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Author(s)	Kok, KH; Ng, MHJ; Ching, YP; Jin, DY
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HUMAN TRBP AND PACT DIRECTLY INTERACT WITH EACH OTHER AND ASSOCIATE WITH DICER TO FACILITATE THE PRODUCTION OF SMALL INTERFERING RNA

Kin Hang Kok¹, Ming-Him James Ng¹, Yick-Pang Ching^{1,2} and Dong-Yan Jin^{1,*}

From the Departments of ¹Biochemistry and ²Anatomy, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong

Running title: Interaction of human TRBP, PACT and Dicer

* To whom correspondence should be addressed: Department of Biochemistry, The University of Hong Kong, 3/F Laboratory Block, Faculty of Medicine Building, 21 Sassoon Road, Pokfulam, Hong Kong. Phone: +852-2819-9491; Fax: +852-2855-1254; E-mail: dyjin@hkucc.hku.hk.

Mammalian Dicer interacts with double-stranded RNA binding protein TRBP or PACT to mediate RNA interference and microRNA processing. TRBP and PACT are structurally related, but exert opposite regulatory activities on protein kinase PKR. It is not understood whether TRBP and PACT are simultaneously required for Dicer. Here we show that TRBP directly interacts with PACT *in vitro* and in mammalian cells. TRBP and PACT form a triple complex with Dicer and facilitate the production of small interfering RNA (siRNA) by Dicer. Knockdown of both TRBP and PACT in cultured cells leads to significant inhibition of gene silencing mediated by short hairpin RNA, but not by siRNA, suggesting that TRBP and PACT function primarily at the step of siRNA production. Taken together, these findings indicate that human TRBP and PACT directly interact with each other and associate with Dicer to stimulate the cleavage of double-stranded or short hairpin RNA to siRNA. Our work significantly alters the current model for the assembly and function of the Dicer-containing complex that generates siRNA and microRNA in human.

RNA interference (RNAi) is an evolutionarily conserved mechanism for gene silencing mediated through small RNAs of approximately 22 nucleotides in

length (1). At least two classes of small RNAs have been described in mammals: microRNAs (miRNA) produced from hairpin precursors and small interfering RNAs (siRNAs) derived from long double-stranded RNAs (dsRNAs) (2). Both miRNAs and siRNAs are generated by RNase III-type nuclease Dicer and they are assembled into effector complex termed RNA-induced silencing complex (RISC) (3, 4). While two Dicer enzymes Dcr1 and Dcr2 have been found in fruit flies and are responsible for the generation of miRNAs and siRNAs, respectively, there exists only one single Dicer in humans, which produces both miRNAs and siRNAs (3). Dcr2 is known to associate with a dsRNA binding protein (dsRBP) termed R2D2, which binds to siRNA and facilitates its passage from Dcr2 to RISC (5, 6).

A new family of dsRBPs which includes Loquacious in *Drosophila* as well as TRBP and PACT in humans has recently been shown to interact with Dicer and be required for its function in RNAi (7-12). Unlike its counterparts in *Drosophila* that have only one dsRBP partner, human Dicer appears to associate with two closely related dsRBPs, TRBP and PACT (10-12). TRBP was originally identified and characterized by its high affinity for TAR, a hairpin RNA encoded by human immunodeficiency virus type 1 (13). PACT was initially cloned as a cellular protein activator of protein kinase PKR (14, 15). Although both PACT and

TRBP bind to PKR and have three similar dsRNA binding domains (dsRBDs), TRBP exerts an inhibitory effect on PKR (16-20).

While both TRBP and PACT interact with and support the function of human Dicer in RNAi (10-12), it is not understood whether their binding with Dicer is simultaneous or mutually exclusive. TRBP and PACT are closely related and both are capable of homodimerization (21, 22). In addition, they share two binding partners, PKR and Dicer (10-12, 16-20). This raises the possibility that TRBP and PACT might function as a protein complex in cells. In this study, we explored the direct interaction between TRBP and PACT as well as its impact on Dicer and RNA silencing.

