An Evolutionary View of the Arms Race between Protein Kinase R and Large DNA Viruses

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To establish productive infections, viruses must counteract numerous cellular defenses that are poised to recognize viruses as nonself and to activate antiviral pathways. The opposing goals of host and viral factors lead to evolutionary arms races that can be illuminated by evolutionary and computational methods and tested in experimental models. Here we illustrate how this perspective has been contributing to our understanding of the interactions of the protein kinase R pathway with large DNA viruses.

Recent studies have identified numerous host cell restriction factors that constitute the first line of defense against viruses. These factors, which include many interferon (IFN)-induced proteins, can act in a generalized manner by recognizing common molecular by-products of viral replication or more exclusively by targeting specific viral proteins. In the former case, viral antagonists that counteract the restriction factor put the host gene “on defense” as it must evolve to evade viral antagonists (1). In contrast, restriction factors with more specificity evolve “on offense” to maintain their ability to recognize viral targets. In both cases, the resulting arms race drives rapid evolution of the host and viral genes as they continually adapt to evade or restore recognition (Fig. 1A). These evolutionary signatures of conflict can be detected and quantified by analyses of the rates of nonsynonymous (*dN*) and synonymous (*dS*) substitutions among orthologous genes, with a *dN/dS* ratio greater than 1 indicating positive selection. Various algorithms enable estimation of the extent to which a gene has been evolving under positive selection, which lineages within a phylogenetic tree have experienced the strongest selection, and even which codons have been under recurrent pressure to evolve. These “hot spots” of evolution often pinpoint critical interacting surfaces between host and viral proteins (1). While this evolutionary framework has been applied to assess a variety of host restriction factors (reviewed in references 1 and 2), here we focus on the evolutionary approaches that are uncovering insights into the molecular conflicts between protein kinase R (PKR) and its viral antagonists in large DNA viruses.

PROTEIN KINASE R: A dsRNA SENSOR WITH BROAD ANTIVIRAL ACTIVITY

PKR is a member of a family of kinases that respond to various cellular stresses by phosphorylating eukaryotic initiation factor 2 α (eIF2 α), resulting in arrest of translation initiation. Unlike the other eIF2 α kinases, PKR is interferon inducible, highlighting its role in the innate immune response. PKR has broad antiviral activity as a result of its activation by double-stranded RNA (dsRNA), a nearly ubiquitous by-product of viral infections. While dsRNA is known to be a necessary intermediate in the life cycle of most RNA viruses, immunological methods have also identified dsRNA in cells infected with DNA viruses (3, 4). Whether this dsRNA arises from viral or cellular transcripts is unclear. Transcriptome sequencing (RNA-seq) data in several systems have identified overlapping complementary viral transcripts

that have the potential to form dsRNA (5–8), suggesting that the pressure to maintain compact genomes may be an Achilles heel for these DNA viruses. However, there are also data suggesting that DNA viruses induce structured host transcripts that can regulate PKR activity (9–11). Regardless, dsRNA is an important pathogen-associated molecular pattern (PAMP) that induces dimerization, autophosphorylation, and activation of PKR.

ROLE OF PKR ANTAGONISTS IN LARGE DNA VIRUSES

The importance of the PKR antiviral response is substantiated by the presence of antagonists in many virus families. Furthermore, these antagonists are often essential for viral replication. For example, deletion of the human cytomegalovirus (HCMV) PKR antagonists IRS1 and TRS1 results in a virus (HCMV[Δ I/ Δ T]) that fails to replicate in cell culture but can be rescued by introduction of the vaccinia virus (VACV) PKR antagonist E3L (12). Moreover, HCMV[Δ I/ Δ T] replicates efficiently in PKR-deficient cells, supporting the conclusion that the replication defect in wild-type cells is due to PKR activity (13; B. Ziehr and N. J. Moorman, University of North Carolina, personal communication). Similarly, deletion of the PKR antagonists from mouse CMV eliminates replication in wild-type cells and mice but has no effect in PKR-null cells and mice (14).

