, residues in the gibbon α E helix, a region of PKR that does not make direct contact with eIF2 α , were also identified as a source of PKR resistance to K3L (**Figure 2**); when substituted with residues from orangutan α E, growth suppression of gibbon PKR greatly increased. Given that the α E helix is oriented away from the eIF2 α interface, this suggests that allostery may be a mechanism for altering K3L recognition.

Next, the authors examined the interplay between mutations in the α G and α E helices. A connection between adaptive substitutions at residues 489 and 492 in the α G helix and ‘toggling’ between Leu and Phe at position 394 in the α E helix was tested by making all possible configurations of substitutions at these residues in both human and gibbon PKR backbones. By mapping the results of yeast assays to phenotype ‘cubes’, the authors discerned that all single, and most double, substitutions maintain K3L resistance in human PKR. In addition, in the background of gibbon PKR, most single substitutions reverse susceptibility of the wild-type configuration. Positive selection in helix α G and changes at site 394, which was not found to be under positive selection, makes clear the diverse evolutionary strategies PKR employs to evade rapidly evolving viral mimics.

Concluding Remark

Recently, Rothenburg *et al.* performed a similar study where they also found that PKR is evolving in response to viral inhibitors [13]. These authors discovered rapid evolution of the kinase domain in a diverse collection of vertebrates ranging from human to zebrafish, including positive selection at specific sites near the eIF2 α binding site. In addition, they found that substitution of PKR residues from other species into human PKR altered its response to poxviruses. These authors made the point that differences in how species respond to viruses has profound implications for studying human diseases in model organisms.

The findings presented by Elde *et al.* focused on the positively selected residues at the PKR-eIF2 α interface. Several other clusters of positively selected residues occur in regions of PKR, including the α O helix of the N-lobe of the kinase domain (**Figure 2**) and the base of the C-lobe. As the investigation of Leu394 revealed, it is possible for distal sites on PKR to have an impact in K3L-resistance of PKR, perhaps due to participation in conformational changes upon eIF2 α binding. The possibility that positively selected sites beyond the PKR-substrate interface may play a role in evading substrate mimicry also merits investigation. This includes residues in the dsRBD, which were also found to be under positive selection.

It appears that PKR evolves in such a way as to continue to interact with and phosphorylate eIF2 α . The structural basis behind this behavior is not entirely clear at present, but it is noteworthy that Ser51 is located in a disordered (and presumably flexible) region of the eIF2 α structure (**Figure 2**), whereas this same region on K3L is rigid [6]. The pliant nature of this linker region in eIF2 α may thus allow the integrity of the PKR-eIF2 α interaction to be maintained despite the rapid evolution of PKR at the binding interface. As positive selection occurs at PKR residues that make contacts with eIF2 α , the flexible linker of eIF2 α may respond by adjustments that allow Ser51 to restore contact with the PKR active site.

Acknowledgements

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