EXPERIMENTAL PROCEDURES

Plasmids and siRNA – Human cDNAs for TRBP and PACT were derived from clones IRAUp969F1229 and DKFZp564I0123 obtained from RZPD (Berlin, Germany). Expression plasmids for TRBP and PACT were based on pcDNA3.1/V5-His (Invitrogen), pCMVTag3B (Stratagene), pGEX-4T1 (Amersham), pMAL-c2X (New England Biolabs) and pET-28a (Novagen). Coding sequences of CFP and YFP were PCR-amplified from plasmids pECFP-C1 and pEYFP-C1 (Clontech). Plasmid pshRL1776 expressing shRL has been described elsewhere (23). Expression vectors for shTRBP, shPACT and shGFP were constructed using pSHAG-1 (24) and pshRL1776 as template. siRL and siDicer were chemically synthesized as previously described (25, 26).

Protein Purification and Protein Analysis – GST-TRBP, MBP-TRBP, His-PACT and GST-PACT as well as the truncated forms of His-TRBP and His-PACT were expressed in *E. coli* BL21 (DE3) strain. GST fusion proteins were purified by glutathione-Sepharose column (Amersham) and eluted with 50 mM reduced glutathione. MBP-TRBP was purified through amylose resin (New England Biolabs) and eluted with

10 mM maltose solution. Polyhistidine-tagged proteins were purified through Ni-chelating column (Qiagen) and eluted with 200 mM imidazole. All fusion proteins were dialyzed overnight at 4°C. To remove contaminating RNA bound to TRBP/PACT, protein purification was performed in the presence of 200 µg of RNase A (Sigma). All proteins were purified to >90% homogeneity as verified by SDS-PAGE analysis. All preparations of purified proteins were free of contaminating RNA as measured by UV absorbance (OD₂₆₀). GST pull down, co-immunoprecipitation and Western blot analysis were carried out as described (27, 28).

Mouse monoclonal anti-V5 antibody was purchased from Invitrogen. Rabbit polyclonal anti-Myc serum was from Sigma. Mouse anti-Dicer and goat anti-T7 antibodies were from Abcam. A rabbit anti-TRBP serum was raised against purified recombinant GST-TRBP-A. Specificity of anti-TRBP antibodies was verified with lysate of TRBP-overexpressing HEK293T cells.

Fluorescence Resonance Energy Transfer (FRET) Imaging – Multi-color fluorescence microscopy was performed as previously described (29, 30). HeLa cells were transfected with pcDNA-CFP-TRBP, pcDNA-YFP-PACT, or both. Cells were fixed with methanol/acetone (1:1, v/v) at 24 hours post transfection. Fixed cells were excited either at 820 nm (for CFP excitation) or 488 nm (for YFP excitation) and signals were recorded at either 435-485 nm (signal for CFP) or 535-590 nm (signal for YFP) on a Zeiss LSM510 META laser scanning microscope. FRET signal was captured at 535-590 nm during excitation at 820 nm. FRET signal between CFP and YFP was corrected using Zeiss LSM software.

Protein Fractionation Analysis – Two C57 mouse testes were dissected and homogenized in 1 ml of MTPBS buffer with protease inhibitor (Roche) for 1 min on ice. Homogenized testes were centrifuged at 14,000 × g for 30 min at 4°C. Protein lysates were treated with RNase A at 50 µg/ml for

15 min at 4°C and then passed through 0.22 µm filter. Filtered lysates were loaded into Superdex 200 gel filtration column (Amersham) and were fractionated at 0.3 ml per min in MTPBS buffer. Fractions (1 ml each) were lyophilized overnight and redissolved in 100 µl of MTPBS buffer. Samples were analyzed by Western blotting with rabbit anti-TRBP, rabbit anti-PACT (Abcam) and mouse anti-Dicer (Abcam) antibodies.

In vitro Dicer Cleavage Assay – dsRNA was transcribed *in vitro* using T7 RNA polymerase (Ambion) and purified with RNeasy reagents (Qiagen). Excess amount of dsRNA was incubated with recombinant human Dicer (Stratagene or Ambion) for 16 hrs at 37°C. Cleavage products were purified by passing through G-25 (Amersham) and then YM-100 (Millipore) columns to remove salts and undigested dsRNA. Purified products were analyzed through urea polyacrylamide gel electrophoresis and stained with ethidium bromide. The purity of recombinant human DICER was verified by SDS-PAGE and a single discrete band of >200 kDa was seen.

