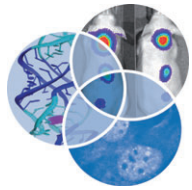
Advanced Review



Dissecting the roles of TRBP and PACT in double-stranded RNA recognition and processing of noncoding RNAs

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HIV TAR RNA-binding protein (TRBP) and Protein Activator of PKR (PACT) are double-stranded (ds) RNA-binding proteins that participate in both small regulatory RNA biogenesis and the response to viral dsRNA. Despite considerable progress toward understanding the structure–function relationship of TRBP and PACT, their specific roles in these seemingly distinct cellular pathways remain unclear. Both proteins are composed of three copies of the double-stranded RNA-binding domain, two of which interact with dsRNA, while the C-terminal copy mediates protein–protein interactions. PACT and TRBP are found in a complex with the endonuclease Dicer and facilitate processing of immature microRNAs. Their precise contribution to the Dicing step has not yet been defined: possibilities include precursor recruitment, rearrangement of dsRNA within the complex, loading the processed microRNA into the RNA-induced silencing complex, and distinguishing different classes of small dsRNA. TRBP and PACT also interact with the viral dsRNA sensors retinoic acid-inducible gene I (RIG-I) and double-stranded RNA-activated protein kinase (PKR). Current models suggest that PACT enables RIG-I to detect a wider range of viral dsRNAs, while TRBP and PACT exert opposing regulatory effects on PKR. Here, the evidence that implicates TRBP and PACT in regulatory RNA processing and viral dsRNA sensing is reviewed and discussed in the context of their molecular structure. The broader implications of a link between microRNA biogenesis and the innate antiviral response pathway are also considered. © 2015 John Wiley & Sons, Ltd.

How to cite this article:
WIREs RNA 2015, 6:271–289. doi: 10.1002/wrna.1272

INTRODUCTION

Double-stranded (ds) RNA has a vital role in normal cellular function. Any structured RNA, such as the ribosome, contains regions of dsRNA, which are often part of higher order tertiary structure. In addition, dsRNA is an intermediate in the biogenesis of short regulatory RNAs such as micro RNAs (miRNAs), endogenous short interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs).[1]

However, dsRNA is also an important replication intermediate for RNA viruses, for instance SARS coronavirus, poliovirus, and hepatitis C virus. It is crucial for cells to distinguish cellular dsRNA from viral dsRNA, and respond appropriately. Differentiation of self versus non-self dsRNA is achieved by proteins that have evolved to recognize chemical features specific to viral dsRNA, such as terminal 5′ triphosphate groups. These proteins are termed pattern recognition receptors (PRRs), and include TLRs (Toll-like receptors) 3, 7, and 8; RIG-I (retinoic acid-inducible gene I);

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Conflict of interest: The authors have declared no conflicts of interest for this article.

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and MDA5 (melanoma differentiation associated protein 5).[2]

The closely related mammalian proteins TRBP [HIV trans-activation responsive (TAR) RNA-binding protein; HGNC symbol, TARBP2; UniProt, Q15633] and PACT (Protein Activator of PKR; PRKRA; O75569) bridge several of these pathways (Figure 1(a) and (b)). TRBP was initially identified through its interaction with the HIV TAR RNA element, and both proteins were found to regulate the response to viral dsRNA through the protein PKR (double-stranded RNA-activated protein kinase; EIF2AK2).[3,4] Subsequently, TRBP and PACT were shown to interact with Dicer (DICER1), the ribonuclease responsible for processing the precursors of miRNAs and siRNAs.[5,6] More recently, PACT has been shown to activate RIG-I (DDX58), another innate immune sensor of viral RNA.[7] However, these different roles are often studied independently, and the links between them remain largely unexplored.

This review will summarize current knowledge about TRBP and PACT, and how they influence small RNA biogenesis and viral sensing.

TRBP AND PACT EACH CONTAIN THREE DOUBLE-STRANDED RNA-BINDING DOMAINS

TRBP and PACT each contain three double-stranded RNA-binding domains (dsRBDs) that are separated by unstructured linker regions[8,9] (Figure 1(c)). Many RNA-binding proteins contain multiple dsRBDs: PKR, ADAR2, and DGCR8 all have tandem dsRBDs, while human Staufen has five.[10–13] The presence of multiple dsRBDs in the same protein is thought to allow greater affinity and specificity, and to allow functional divergence of individual domains.[14,15]

Although all dsRBDs share the core α-β-β-β-α fold (Figure 2), they can be divided into two subgroups depending on sequence conservation. The type A dsRBD is the canonical form, which shows amino acid conservation in three regions involved in dsRNA binding (Figure 2(a)). Type B dsRBDs show conservation only at the C-terminal end of the domain, and are generally unable to bind dsRNA, despite retaining the same overall three-dimensional (3D) structure.[8,18] PACT and TRBP contain both classes of dsRBD (Figure 2(a)).

Some dsRBDs have additional structural elements that contribute to their function: for example, Rnt1p dsRBD from *Saccharomyces cerevisiae* has an additional α-helix that contributes to domain stability and RNA binding,[19] while dsRBDs from the *Caenorhabditis elegans* TRBP homolog, RDE-4, have recently been shown to contain numerous additional helixes and extended loop regions[20] (see Box 1).

BOX 1

HOMOLOGS OF TRBP AND PACT: *C. elegans* RDE-4

C. elegans RDE-4 forms a complex with homologs of Dicer and Argonaute, and is required for dsRNA-mediated RNA interference (RNAi), but not miRNA-mediated silencing.[21] RDE-4 contains three dsRBDs and binds nonspecifically to dsRNA, with an affinity for siRNA similar to that reported previously for TRBP.[22–24] The first two dsRBDs appear to function independently, with the second having a higher affinity for dsRNA, as was shown for TRBP.[20,22] RDE-4 has a higher affinity for longer dsRNAs, which may be due to cooperative binding, or an effect related to the overlap in potential binding sites.

As in TRBP and PACT, the C-terminal dsRBD of RDE-4 is required for interaction with Dicer, and also mediates dimerization.[24] In addition, the linker between domains 1 and 2 is required for dsRNA processing by Dicer.[25] The first two dsRBDs of RDE-4 have additional structural elements that extend into this linker region.[20] It will be interesting to see how these additional elements affect the function of the domains—it has already been shown that domain 1 is required for binding siRNAs, while having only a small influence on binding to longer dsRNA.[25]

TRBP and PACT Domains 1 and 2 Bind dsRNA

Domains 1 and 2 of both TRBP and PACT are type A dsRBDs, and all bind dsRNA.[22,26] In type A dsRBDs, three distinct regions participate in dsRNA binding (Figure 2(a) and (b)). Unlike dsDNA, dsRNA adopts an A form helix in which the major groove is deep and narrow, limiting access to the bases and therefore to sequence-specific information. dsRBDs bind across two adjacent minor grooves and the intervening phosphate backbone, burying ~780 Å^2 of the domain surface. In general, it is thought that dsRBDs bind nonspecifically to dsRNA. On discovery, TRBP was thought to specifically recognize certain structured RNAs, such as HIV-1 TAR RNA[27,28]; however, later evidence has shown that TRBP and PACT interact with a broad range of targets.[22,23]