Even in systems where PKR antagonists appear not to be absolutely essential, they still often contribute to replication and pathogenicity. Guinea pig CMV depleted of its PKR antagonist, gp145, replicates poorly in cell culture, has reduced virulence in animals, and appears to be a safe and potentially effective vaccine for preventing congenital transmission in guinea pigs (15). The fact that it replicates at all suggests that the guinea pig PKR defense system may be less effective than that in humans or mice, or that guinea pig CMV has a second, as yet unidentified, PKR antagonist. Herpes simplex virus 1 (HSV-1) with a deletion of γ 34.5, one of its two known PKR antagonists, is avirulent in wild-

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FIG 1 Host-virus arms race and functional consequences. (A) Schematic representation of the evolutionary steps taken as a host gene evolves “on defense” to evade a viral antagonist. (B) Rapid evolution has led to the divergence of PKR in primates and contributes to cross-species barriers to infection. While HCMV_{TRS1} can readily antagonize human PKR, it has limited activity against Old World monkey (OWM) PKRs. In contrast, RhCMV_{TRS1} can antagonize some Old World monkey PKRs but not human PKR.

type mice, even when directly inoculated into the central nervous system (CNS) (16); however, it does replicate in some cell types and, notably, is fully virulent in PKR-null mice (17). VACV lacking its PKR antagonist, E3L (VACVΔE3L), replicates poorly in many cell types and in mice, but this defect is ameliorated at least partially in PKR-deficient cells and animals (18–20). Like HSV-1, VACV encodes at least one other PKR antagonist (K3L), which may explain why VACVΔE3L is able to replicate at all. Although some PKR antagonists have additional activities that might account for the presence of more than one antagonist in a single virus, it is also possible that the encoding of multiple antagonists increases the total potency of PKR antagonism or that each antagonist is effective in alternative settings, such as different cell types. The fact that several large DNA viruses encode more than one antagonist underscores how critical overcoming the PKR pathway has been during viral evolution.

PKR VERSUS VIRAL ANTAGONISTS: EVOLUTIONARY SIGNATURES OF CONFLICT

The broad antiviral activity of PKR and the diversity of its viral antagonists predict that PKR has engaged in multiple evolutionary arms races. Consistent with this hypothesis, two groups reported that PKR has a strong signature of positive selection (21, 22). Similar findings of positive selection have been found for retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) (23), revealing that multiple dsRNA sensors have been engaged in arms races with viruses. In the case of PKR, rapidly evolving codons were identified in both the N-terminal half of PKR (though not in the dsRNA-binding residues) and throughout the C-terminal kinase domain, consistent with multiple regions of PKR being targets of viral antagonists (21, 22). The sets of rapidly evolving codons identified by each group were surprisingly distinct, which is likely due to differences in the number and evolutionary breadth of the sequences used for the analyses and in the algorithms employed (24). Nevertheless, both papers conclude that the broad antiviral activity of PKR has made it a target of diverse viral antagonists, resulting in rapid evolution of PKR.

Given the broad diversity of PKR proteins across species, it is somewhat surprising that human PKR has so few polymorphisms. In fact, the frequency of missense mutations at any codon in over

100,000 human PKR alleles is less than 0.02%; this is in contrast to missense mutations existing at frequencies from 1 to 15% in PKR for other great ape species and frequencies of up to 22 and 50% in human RIG-I and MDA5, respectively (unpublished observation using data from reference 25 and the Exome Aggregation Consortium [ExAC], Cambridge, MA, November 2015 [<http://exac.broadinstitute.org>]). The low variation in human PKR may be a consequence of rapid population expansion following a bottleneck event or of a selective sweep occurring sometime after humans diverged from other hominoids.

Consistent with the arms race paradigm, rapid evolution of PKR appears to have driven the evolution of its viral antagonists. For instance, analysis of the PKR antagonist K3L from variola major and VACV revealed a *dN/dS* ratio of 2.8, while fewer than 10% of other orthologs in these viruses show signatures of positive selection (21). Similarly, recent comparisons of more than 100 isolates of HCMV revealed that TRS1 is evolving under positive selection (26), which is surprising considering that PKR is so invariant among humans. However, TRS1 has been reported to have multiple other functions in addition to PKR antagonism, so it is possible that one or more of these activities or the host adaptive immune response is driving TRS1 evolution (27). Regardless, these evolutionary analyses indicate that the host viral arms race has significantly impacted the evolution of PKR and its viral antagonists.