Northern Blot Analysis – RNA was isolated from cultured cells with *mirVana* miRNA isolation kit (Ambion). RNA on denaturing polyacrylamide gel was transferred onto ZetaProbe membrane (BioRad) using a semi-dry Transblot apparatus (Hoefer). RNA on the membrane was crosslinked and then hybridized in ULTRAhyb-Oligo solution (Ambion) with ³²P-labeled RNA oligos that can hybridize to siRL.

Luciferase Assay – *Renilla* luciferase activity was determined as described (23, 31) using the Dual-Luciferase[®] reagents (Promega). Transfection efficiencies were normalized to a control plasmid expressing firefly luciferase.

RESULTS

TRBP and PACT Interact Directly with Each Other – Because TRBP and PACT are related both structurally and functionally, we

asked whether they bind to each other directly. We first performed GST pull down experiments with recombinant proteins purified from *E. coli*. We found that a polyhistidine-tagged PACT (His-PACT) bound with high affinity to GST-TRBP, but not to GST (Fig. 1A, lane 2 compared to lane 1, and lane 5 compared to lane 4). In agreement with the previous finding on homodimerization of PACT (22), we also observed the retention of His-PACT in the GST-PACT resin (Fig. 1A, lanes 3 and 6). To confirm the direct binding between TRBP and PACT, we employed another TRBP fusion protein MBP-TRBP. We noted that MBP-TRBP specifically bound to GST-PACT resin, but not to GST alone (Fig. 1B, lane 4 compared to lane 2). In keeping with the concept of TRBP homodimerization (21), homophilic interaction between GST-TRBP and MBP-TRBP was also seen in this experiment (Fig. 1B, lane 6 compared to lane 2). Thus, our results from *in vitro* affinity binding assay consistently support that TRBP and PACT can not only form homodimers, but also interact directly with each other.

TRBP and PACT contain three dsRBDs (Fig. 1C) that have differential activities in regulating PKR (17, 18) and in the formation of homodimers (21, 22). To determine the roles of the three dsRBDs in mediating TRBP-PACT interaction, we constructed two sets of truncated mutants and interrogated them for binding activities with TRBP or PACT using GST pull down assay. We found that PACT-A containing the first dsRBD was capable of binding to GST-TRBP (Fig. 1C, lane 1), while the interaction with GST-PACT was mediated by TRBP-A and TRBP-B corresponding to the first and second dsRBDs, respectively (Fig. 1C, lanes 4 and 5).

Next we carried out co-immunoprecipitation experiment to further investigate the interaction of TRBP with PACT in human cells. Immunoprecipitation and Western blotting were performed reciprocally with antibodies recognizing Myc-tagged TRBP (Myc-TRBP) and V5-

tagged PACT (V5-PACT). Since both Myc-TRBP and V5-PACT were found in the precipitates (Fig. 1D, lanes 4 and 8 compared to lanes 3 and 7, respectively), the two entities plausibly formed a protein complex inside human cells in culture. To rule out the possibility that TRBP and PACT associate as a result of lysate preparation, Myc-TRBP and V5-PACT were also expressed in separate cells. Precipitation was then performed immediately after mixing the lysates of different cells. Because similar results were also obtained in this setting (data not shown), the interaction of TRBP and PACT was unlikely an artifact generated during the preparation of cell lysates.

In a third approach, we used FRET imaging to analyze the interaction between TRBP and PACT. FRET imaging can detect the interaction between two proteins differentially fused with fluorescent tags. The FRET signal is emitted only when the two proteins are within a distance of less than 10 nm (32). In our experiment, both CFP-TRBP and YFP-PACT were found to localize in the cytoplasm (Fig. 2, panels 1 and 5). No FRET signal was measurable when CFP-TRBP alone or YFP-PACT alone was expressed (Fig. 2, panels 3 and 6). In contrast, when cells co-expressing CFP-TRBP and YFP-PACT were recorded simultaneously in the CFP, YFP and FRET channels, a significant FRET signal was observed (Fig. 2, panel 9). These results lent further support to the notion that TRBP and PACT interact directly with each other within human cells.