However, several well-studied dsRBD-containing proteins do act on specific targets. For

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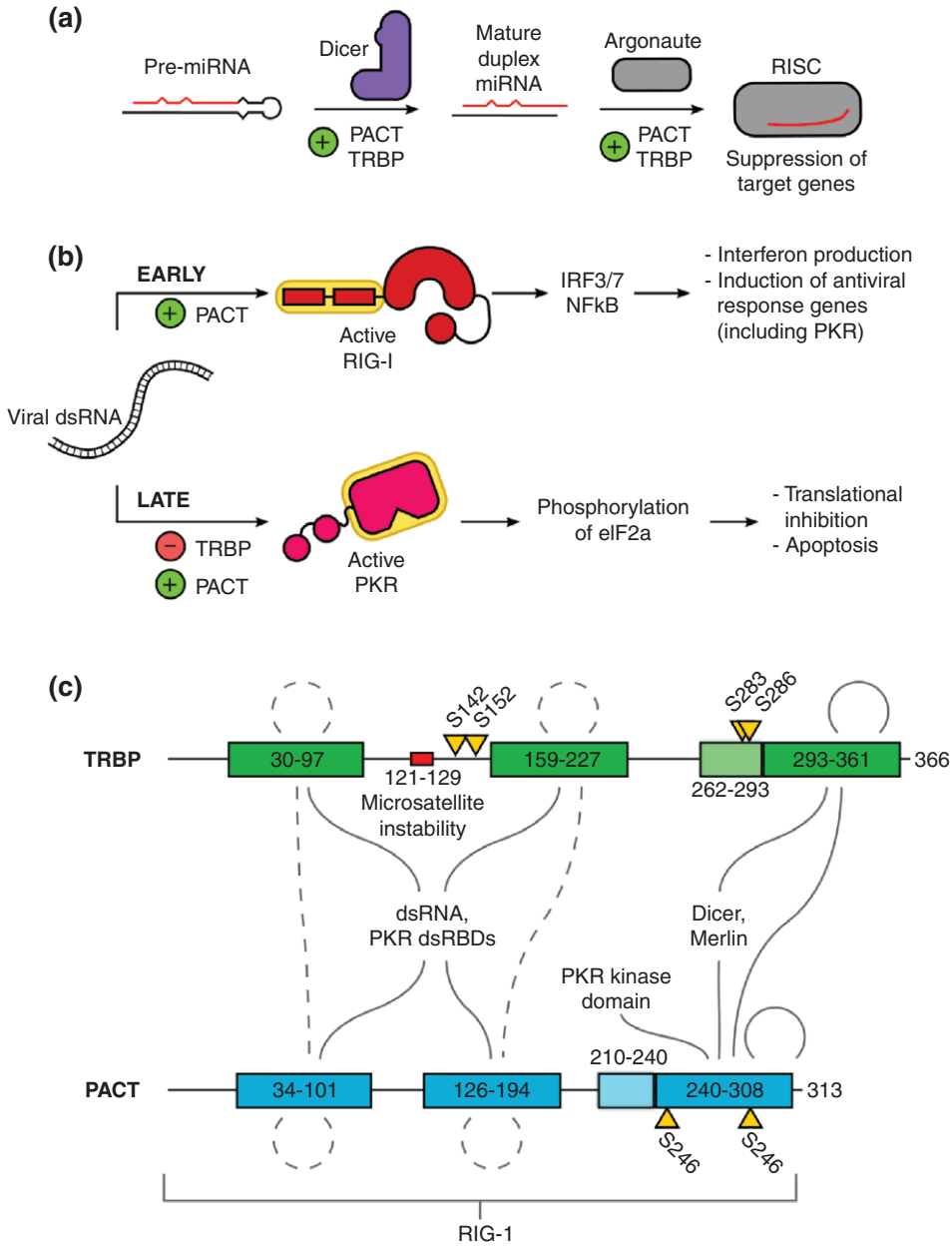

FIGURE 1 | Functions and domain composition of TAR RNA-binding protein (TRBP) and Protein Activator of PKR (PACT). (a) Precursor (pre-)microRNAs (miRNAs) are RNA hairpins that are produced in the nucleus, and exported to the cytoplasm. They contain the ~22 nt sequence of the mature miRNA, indicated in red. The endonuclease Dicer removes the terminal loop to give an RNA duplex, one strand of which is loaded into an Argonaut (Ago) protein to form RNA-induced silencing complex (RISC). TRBP and PACT are implicated in both Dicing and RISC loading. (b) PACT and TRBP have roles in at least two viral response pathways. First, PACT can facilitate activation of retinoic acid-inducible gene I (RIG-I) by viral double-stranded RNA (dsRNA) (distinguished from cellular dsRNA by distinct molecular features, discussed in section *TRBP and PACT mediate innate immune surveillance*). This begins a signaling cascade that results in the production of interferon and other antiviral genes. PACT and TRBP also regulate PKR, a kinase that targets the translation initiation factor eIF2α to inhibit protein production and promote apoptosis. TRBP inhibits PKR, while PACT can activate it in response to cellular stress. (c) Both PACT and TRBP contain three double-stranded RNA-binding domains (dsRBDs). The first two domains can bind dsRNA, while the third cannot. Interactions with many other proteins have been documented, particularly for the third domain. Solid lines indicate direct protein–protein interactions, while dashed lines indicate interactions that may be mediated via dsRNA. The third dsRBD potentially has an N-terminal extension, based on sequence conservation. Each protein has a number of phosphorylation sites (marked by yellow triangles) that regulate function under certain conditions. A region of TRBP implicated in cancers exhibiting microsatellite instability is indicated in red.