CONSEQUENCES OF THE PKR ANTAGONIST ARMS RACES

The rapid evolution and divergence of PKR and its antagonists have consequences for cross-species viral transmission events (Fig. 1B). For instance, HCMV TRS1 has coevolved with human PKR and can readily antagonize it but fails to antagonize Old World monkey PKRs, likely contributing to the inability of HCMV to replicate in monkey cells (18). In contrast, rhesus CMV (RhCMV) TRS1 antagonizes some Old World monkey PKR alleles but has limited activity against human PKR. In addition, RhCMV TRS1 and HCMV TRS1 block PKR pathway activation at different steps. While HCMV TRS1 blocks prior to PKR phosphorylation, RhCMV TRS1 allows PKR phosphorylation but prevents phosphorylation of eIF2α, the downstream substrate of PKR (18). Thus, the evolutionary adaptations in both PKR and TRS1 have led to qualitatively different mechanisms of antagonism, likely reflecting alterations in the protein-protein interfaces.

Despite these clear differences between HCMV and RhCMV TRS1, the species specificity of PKR antagonism is surprisingly complex. For example, even though humans and rodents are much more distantly related than humans and Old World monkeys, HCMV TRS1 functions in mouse cells and the PKR antagonists from mouse and guinea pig CMV function in human cells (28–30). In addition, although RhCMV TRS1 does not inhibit human PKR in VACVΔE3L rescue and reporter gene expression assays (18; unpublished data), RhCMV replicates in human fibroblasts (HFs), suggesting that RhCMV TRS1’s weak activity against human PKR may be sufficient for RhCMV replication in HFs. The explanations for these puzzling observations will require further investigation.

The species-specific differences driven by the host-virus arms race can guide experimental dissection of these interactions and mechanisms. For example, primate PKR alleles vary in their resistance to K3L (21), the VACV eIF2α mimic that competitively inhibits PKR activity (31). Analyses of chimeric PKR alleles re-

vealed that critical differences in K3L resistance mapped to residues under positive selection within the α G helix (21). In fact, a single amino acid present in human PKR was sufficient to confer resistance to the otherwise susceptible gibbon PKR allele. However, other residues, including one not identified as under positive selection, also contribute to K3L resistance, reflecting the intricacies arising from PKR having adapted to numerous extant and extinct viruses. Nonetheless, the utility of an evolution-guided approach for identifying critical interacting surfaces of restriction factors and their viral antagonists in other systems suggests that this approach will be useful for elucidating the mechanisms of antagonism used by other PKR antagonists.

APPLICATIONS OF EXPERIMENTAL EVOLUTION AND NEXT-GENERATION SEQUENCING

The species-specific functional differences between antagonists can also be leveraged to evaluate how viruses might adapt to overcome restriction factors. The VACV PKR antagonist K3L has limited activity against human PKR (21). To investigate how VACV could adapt to this resistant PKR variant, a VACV expressing K3L as the only PKR antagonist was serially passaged through human cells. Deep-sequencing and functional assays of progeny viruses revealed that improved viral fitness resulted from amplification of the K3L locus (32). This amplification not only augmented K3L expression but also increased the genomic space available for sampling mutations. In fact, a mutant K3L with improved activity against human PKR emerged, which enabled collapse of the amplified locus. These experiments suggested a “gene accordion” model to explain how large DNA viruses may rapidly adapt despite their low mutation rates (32). A similar cycle of gene amplification, mutation, and collapse was observed upon serial passage of a VACV expressing RhCMV TRS1 as its sole PKR antagonist in cells containing a relatively resistant PKR allele (33). In this case, the RhCMV TRS1 locus amplified, but subsequent collapse of the locus appeared to be enabled by mutations in other loci affecting genes not previously implicated in PKR pathway antagonism (34).

The strategy of using experimental evolution of a virus in a setting in which it is poorly adapted to overcome a host restriction factor is potentially broadly applicable to studies of other host-virus conflicts. The increasing ease of deep-sequencing technologies is particularly useful for studies of large DNA viruses, where it enables efficient identification of adaptive mutations and mechanisms, such as the genetic “accordion.” Furthermore, this system could be developed to reveal unidentified viral antagonists by serially passaging viruses through cells overexpressing a host restriction factor or expressing a resistant ortholog to expose antiviral effects that would otherwise be masked by effective viral antagonists.

CONCLUSIONS

Evolution-guided analyses coupled to functional studies have led to many significant insights into the conflicts between host restriction factors and their viral antagonists. This approach has applications in many viral systems to identify participating factors and mechanisms underlying host-virus interactions. Moreover, these types of analysis may be useful for evaluating the strength and limitations of animal models. Finally, with sensible consideration of potential issues arising from identification of gain-of-function mutations, the power of experimental evolution coupled to deep sequencing will be valuable for evaluating the risks and barriers to

cross-species transmission events and for dissecting the mechanisms underlying host-virus conflicts.

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