Formation of TRBP-PACT-Dicer Complex in vitro and in Cultured Cells – The direct interaction between TRBP and PACT (Fig. 1 and Fig. 2) as well as the association of TRBP/PACT with Dicer (7-12) prompted us to ask whether TRBP, PACT and Dicer might function as a protein complex. In this regard, while the roles of TRBP and PACT in RNAi have been well described (10-12), two important questions remain unanswered. First, since the association of PACT with Dicer has been demonstrated by co-fractionation and co-

immunoprecipitation only (12), it is not known whether PACT directly interacts with Dicer or through TRBP. Second, it is not understood whether TRBP and PACT bind to Dicer simultaneously or exclusively. To address these questions, we performed *in vitro* protein affinity binding assays using bacterially produced TRBP, PACT and Dicer (Fig. 3A). Because recombinant Dicer was retained in the GST-PACT-bound resin as efficiently as in the resin containing GST-TRBP (Fig. 3A, lane 1 compared to lane 4), the physical interaction between PACT and Dicer is direct and unlikely mediated through another protein. We then queried for the formation of triple complex by loading MBP-TRBP and Dicer sequentially to the GST-PACT resin. In this experiment, GST-PACT was first saturated with an excess of MBP-TRBP and the unbound MBP-TRBP was then removed by extensive washing (Fig. 3A, lane 2). Next, Dicer was added and found to be retained in the resin (Fig. 3A, lane 3). Likewise, when we saturated GST-TRBP resin with excess His-PACT, washed away all free His-PACT and incubated the GST-TRBP-His-PACT-bound resin with Dicer, Dicer was also retained in the resin (Fig. 3A, lane 6 compared to lane 5). Reciprocally, MBP-TRBP or His-PACT was found in the resin if the GST-PACT or GST-TRBP resin was first saturated with Dicer (data not shown). Collectively, our results are consistent with the formation of a stable triple complex of TRBP, PACT and Dicer.

To verify that a protein complex containing TRBP, PACT and Dicer is also formed in cultured human cells, we performed reciprocal immunoprecipitation and Western blotting (Fig. 3B). While both Dicer and V5-PACT were found in the anti-Myc precipitate that contained Myc-TRBP (Fig. 3B, lane 4 compared to lanes 1-3), both Dicer and Myc-TRBP were also detected in the precipitate containing V5-PACT (Fig. 3B, lane 8 compared to lanes 5-7). Thus, TRBP and PACT likely bound with Dicer as a functional complex.

We next compared the binding activities of truncated TRBP and PACT mutants (Fig.

3C). While the first two dsRBDs of TRBP (TRBP-A and TRBP-B) were responsible for interaction with TRBP and PACT, the third dsRBD (TRBP-C) was accounted for binding with Dicer. This is generally consistent with previous findings (8, 11). On the other hand, the first dsRBD of PACT (PACT-A) was necessary for binding to TRBP and PACT, whereas the second and third dsRBDs (PACT-B and PACT-C) were required for interaction with Dicer. Thus, TRBP and PACT used different domains to mediate their interaction with partner proteins. Interestingly, all truncated forms of TRBP and PACT were found to associate with Dicer in cultured cells, suggesting that some mutants incapable of binding with Dicer directly *in vitro* might interact with it indirectly through its TRBP or PACT partner. Moreover, when we pre-saturated recombinant Dicer with excess TRBP-C or PACT-C, other forms of TRBP or PACT was no longer able to bind with Dicer (data not shown), suggesting that TRBP and PACT likely compete for the same binding site(s) in Dicer.