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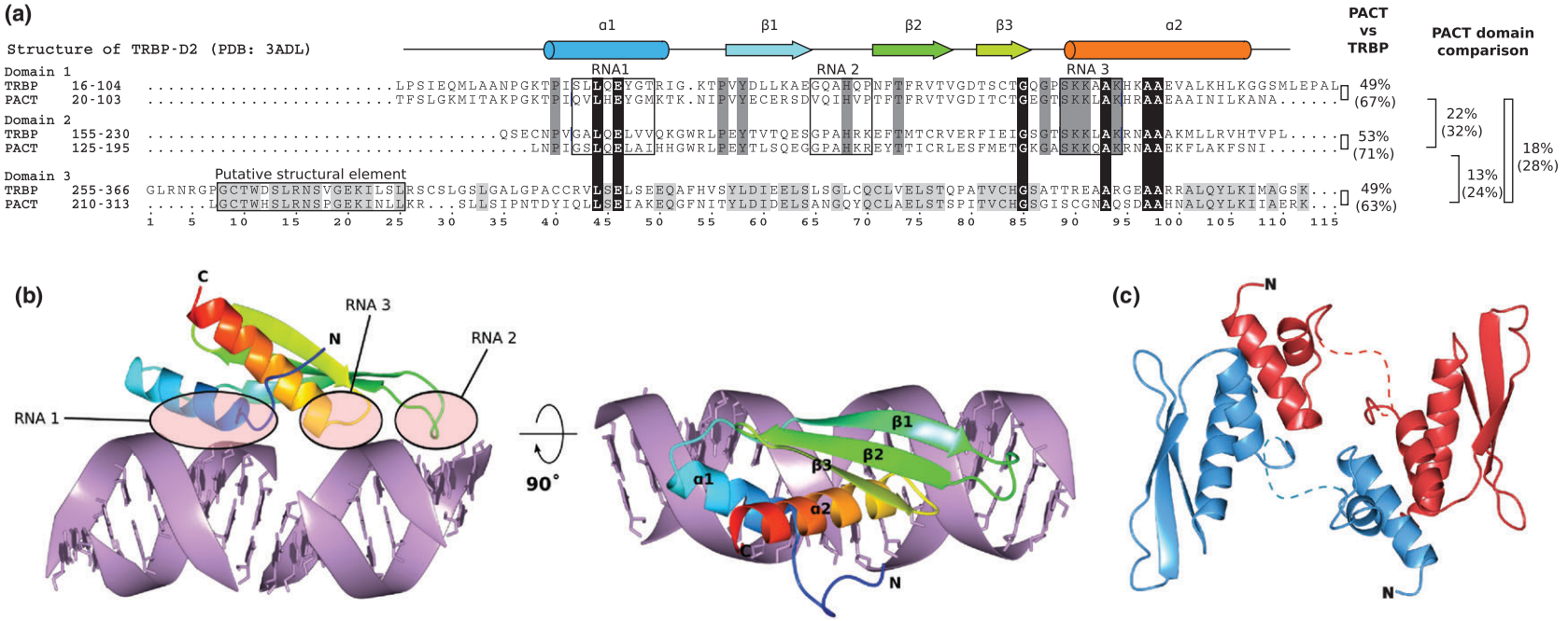

FIGURE 2 | (a) Sequence alignment of the double-stranded RNA-binding domains (dsRBDs) of TAR RNA-binding protein (TRBP) and Protein Activator of PKR (PACT). The top line shows the secondary structure of a 'typical' dsRBD, taken from the three-dimensional (3D) structure of TRBP domain 2 (PDB accession: 3ADL). Residues conserved between all domains are highlighted in black; those conserved between domains 1 and 2 are highlighted in dark gray; while those conserved in the third domain are shown in light gray. The regions of domains 1 and 2 that bind RNA [located in helix α1, the loop between β strands 1 and 2 (loop-β12), and helix α2] are boxed, as is a conserved region upstream of domain 3, which may represent a structural element additional to the standard dsRBD fold. On the right, the % identity (% similarity) shows that equivalent dsRBDs between TRBP and PACT are more similar than dsRBDs within the same protein. The sequences were aligned using Multalin,[16] and rendered using ESPript[17] (http://espript.ibcp.fr). (b) Two views of TRBP-D2 bound to two molecules of 10 bp double-stranded RNA (dsRNA) (PDB accession 3ADL). The RNA-interacting regions shown in part (a) are highlighted. Several parts of the dsRNA-binding interface are flexible, most notably loop-β12 in the second RNA-interacting region, which contains a highly conserved histidine residue. (c) Structure of a Staufen-D5 dimer (PDB accession 4DKK). The N-terminal extension (consisting of two α-helixes) is thought to interact with the dsRBD core of a second molecule. The linker between the dsRBD core and the extended region is not visible in the crystal structure, and is indicated here with dashed lines.

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example, Staufen can regulate translation and decay of specific mRNAs, while ADAR proteins can convert adenosine to inosine at precise positions in a variety of dsRNAs.[29,30] In both cases, RNA recognition is believed to occur through tertiary structures, such as bulges and loops.[19,31] Some specificity may also arise through contact with the edges of bases in the minor groove, and through the combination of dsRBDs with weak sequence or secondary structure preferences.[15,18] Current evidence does not rule out PACT and TRBP having a degree of substrate specificity.

TRBP and PACT Bind Protein Partners Primarily via Their Third Domain

The C-terminal dsRBDs of both TRBP and PACT (hereafter referred to as TRBP-D3 or PACT-D3) are type B dsRBDs. They do not bind RNA, but are instead required for interaction with other proteins. It is thought that many type B dsRBDs mediate protein–protein interactions: for example, *Drosophila* Staufen domain 5 binds to Miranda to enable mRNA localization.[32,33] However, currently, there is no structural information available about how dsRBDs interact with other proteins.

Both PACT-D3 and TRBP-D3 bind to Dicer,[5,6,34,35] while PACT-D3 has also been shown to interact with the virus-sensing protein PKR.[7,9] TRBP-D3 is not thought to interact with PKR,[36] suggesting that binding to Dicer and PKR is mediated by different regions of PACT-D3. These interactions are discussed further in sections *TRBP and PACT Form Part of the Small RNA Biogenesis Pathway* and *TRBP and PACT Mediate Innate Immune Surveillance of dsRNA* below. Other binding partners have been suggested for TRBP and PACT, such as the tumor suppressor Merlin,[37] but the nature of their interactions has not yet been studied in detail.

Although several dsRBDs have been reported to dimerize (e.g., the dsRBDs from PKR[38] and ADAR2[39]), it has been difficult to distinguish between direct binding and indirect association via dsRNA.[40] There is substantial biochemical evidence that PACT-D3 can homodimerize or heterodimerize with TRBP-D3.[41–43] While there is no direct biophysical evidence that TRBP-D3 homodimerizes, full-length TRBP can form dimers,[23] though a construct lacking TRBP-D3 does not.[22] Dimerization of PACT and TRBP has been suggested to modulate PACT activation of PKR,[43] but could also increase their affinity for dsRNA, by bringing together four type A dsRBDs.[26]

The recent 3D structure of dsRBD 5 of Staufen (Staufen-D5) gave the first high-resolution information about a dsRBD dimer[32] and revealed several features that distinguish type B dsRBDs, including the absence of the conserved histidine residue in loop-β1,2, the presence of large negatively charged patches on the canonical dsRNA-binding surface, and a region of extra-dsRBD structure, which is required for dimerization. As the linker between the core domain and the extended region is flexible, it was proposed that dimerization occurs via a domain-swapping mechanism (Figure 2(c)).

TRBP-D3 and PACT-D3 both lack the key histidine residue and have negatively charged residues at sites that would correspond to the dsRNA-binding surface. Furthermore, both domains are preceded by a highly conserved 20 amino acid sequence, which may suggest a similar dsRBD/dsRBD interaction to that seen in Staufen-D5. Interestingly, the conserved residues between TRBP-D3 and PACT-D3 are predicted to cluster on the opposite surface to the typical dsRNA-binding face, which potentially suggests the location of a conserved protein interaction site.