Formation of TRBP-PACT-Dicer Complex in Mouse Testicular Tissue – Consistent with a previous report (33), we found that TRBP and PACT were abundantly expressed in male germ cells (data not shown). Thus, we investigated the *in vivo* formation of TRBP-PACT-Dicer complex in mouse testicular tissue. As a first step, the specificity of polyclonal antibodies against endogenous TRBP, PACT and Dicer, which we prepared or purchased, was verified with human and mouse tissues as well as transfected cells. All three antibodies reacted with target proteins in human and mouse tissues and cells in a highly specific manner (data not shown). We then fractionated extracts of mouse testes through Superdex 200 gel filtration column and analyzed the fractions by Western blotting with anti-TRBP, anti-PACT and anti-Dicer antibodies (Fig. 4A). TRBP, PACT and Dicer were detected in fractions 40-43, consistent with the presence of a TRBP-PACT-Dicer complex displaying a

molecular mass of more than 440 kDa. The detection of multiple TRBP or PACT species in mammalian cells was consistent with previous reports (10, 11, 34).

In keeping with the co-fractionation results, we also detected PACT and Dicer in the protein complex precipitated with anti-TRBP (Fig. 4B, lanes 3 and 7). Thus, our results suggested that endogenous TRBP, PACT and Dicer proteins associate to form a triple complex in mouse testicular tissue.

Roles of TRBP and PACT in Dicer-dependent Production of siRNA – The formation of a stable TRBP-PACT-Dicer complex led us to explore the influence of TRBP and PACT on Dicer function. In previous studies, comparison of the siRNA/miRNA-producing activities of TRBP-Dicer or PACT-Dicer with Dicer alone suggest that neither TRBP nor PACT is required for the RNA cleavage reaction catalyzed by Dicer (10, 12). However, a *Drosophila* homolog of TRBP and PACT named Loquacious can stimulate the specific pre-miRNA processing activity of Dicer-1 (8, 35). Moreover, a dsRBP partner of *Caenorhabditis elegans* Dicer termed RDE-4 also functions in concert with Dicer at the step of siRNA production (36). To clarify whether human TRBP, PACT or TRBP-PACT complex could facilitate RNA processing by Dicer, we monitored the cleavage of dsRL566, a 566-bp long dsRNA corresponding to a fragment of *Renilla* luciferase mRNA. In reactions containing the same amount of recombinant Dicer and escalating amounts of GST-TRBP, His-PACT or GST-TRBP plus His-PACT, a significant increase in the production of siRNA could be appreciated (Fig. 5). Notably, the increase in siRNA yield attributed to GST-TRBP plus His-PACT is comparable to the elevation ascribed to the same amount of GST-TRBP or His-PACT (Fig. 5, lanes 10-12 compared to lanes 4-6 and 7-9). These results indicate the facilitation of Dicer-mediated production of siRNA by TRBP and PACT. In addition, a protein complex consisted of TRBP and PACT is at least equally active in facilitating

the production of siRNA by Dicer when compared to TRBP or PACT alone.

In order to assess the roles of endogenous TRBP and PACT in RNA silencing in human cells, we knocked down the expression of TRBP or PACT effectively and specifically using short hairpin RNAs (shRNAs) in HEK293T cells (Fig. 6A and 6B). We then performed Northern blot analysis to confirm that the production of siRNAs corresponding to the *Renilla* luciferase mRNA (siRL) was significantly diminished in TRBP- or PACT-depleted cells (Fig. 6C, lanes 3 and 4 compared to lane 2). Notably, the combined effect of shTRBP and shPACT was even more pronounced than that of shTRBP or shPACT alone (Fig. 6C, lane 5 compared to lanes 3 and 4). Consistent with these results and with previous reports (10-12), depletion of TRBP or PACT by shRNAs also led to substantial inhibition of the gene-silencing activity of an shRNA targeting *Renilla* luciferase mRNA (shRL), which has been shown (23) to be highly effective and specific in blocking the expression of *Renilla* luciferase (Fig. 6D, columns 3-5 compared to columns 1 and 2, and columns 8-10 compared to columns 6 and 7). The observed effect was unlikely due to non-specific saturation of the RNAi machinery by shRNA, since shGFP at the highest dose had no influence on shRL activity (Fig. 6D, column 16). The effectiveness of this shGFP in silencing GFP expression has been shown previously (23) and its abundant expression in HEK293T cells was confirmed by Western blotting (data not shown). Again, the combined effect of shTRBP and shPACT was greater than that of shTRBP or shPACT alone (Fig. 6D, columns 13-15 compared to columns 3-5 and 8-10). This was most evident at the lowest dose of shRNAs. In fact, the inhibitory activity of shTRBP + shPACT at the lowest dose was significantly higher than that of shTRBP alone or shPACT alone at the same dose (Fig. 6D, columns highlighted by # and *; $P < 0.005$ by *t*-test). Thus, simultaneous knockdown of TRBP and PACT in cultured human cells

had a significant impact on RNAi. In other words, TRBP and PACT likely cooperate with each other in facilitating the execution of RNA silencing.

To address the concern of other non-specific off-target effects, we performed additional control experiments by adding back TRBP or PACT. Because enforced re-expression of TRBP was able to reverse the effect induced by shTRBP at the highest dose (Fig. 6D, column 17 compared to column 5), the inhibition of shRL-induced silencing by shTRBP was specific. Likewise, reversal of shPACT-induced inhibition by PACT (Fig. 6D, column 18 compared to column 10) verified the specificity of effect.

In contrast to the above results obtained with shRL, depletion of endogenous TRBP and/or PACT in HEK293T by shTRBP/shPACT had no influence on the silencing of *Renilla* luciferase expression induced by siRL, a synthetic siRNA targeting the same sequence on *Renilla* luciferase mRNA as shRL (Fig. 6E). As a positive control, depletion of Dicer by siRNA was effective in rescuing the siRL activity significantly (Fig. 6E, column 16), as demonstrated previously by others (26). Our results are surprising since TRBP and PACT have previously been shown to be required for siRNA-mediated silencing (10, 12). Nevertheless, taking into account the effects of shTRBP/shPACT on shRL (Fig. 6D) and siRL (Fig. 6E), we argued that TRBP and PACT function primarily at the step of siRNA production.

DISCUSSION

In this study, we established the direct interaction between human dsRBPs TRBP and PACT by using *in vitro* affinity binding assay (Fig. 1). This interaction was further verified by co-immunoprecipitation and FRET imaging (Fig. 1 and Fig 2). In addition, we demonstrated the direct interaction of PACT with Dicer and the formation of a TRBP-PACT-Dicer complex *in vitro*, in cultured human cells and in mouse testicular tissue (Fig. 3 and Fig. 4).

Finally, we provided the first evidence that human Dicer associated with TRBP and PACT is more active than Dicer alone in processing long dsRNA into siRNAs (Fig. 5). Accordingly, depletion of endogenous TRBP and PACT resulted in an inhibition of shRNA-induced gene silencing primarily at the step of siRNA production (Fig. 6). Our findings suggest a new model for the assembly and function of human Dicer complex in which TRBP and PACT directly interact with each other and bind simultaneously to Dicer to facilitate the production of siRNA (Fig. 7).

While more than one Dicer have been found in *Drosophila* and plants (1, 37, 38), there is one single Dicer in humans and it is distinct from other members in the family of Dicer-like proteins by associating with two dsRBPs, TRBP and PACT (10-12). Our demonstration of the direct interaction between TRBP and PACT suggests another level of complexity in the regulation of RNAi in human cells. This is the first example of an interaction between two Dicer-associated dsRBPs. TRBP and PACT are two members in the family of dsRBPs that have diverse cellular functions (39). Several other dsRBPs have also been known to interact with Dicer-like proteins or with other RNase III endonucleases such as Drosha (5-8, 35, 40). Thus, it will be of interest to determine whether any of these dsRBPs might be binding partners. We demonstrated that TRBP and PACT use different domains to interact with each other and with Dicer (Fig. 3C). Noteworthy, all truncated mutants of TRBP and PACT can directly or indirectly bind with Dicer within cultured cells. Since there exist multiple naturally occurring isoforms of TRBP and PACT that contain only the first one or two dsRBDs (8, 11), further characterization of these isoforms for influence on Dicer function is warranted. In addition, because TRBP and PACT use the same dsRBD(s) to mediate homodimerization and interaction with each other (Fig. 3C), it is possible that TRBP and PACT function as a stable heterodimer *in vitro* and *in vivo*. Dicer has

been shown to form an intramolecular pseudodimer (41). Thus, additional biochemical and biophysical analyses are required to elucidate the protein stoichiometry and structure of TRBP-PACT-Dicer complex. In this regard, it will also be of importance to compare the specific activities of TRBP and PACT homo- and heterodimers in terms of dsRNA-binding, interaction with Dicer and modulation of PKR.