There is also evidence that the type A dsRBDs of TRBP and PACT mediate protein–protein interactions. Domains 1 and 2 of PACT are reported to directly interact with the dsRBDs of PKR.[9,44] PKR also dimerizes partly through its dsRBDs,[38] and PACT can still activate PKR if its first two domains are replaced with those from PKR.[9] The first two dsRBDs of PACT and TRBP have also been reported to homodimerize and heterodimerize,[41,43] which suggests there may be a common dimerization mechanism between these domains. However, current evidence does not exclude the possibility that these interactions are indirect or mediated by dsRNA.

TRBP AND PACT FORM PART OF THE SMALL RNA BIOGENESIS PATHWAY

RNAi is a highly conserved process (see Figure 1(a)) in which short RNA molecules cause translational (or transcriptional) silencing of complementary messenger RNAs (mRNAs). The cellular pathways that underpin RNAi have been discussed in detail by several recent reviews.[1,45,46] Here, we will provide only a brief summary of RNAi in mammals.

Somatic cells contain two major classes of small regulatory RNA: miRNA and siRNA. Both are approximately 22 nt long, but differ in their biogenesis and target recognition. miRNAs are derived from RNA hairpins that usually contain several mismatched bases, while siRNAs are processed from longer dsRNA molecules. siRNAs are typically highly complementary to their targets, whereas miRNAs require a 6–8 nt 'seed' region to be perfectly matched, with other positions contributing only weakly to

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target specificity. Although there are relatively few reports of endogenous mammalian siRNAs, dsRNA can be introduced exogenously either as RNA hairpins (shRNA) or as short dsRNA duplexes.

In the canonical miRNA biogenesis pathway, miRNAs are transcribed as long primary (pri-) miRNAs that contain a 60–80 nucleotide hairpin structure. The pri-miRNA is processed in the nucleus by Drosha/DGCR8 (the 'microprocessor' complex) to a shorter precursor (pre-) miRNA. The pre-miRNA is exported from the nucleus by Exportin 5, and further cleaved by a protein complex containing the ribonuclease Dicer, one of four Argonaute proteins (Ago1–4), and either TRBP or PACT (Figure 3(a)–(c)). This second processing step removes the terminal hairpin loop, leaving an RNA duplex of ~22 bp in length.

One strand of the dsRNA Dicer product is removed, while the other (the guide strand) is loaded into Argonaute. This Ago:miRNA complex, together with its protein partners, is referred to as the RNA-induced silencing complex (RISC). Despite recent progress, the mechanism of strand-selective RISC loading remains to be determined[47,48]; however, many miRNAs can contribute either strand to RISC.[49] Once loaded, the guide strand targets RISC to complementary mRNAs, which results in either suppression of translation, enhanced degradation and/or cleavage of the mRNA. Although only Ago2 has the ability to cleave mRNAs, miRNAs and siRNAs appear to distribute among all four Argonautes with little specificity.[50,51]

How Do TRBP and PACT Affect Processing by Dicer and the Formation of Active RISC?

There is now clear evidence that TRBP and PACT can associate with Dicer, both from immunoprecipitation experiments[5,6,35,52,53] and from *in vitro* reconstitution of the complex from the individually purified components.[54,55] Furthermore, there is strong support for the idea that PACT and TRBP are involved in processing of pre-miRNAs and RISC loading.[5,58] However, the molecular mechanism by which TRBP and PACT facilitate miRNA and siRNA processing remains unclear. It has long been established that Dicer alone is able to cleave pre-miRNA to mature miRNA *in vitro*,[56] and there is evidence that some substrates can be loaded into Ago2 *in vitro* in the absence of TRBP or PACT.[48,57,58]

The importance of tackling this question is underscored by the evidence linking miRNA biogenesis in general, and TRBP in particular, to a number of different cancers.[59–63] In particular, tumors exhibiting microsatellite instability are prone to frameshift mutations in TRBP (see Figure 1(c)), leading to impaired

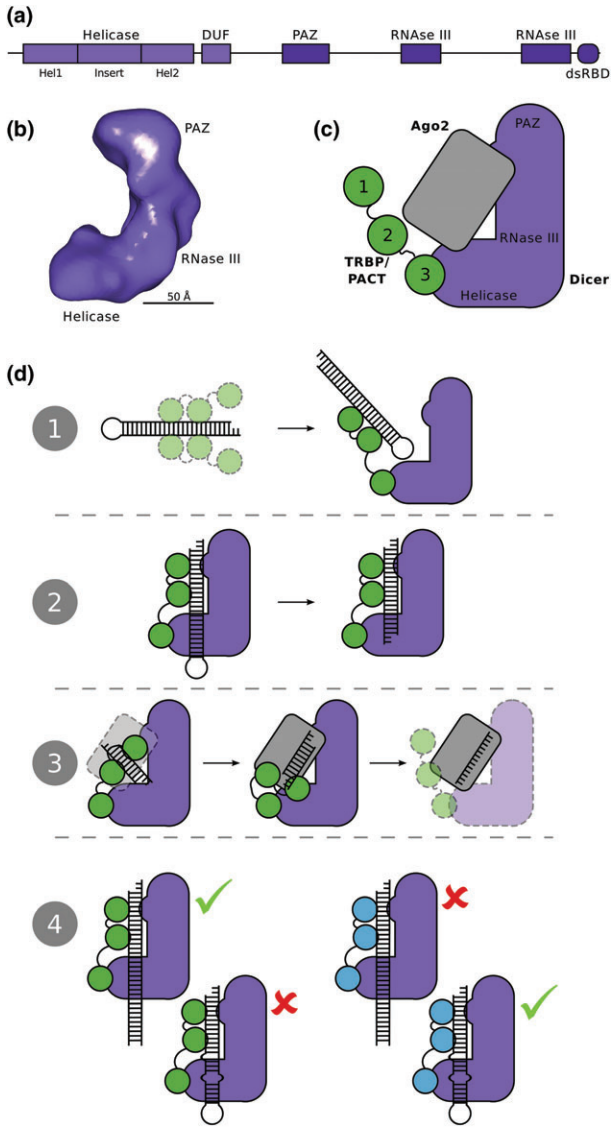

FIGURE 3 | (a) Layout of domains within Dicer. (b) Reconstruction of the Dicer–TAR RNA-binding protein (TRBP) complex from cryo-electron microscopy (EM) data, with ~15 Å resolution (EM data bank accession EMD-1646).[64] The locations of the RNase III and helicase domains are inferred from epitope-tagged Dicer.[65] The position of TRBP is not resolved. (c) A schematic of a minimal RNA-induced silencing complex (RISC)-loading complex of Dicer, Ago2, and TRBP/PACT (Protein Activator of PKR) based on cryo-EM data.[66] All components are approximately to scale. It is unknown whether all three components assemble prior to double-stranded RNA (dsRNA) binding, or if the complex is more dynamic. *In vivo*, it is likely that other proteins associate with the RISC-loading complex.[67,68] (d) At least four possible roles for TRBP/PACT can be envisaged (see section *How Do TRBP and PACT Affect Processing by Dicer and the Formation of Active RISC?*): (1) TRBP/PACT may help recruit dsRNA to Dicer; (2) TRBP/PACT may aid alignment of dsRNA for cleavage by Dicer; (3) TRBP/PACT may help dsRNA unwinding and/or loading into Argonaute proteins; or (4) TRBP/PACT may favor processing and loading of different substrates into RISC (the substrates shown are illustrative only).