Both TRBP and PACT have been shown to interact with PKR and to regulate its activity (14-20). Moreover, their interaction with PKR has functional implications in inflammation, stress response, viral infection and oncogenesis (20, 34, 42-44). While TRBP and PACT directly interact with each other (Fig. 1), it remains to be understood whether they associate *in vivo* with PKR as a TRBP-PACT heterodimer and how a TRBP-PACT complex might influence PKR activity. TRBP and/or PACT are also critically involved in HIV biology (13, 21, 43, 45), transcriptional gene silencing (46), translational control (33, 44) and ear development (47). Plausibly, the interaction between TRBP and PACT may provide a platform or a common regulatory point for multiple pathways including RNA silencing and PKR signaling.

While TRBP and PACT have been shown to be necessary for RNA silencing in mammalian cells (10-12), their exact roles in Dicer and RISC function are obscure. While one report has suggested that depletion of TRBP by siRNAs reduces the level of mature miRNA (10), in another study this reduction has not been observed and TRBP depletion has been demonstrated to affect pre-miRNA processing *in vitro* (11). We showed that recombinant Dicer associated with TRBP and/or PACT is more active than Dicer alone in cleaving dsRNA into siRNAs (Fig. 5). Consistent with these results, we observed that knockdown of TRBP and PACT suppressed siRNA production and shRNA-mediated gene silencing in cultured cells, but had no influence on siRNA-induced silencing (Fig. 6). While we do not

exclude the possibilities that TRBP and PACT are required for other aspects of Dicer function and RISC assembly as previously shown by others (10-12), our data suggest that they function primarily in facilitating the production of siRNAs. Although the knockdown of Dicer could effectively rescue the siRNA-induced silencing effect in our experiment (Fig. 6), at this point we still do not understand fully whether the use of a highly effective siRNA and a different cell line could possibly account for the discrepancies between our results and those from other groups (10-12, 48, 49). However, our finding that purified TRBP and PACT proteins facilitate the cleavage of dsRNA by Dicer (Fig. 5) is generally consistent with the previous observations that depletion of TRBP inhibits pre-miRNA processing *in vitro* (11) and that Loquacious *in Drosophila* stimulates pre-miRNA processing by Dicer-

1 (7, 35). This function of TRBP and PACT is also reminiscent of the role of Dicer-associated RDE-4 in *C. elegans* (36, 50). We are currently in the process of comparing the pre-miRNA-cleaving activities of Dicer and TRBP-PACT-Dicer. This and other experiments will help clarify the roles of TRBP and PACT in siRNA and miRNA production.

Our *in vitro* Dicer cleavage experiments did not show cooperation between TRBP and PACT (Fig. 5), whereas the knockdown experiments indicated a synergistic effect (Fig. 6). One possibility is that other proteins or factors in the cell could be required for the cooperation of TRBP with PACT. Alternatively, the protein concentrations used in the reconstituted cleavage reaction might not be optimal for the cooperation to occur. Further experiments are required to clarify the discrepancy.

FOOTNOTES

The abbreviations used are: dsRNA, double-stranded RNA; dsRBD, dsRNA binding domain; dsRBP, dsRNA binding protein; miRNA, microRNA; RNAi, RNA interference; RISC, RNA-induced silencing complex; shRNA, short hairpin RNA; siRNA, small interfering RNA; FRET, fluorescence resonance energy transfer; MBP, maltose binding protein; GST, glutathione S transferase;