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miRNA production.[60] The drug enoxacin has recently been investigated as a cancer therapeutic owing to its effect on miRNA biogenesis, and is believed to act through TRBP.[69,70] Although several studies have linked PACT expression to cancer, the effect is not as well documented as for TRBP.[71,72]

There are several points along the miRNA biogenesis and RISC loading pathway at which TRBP and PACT could act (see also Figure 3(d)):

(1) Recruitment of substrates to Dicer

   (a) TRBP and/or PACT may increase the affinity of the Dicer complex for dsRNA, or
   (b) more specifically increase Dicing rate through favoring binding of substrates over products.

(2) Facilitating efficient substrate cleavage by Dicer

   TRBP and/or PACT could ensure that the substrate dsRNA or pre-miRNA has the optimum orientation for Dicer cleavage.

(3) Removing the Dicer product

   (a) TRBP and/or PACT may aid unloading of Dicer or loading of Dicer products into Argonaute proteins,
   (b) in particular affecting which strand is loaded.

(4) Controlling which type of dsRNA is loaded into Argonaute

   TRBP and/or PACT may preferentially recognize different subsets of miRNA or siRNA (or their precursors), resulting in differences in processing or RISC loading.

We will now discuss evidence from structural, biochemical, biophysical, and molecular cell biology studies in the context of these possible mechanisms.

Evidence from Structural Studies

As described above, TRBP and PACT have been shown to interact with Dicer, a 1922-amino acid type III ribonuclease (Figure 3). The mechanism by which Dicer controls the length of small RNAs was elegantly resolved in an early crystallographic study.[64,73] The Dicer PAZ domain binds one end of the pre-miRNA, while a 'ruler' domain positions the PAZ domain at a distance from the RNase III site that corresponds to roughly 22 base pairs of dsRNA. This simple structural mechanism appears to function independently of binding partners as isolated Dicer can still cleave dsRNA targets.

While a considerable amount is known about the structure–function relationship of the RNase III/PAZ region of Dicer, much less is known about the N-terminal DExD/H helicase domain. The helicase domain consists of Hel1 and Hel2 regions separated by a 100-amino acid insert region (Figure 4(a)), which has no sequence homologs outside of the Dicer family. Yeast-2-hybrid screens have identified the insert region as the site of TRBP (and presumably PACT) binding.[35] This domain does not appear necessary for dsRNA cleavage *in vitro*,[74] nor does processing of pre-miRNAs require ATP.[75]

Atomic resolution structures of larger constructs of human Dicer or complexes containing TRBP or PACT are not currently available. The 20–30 Å resolution cryo-electron microscopy studies of a Dicer alone,[65] in complex with TRBP,[64] or TRBP and Ago2 (a minimal RISC-loading complex)[66] have been reported. They reveal an L-shaped overall structure with the helicase located in the base, and the RNase III and PAZ domains located in the long arm. However, these studies have been unable to identify precisely where and how TRBP interacts with Dicer.

Because only the C-terminal domains (CTDs) of TRBP or PACT bind to Dicer, it appears likely that domains 1 and 2 have considerable freedom of movement, consistent with roles 1–3 outlined above. To exclude proposed roles for TRBP, it will be necessary to determine more precisely where it interacts with Dicer. Similarly, a greater understanding of how flexible TRBP remains when bound to Dicer and dsRNA would allow possible roles to be considered or discounted. It is worth noting that several papers have suggested that Dicer and/or associated proteins undergo conformational shifts[66,76] and that these complexes contain multiple dsRNA-binding sites.[77] This raises the possibility that the role of TRBP may be more complex than currently believed.

Evidence from Biochemical and Biophysical Studies

A number of *in vitro* studies have shown that the complex of Dicer with TRBP or PACT has a higher affinity for dsRNA than Dicer alone by several orders of magnitude.[55,78,79] However, these studies report a more modest effect on small RNA processing rate, ranging from a fivefold increase in processing rate to a fivefold reduction. With the caveat that the reaction conditions may differ considerably from those present *in vivo*, this suggests TRBP and PACT fulfill role

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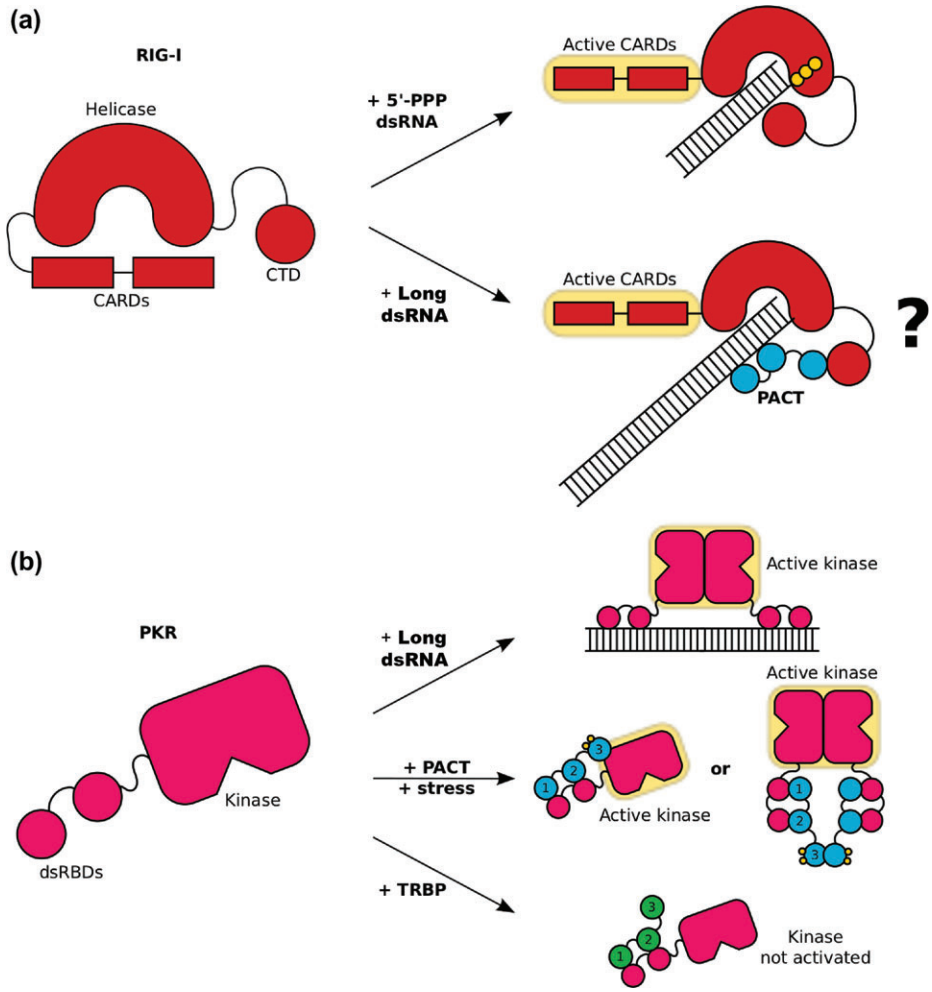

FIGURE 4 | (a) In the absence of appropriate ligands, retinoic acid-inducible gene I (RIG-I) has an inactive conformation, in which the helicase domain binds to the caspase recruitment domains (CARDs). 5′-Triphosphate double-stranded RNA (dsRNA) binds to the C-terminal domain (CTD) and helicase domain, which displaces the CARDs and results in signaling. It is less clear how Protein Activator of PKR (PACT) enables RIG-I activation: one possibility is that it increases RIG-I binding to additional ligands such as long dsRNA that lacks a 5′-triphosphate. (b) PKR can bind to long dsRNA through two N-terminal double-stranded RNA-binding domains (dsRBDs). This brings PKR molecules together to form dimers, which can then autophosphorylate and become active. PACT (when phosphorylated during cellular stress) can also activate PKR, although the mechanism is unclear. The two main hypotheses are: PACT-D3 contacts the kinase domain, somehow favoring activation, or PACT dimers can bind two molecules of PKR, promoting their dimerization and activation. TRBP acts as an inhibitor of PKR, either because its third domain cannot interact with PKR's kinase domain or because its third domain exhibits weaker dimerization.

1a above, but not 1b. Furthermore, TRBP has been reported to bind a pre-miRNA and the duplex Dicer product with similar affinities,[22,55] which is inconsistent with a role in modulating Dicer processing rate due to preferential binding of substrate over product.

The measured affinity of TRBP (without Dicer) for short dsRNA has varied considerably between different studies.[22,23,55] While this may simply represent variation in sample preparation and technique, it could also be due to the different RNAs studied, which would be consistent with TRBP and PACT showing a degree of specificity for different small RNAs (role 4). Additional evidence comes from studies of reconstituted complexes of Dicer. Dicer/TRBP complexes show different affinities for different pre-miRNAs and siRNAs, although Dicer itself is at least partially responsible for this specificity.[55,78] A complex containing Dicer and PACT showed considerably slower processing of short dsRNA substrates than a Dicer/TRBP complex, even though they seemed to have similar processing rates for pre-miRNAs.[79] These data potentially conflict with an earlier study, which concluded that both PACT and TRBP increase processing of long dsRNAs by Dicer.[80] This discrepancy could reflect

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a genuine difference in how Dicer complexes process dsRNAs of different lengths; further experiments using matched reaction conditions will be required to resolve this issue.

Both *in vitro* and *in vivo*, cleavage of pre-miRNAs by Dicer can give a range of products with different lengths, termed iso-miRs.[81,82] The size distribution of iso-miRs varies depending on the pre-miRNA. The Dicer/TRBP complex produces different length products compared with Dicer alone,[82,83,78,79] supporting the idea that TRBP helps position RNA for cleavage by Dicer (role 2). PACT has not yet been observed to affect iso-miR distribution.

In *Drosophila*, current models suggest that the TRBP homolog R2D2 (see Box 2) plays a significant role in selecting which strand of the miRNA/siRNA is loaded into Argonaute. There is some evidence that this is also the case in humans. The thermodynamic stability of the miRNA or siRNA duplex produced by Dicer is dependent on the nucleotide sequence. Experiments with photo-cross-linking dsRNA concluded that TRBP has some propensity to bind to the most thermodynamically stable end of the siRNA.[47] Furthermore, strand-specific crosslinking to the helicase domain of Dicer required TRBP or PACT and depended on thermodynamic stability, terminal nucleotides, and mismatched bases.[77] However, strand selectivity is not solely determined at this step: a study that measured which strand was loaded into RISC concluded that Ago2 alone had some strand selectivity (dependent on substrate), though TRBP and PACT could enhance this.[48]

Lastly, it has recently been shown that TRBP can diffuse along dsRNA *in vitro*.[96] Although it is not yet clear what effect this would have on the Dicer complex, one-dimensional diffusion may allow the dsRNA to explore a greater range of positions and orientations within the complex. Together with the flexibility of TRBP, this could potentially facilitate RNA positioning for Dicer processing or RISC loading.

BOX 2

RNA INTERFERENCE IN *D. melanogaster*

Drosophila melanogaster has emerged as an important model system for research into RNAi, often revealing interactions and mechanisms that are later shown to occur in humans. Unlike mammals, *Drosophila* uses siRNA as an antiviral defense, termed exogenous (exo-) siRNA.[84] In addition, an endogenous (endo-) siRNA pathway exists to target mobile genetic elements.[85] In general, the miRNA pathway utilizes the proteins Dicer-1 and Ago1, while siRNA pathways rely on Dicer-2 and Ago2.[86]

Several TRBP homologs have been identified in *Drosophila*, most notably the proteins R2D2 and Loquacious (Loqs). R2D2 forms a complex with Dicer-2 and assists strand-specific loading of siRNA into Ago2,[87,88] while Loqs plays a role in processing of pre-miRNAs by Dicer-1.[89,90] At least four isoforms of Loqs have been identified, which differentially process specific miRNAs, lead to altered iso-miR distributions, and even interact with different Dicer proteins.[83,91,92] At the whole organism level, reduction of Loqs expression causes infertility owing to terminal differentiation of germ cells, while complete knockout is lethal.[83,93,94] While TRBP and PACT share several properties with *Drosophila* dsRBPs,[78,95] currently, there is no clear evidence for a similar delineation of function on the protein level in the mammalian system.

Evidence from Molecular Cell Biology Studies

The initial evidence that TRBP and PACT bound Dicer came from immunoprecipitation experiments using HEK293 cells.[5,6,53] In one case, epitope-tagged Dicer was used to recover a complex containing TRBP and Ago2 that was able to process pre-let-7 to mature let-7, and cleave target mRNAs.[52] The same studies used RNAi to investigate the function of TRBP and PACT, but gave somewhat contradictory results. However, one common feature is that knocking down any of the four components (Dicer, Ago2, TRBP, or PACT) resulted in a decrease in levels of mature miRNA.[6,53,82]

There are some hints that PACT and TRBP may act on different sets of miRNAs and/or siRNAs (role 4). In one study, knocking down TRBP dramatically reduced the effectiveness of exogenous siRNA while having a small effect on levels of an inducible miRNA, whereas targeting PACT had the opposite effect.[53] It is currently unclear whether knockdown of PACT or TRBP differentially affects a certain subset of miRNAs.

It has been reported that TRBP contains multiple phosphorylation sites (Figure 1(c)), which modulate its stability. When TRBP with phospho-mimic mutations at these sites was transfected into human cells, miRNA production was shown to increase relative to controls.[97] The mechanism by which this occurs is unclear, as *in vitro* tests showed negligible differences in Dicer processing between complexes containing wild-type and phospho-mimic TRBP.[55] Transfection with phospho-mimic TRBP also caused a relative

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downregulation of the let-7 miRNA family, hinting that TRBP may differentially affect at least some miRNAs (role 4).[97] Although PACT also undergoes phosphorylation at several sites, the effects have never been studied in the context of miRNA biogenesis. Post-translational modification of TRBP and PACT may indicate that their functions are context dependent, and are not constitutively required.

To determine whether TRBP and PACT aid Dicer processing (roles 1 and 2) or RISC loading (role 3), several studies have examined how depletion of these proteins affects silencing mediated by shRNA (which requires processing by Dicer) or duplex siRNA (which can in principle be incorporated directly into RISC). The consensus is that both are affected, implying an important role in RISC loading, while not ruling out a supporting contribution to Dicer processing.[5,53,80] Although one of these studies reports siRNA-mediated silencing to be unaffected by depletion of TRBP or PACT, the effectiveness of TRBP/PACT knockdown was not measured.[80] It is therefore possible that TRBP and PACT were not effectively depleted, owing to saturation of the RNAi machinery with luciferase siRNA.

It is worth noting that it can be challenging to use RNAi to knock down proteins involved in small RNA processing, as the effectiveness of RNAi is itself dependent on the presence and activity of these proteins. The recent development of CRISPR/Cas genome editing may allow future studies to avoid this problem.[98] The interpretation of experimental data is also complicated by the possibility that these proteins may stabilize one another. Several studies have reported that knocking down TRBP also reduces Dicer levels, confounding interpretation in terms of TRBP alone,[5,60,97] especially because this effect has not been unanimously reported.[6] A similar stabilizing effect on Dicer was seen for PACT—but not for TRBP—in a separate study.[53]

The majority of both TRBP and PACT is found in the cytoplasm, particularly in the perinuclear space, though a smaller amount is present in the nucleus.[41,80,99] Förster resonance energy transfer experiments using fluorescently tagged TRBP and PACT provide evidence that they interact *in vivo*.[80] The distribution of TRBP and PACT mirrors that of Dicer and Ago2, which are also predominantly cytoplasmic with a small nuclear fraction. Dicer, Ago2, TRBP, and PACT have lower diffusion rates in the cytoplasm than in the nucleus.[99,100] This observation has been interpreted as evidence for a large cytoplasmic complex that is disassembled in the nucleus. Alternatively, it is consistent with the idea that these proteins are anchored to a larger cytoplasmic feature, such as P-bodies or the rough endoplasmic reticulum (ER). This latter possibility is consistent with reports of co-localization of RISC components with ribosomes or the ER.[101–103]

TRBP AND PACT MEDIATE INNATE IMMUNE SURVEILLANCE OF dsRNA

RNA viruses produce dsRNA in the course of their lifecycle, either as genomic material or as a replication intermediate. Viral dsRNA has features that distinguish it from cellular dsRNA, which may include its longer length, lack of the 5′ cap characteristic of eukaryotic mRNAs, and absence of mismatches. The innate immune system can recognize these features through PRRs, including RIG-I, MDA5, and TLR3.

The PRRs used by mammalian cells can be divided into 'early' responders, which stimulate production of interferons and proinflammatory cytokines, and 'late' responders, which directly block viral proliferation through translation inhibition and RNA degradation (Figure 1(b)). These pathways have been reviewed in more detail elsewhere.[2] TRBP and PACT have roles in several of these pathways, and have been implicated in the response to a number of viruses (summarized in Table 1).

PACT Can Stimulate RIG-I Activation

RIG-I is an early response PRR that detects dsRNA with a 5′ triphosphate group (5′ppp-dsRNA).[104] RIG-I is ubiquitously expressed, and is one of the main viral RNA sensors in nonimmune cells.[2] The mechanism of RIG-I activation has been characterized at the structural level[105–107] (Figure 4(a)). In the absence of 5′ppp-dsRNA, the tandem caspase recruitment domains (CARDs) responsible for signaling are bound to a DExD/H helicase domain, and are inactive. 5′ppp-dsRNA binds to the helicase and CTDs of RIG-I, displacing the CARDs. The CARDs then initiate a signaling pathway that leads to the activation of interferon regulatory factor 3 (IRF3), increasing transcription of type 1 interferon and other antiviral genes (Figure 1(c)). Several recent reviews have described RIG-I function in more detail.[107–109]

Evidence has emerged that PACT can activate RIG-I in response to a number of viruses[7,110,111] (Table 1). Although the helicase domains of RIG-I and Dicer are part of the same subfamily,[112] it is unlikely that PACT binds these two proteins in the same manner: there is no evident sequence homology in the helicase insert domain, which forms the PACT-binding site in Dicer. This is consistent with a report that

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TABLE 1 | Evidence Linking TRBP and PACT to Viral Sensing.

Virus	Details	Reference
Viruses linked to TRBP and PACT		
Newcastle disease virus (NDV)	Overexpressing PACT increased type 1 interferon production from NDV infection	113
Sendai virus	RIG-I activation during infection was enhanced by PACT	7
Herpes simplex virus 1 (HSV-1)	HSV-1 protein Us11 reduces type 1 interferon production due to PACT. Viral proteins block PACT interaction with PKR and/or RIG-I	114,115
Ebolavirus	Viral proteins VP30 and VP35 interfere with RNAi and interact with TRBP, PACT, and/or Dicer. PACT overexpression inhibits viral replication, while VP35 prevents PACT from activating RIG-I	110,116
Influenza	Viral proteins interact with PACT, resulting in increased viral replication	117
MERS-CoV	Viral protein 4a inhibited PACT activation of RIG-I, resulting in reduced interferon production	111
HIV-1	TRBP blocks PKR activation by HIV transcripts. HIV was suggested to disrupt RNAi by sequestering TRBP, but this is disputed	118–120
Viruses found not to depend on TRBP or PACT		
Sindbis virus; encephalomyocarditis virus (EMCV)	Overexpressing PACT had no effect on type I interferon response	113
EMCV; vesicular stomatitis virus (VSV); Sendai virus	Found no difference between PACT knockout and wild-type cells	121

TRBP, TAR RNA-binding protein; PACT, Protein Activator of PKR; RIG-I, retinoic acid-inducible gene I; RNAi, RNA interference.

PACT binds to the CTD of RIG-I.[7] There have been no reports of TRBP interacting with RIG-I.

Many unanswered questions surround the role of PACT in RIG-I activation. It is unclear under which conditions PACT activates RIG-I, and the mechanism is not known. To date, the interaction has been most studied in the context of Sendai virus,[7,110] a negative sense single-stranded RNA (ssRNA) virus that infects rodents. In this case, PACT can act to enhance activation of IRF3 and transcription of genes under the control of IFN-β. A similar effect was seen for Ebola virus (also a negative sense ssRNA virus), and overexpressing PACT was able to slow viral replication.[110] To counter this effect, Ebola virus encodes a protein that is reported to disrupt the interaction between PACT and both Dicer and RIG-I.[110,116] An earlier study found that PACT increased type 1 interferon production in response to Newcastle disease virus, which also has a negative sense ssRNA genome.[113] It now appears plausible that this effect was mediated by RIG-I, although it had not been identified at the time.

One hypothesis is that PACT can extend the range of ligands that can activate RIG-I. Overexpression of PACT did not enhance RIG-I activation by 5′-ppp dsRNA, but did enhance the response to the dsRNA analog poly-I:C.[7] However, further work will be needed to confirm this, and to disentangle the effects of the other RIG-I (and Dicer)-related helicases, MDA5 and LGP2. MDA5 functions similarly to RIG-I, but is activated by longer dsRNA, while LGP2 lacks the CARD signaling domains, and is thought to inhibit RNA sensing.[108] The CTDs of RIG-I, MDA5, and LGP2 are well conserved and so it is plausible that PACT could interact with all of them.

PACT Activates PKR, While TRBP Inhibits It

PKR is a kinase that acts to block translation in response to viral dsRNA, or to other cellular stresses such as oxidative stress, accumulation of misfolded proteins in the ER, or external signals from cytokines and growth factors.[122] It has low basal expression, but is strongly induced by interferon,[123] making it a 'late' responder to viruses.

PKR has two N-terminal type A dsRBDs and a kinase domain (Figure 4(b)). The first two domains bind dsRNA and act as a scaffold to bring PKR

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molecules close together, and increase the likelihood of dimerization of the kinase domain.[124] After dimerization the kinase domain autophosphorylates,[125] then phosphorylates eukaryotic translation initiation factor 2α (eIF2α), in turn blocking translation. The kinase domain is present in a number of other proteins that phosphorylate eIF2α in response to various stresses.[122] PKR function has been reviewed in more detail elsewhere.[122,124,126]

TRBP and PACT can regulate PKR: TRBP has an inhibitory effect, while PACT is a conditional activator[3,4,127] (Figure 4(b)). Domain swap and mutational analyses indicate that the first two dsRBDs of PACT and TRBP are functionally interchangeable, and interact with the dsRBDs of PKR.[9,36,128] There is some evidence that this interaction is independent of dsRNA, indicating that dsRBDs bind one another directly.[128] However, there is some disagreement on this issue,[129] and it has never been explicitly examined biophysically.

The differences between PACT and TRBP stem from their C-terminal dsRBD. In response to cellular stresses, PACT-D3 is phosphorylated at S246 and S287 by an unknown kinase (or kinases), leading to activation of PKR.[127] Two models have been proposed to explain how PACT enables PKR activation. First, binding of phosphorylated PACT-D3 to PKR could cause a conformational change that promotes activation.[9] Supporting this model, PACT-D3 alone has been reported to interact weakly with PKR.[9,130] Alternatively, PACT dimers might bind two molecules of PKR, enhancing PKR dimerization.[43] This is consistent with evidence that PACT-D3 phosphorylation promotes homodimerization and disfavors heterodimerization with TRBP.[42,43]

In contrast, TRBP-D3 has an inhibitory effect on PKR, which appears to be important for preventing inappropriate activation of PKR.[36,131] A similar inhibitory effect is also observed for truncated PKR or PACT constructs containing only their first two dsRBDs,[9,132] which suggests that rather than a specific effect of TRBP-D3, anything that binds to the dsRBDs of PKR and disrupts PKR dimerization will cause inhibition. To our knowledge, TRBP phosphorylation (discussed in section *Evidence from Biochemical and Biophysical Studies*) has not been studied in the context of PKR inhibition.

It is worth noting that many studies prior to 2009 used PACT constructs containing a frameshift mutation that replaces the last 13 amino acids (including part of a predicted helix) with 5 unrelated amino acids.[4,9,36,41,130,132–134] This mutant constitutively activated PKR and disrupted PACT-D3 dimerization.[131]

CONCLUSION

It is more than 15 years since TRBP and PACT were found to interact with PKR, and almost 10 years since their interaction with Dicer was uncovered. It is therefore somewhat surprising that (with a few exceptions[116,120]) there has been so little crosstalk between these two areas of study. RNAi is a key defense against viruses in plants and invertebrates, and while this is no longer the case in mammals,[135] it is intriguing that small RNA biogenesis and viral sensing are still linked through PACT and TRBP. It remains to be seen whether this is an evolutionary accident, or whether these two proteins play a genuine linking role that has yet to be discovered. Interestingly, the helicase domains of Dicer and RIG-I are from the same family, termed RIG-I-like helicases, which also hints at an evolutionary or functional link between the two processes.[112,136] A more mundane consequence is that results from *in vivo* experiments must always be interpreted with all the roles of TRBP and PACT in mind, even if the investigation is intended to focus on only one.

Although much has been learnt about the function of PACT and TRBP in miRNA biogenesis, two important questions remain unanswered: why does miRNA biogenesis utilize dsRBD-containing proteins, and why do vertebrates have two dsRBD-containing proteins which appear to be partially redundant? dsRBD-containing proteins interact with Dicer proteins in plants, insects, nematodes, and mammals, suggesting that there is some evolutionary pressure to conserve them. Results from *Drosophila* demonstrate that dsRBD-containing proteins may function at multiple steps in small RNA biogenesis pathways, rather than having a single role. While it is possible that this is also true for PACT and TRBP, it currently appears more likely that they function at the same step, potentially on different substrates. To work out which sequence or structural features lead to differential processing, it will likely be necessary to measure the affinities and processing rate of Dicer/TRBP and Dicer/PACT on a wider range of small RNAs. A complementary approach would be to examine on a genome-wide scale which miRNAs are differentially affected when TRBP or PACT are depleted from the cell.

Filling in the details of the role(s) of TRBP and PACT in miRNA biogenesis will help to unravel the diverse phenotypes associated with defective expression of these proteins and their homologs. These range from infertility and growth defects in mice and flies,[93,95,137] to early onset dystonia/Parkinson's disease and cancer in humans.[60,138] Biochemical and biophysical experiments must be complemented by cell and whole organism work to tease apart

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these complex conditions. Although misregulation of particular microRNAs may play a large role, it is plausible that additional functions for TRBP and PACT will emerge.

Note Added in Press

We would like to alert the reader to two important studies into TRBP and PACT that were published after submission of this review: Kim and colleagues produced knock-outs of TRBP and/or PACT in HeLa cells and found altered Dicer cleavage in a subset of pre-miRNAs, but no effect on steady-state miRNA levels or Dicer stability. They also showed hyperphosphorylation of TRBP by JNK during M phase. Wilson et al. reported the 3D structure of a fragment of Dicer in complex with TRBP domain 3. A Dicer mutant deficient for TRBP and PACT binding was shown to alter strand selection and iso-miR distribution for certain miRNAs. Please see Further Reading.

ACKNOWLEDGMENTS

The authors would like to acknowledge Herman Fung and Dr. Matthew Warner for critical reading of this manuscript. APH is a recipient of a PhD studentship from the Wellcome Trust-funded CIDCATS programme at the Univeristy of York (WT095024MA).

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