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FIGURE LEGENDS

FIGURE 1. TRBP interacts directly with PACT. *A*, binding between GST-TRBP and His-PACT. Indicated GST fusion proteins were mixed with His-PACT (lane 1-6) at 4°C for 4 hrs. Proteins bound to glutathione-Sepharose beads were washed with 0.2 M (lanes 1-3) or 0.5 M NaCl (lanes 4-6) and then eluted with SDS loading buffer. Eluted proteins and 5% of the input proteins were analyzed by Western blotting with anti-His (α -His) and anti-GST (α -GST) antibodies. GST-T: GST-TRBP. GST-P: GST-PACT. His-P: His-PACT. *B*, binding between GST-PACT and MBP-TRBP. Indicated GST fusion proteins were mixed with MBP-TRBP (MBP-T; lanes 2, 4 and 6) or His-PACT (His-P; lanes 1, 3 and 5). Proteins bound to glutathione-Sepharose beads were washed with 0.5 M NaCl, eluted and analyzed with anti-His (α -His) and anti-MBP (α -MBP) antibodies as in *A*. *C*, domain mapping. The three dsRBDs in TRBP and PACT were depicted. GST pull down was carried out with the indicated GST fusion and His-tagged proteins as in *A*. Western blotting was performed with anti-His antibodies. TRBP-A/B/C contain amino acids 1-98, 104-227 and 234-359 of TRBP, while PACT-A/B/C correspond to amino acids 1-120, 133-234 and 247-314 of PACT. *D*, reciprocal co-immunoprecipitation. HEK293T cells were transfected with plasmids expressing the indicated combinations of Myc-TRBP (Myc-T) and V5-PACT (V5-P) proteins. Co-immunoprecipitation was carried out in the presence of RNase A (25 μ g/ml) with anti-Myc (Myc-IP; lanes 3 and 4) and anti-V5 antibodies (V5-IP; lanes 7 and 8). Input proteins and immunoprecipitates were analyzed by Western blotting with anti-Myc (α -Myc) and anti-V5 (α -V5) antibodies. Arrows point to the heavy chain of immunoglobulins (Ig). Similar results were obtained when Myc-T and V5-P proteins were expressed in separate cells and the lysates of different cells were mixed immediately before immunoprecipitation was carried out (data not shown).

FIGURE 2. FRET analysis of the interaction between TRBP and PACT in HeLa cells. Cells were transfected to express CFP-TRBP (panels 1-3), YFP-PACT (panels 4-6) and CFP-TRBP + YFP-PACT (lanes 7-9). In CFP channel, cells were excited at 820 nm and emission was recorded at 435-485 nm (panels 1, 4 and 7). In YFP channel, cells were excited at 488 nm and emission was recorded at 535-590 nm (panels 2, 5 and 8). When cells were excited at 820 nm, corrected FRET signal was simultaneously recorded at 535-590 nm (panels 3, 6 and 9). Results were representative of 100 transfected cells. Bar, 20 μ M.

FIGURE 3. Formation of a triple complex of TRBP, PACT and Dicer *in vitro* and in cultured cells. *A*, triple complex formation *in vitro*. The indicated combinations of proteins were loaded onto glutathione-Sepharose beads. In lanes 3 and 6, MBP-TRBP/His-PACT and Dicer were loaded sequentially and unbound proteins were removed by extensive washing with 0.5 M NaCl. Bound proteins were eluted and analyzed by Western blotting with anti-Dicer, anti-MBP, anti-His and anti-GST antibodies. MBP-T: MBP-TRBP. His-P: His-PACT. GST-P: GST-PACT. GST-T: GST-TRBP. *B*, triple complex formation in cultured cells. HEK293T cells were co-transfected with plasmids expressing the indicated combinations of T7-Dicer, Myc-TRBP (Myc-T) and V5-PACT (V5-P) proteins. Co-immunoprecipitation was carried out in the presence of RNase A (25 μ g/ml) with anti-Myc (Myc-IP; lanes 3-4) and anti-V5 (V5-IP; lanes 7-8) antibodies. Input proteins and immunoprecipitates were analyzed by Western blotting with anti-T7 (upper panel), anti-Myc (middle panel) and anti-V5 (lower panel). Target proteins are highlighted with “*”, “#” and “▶”. Arrows point to the heavy chain of immunoglobulins. Similar results were obtained when Myc-T, V5-P and T7-Dicer proteins were expressed in separate cells and the lysates of different cells were mixed immediately before immunoprecipitation was carried out (data not shown). *C*, summary of

protein-protein interactions. All interactions were based on *in vitro* GST pull down assay except that the binding to Dicer in cells was defined by co-immunoprecipitation of proteins from extracts of transfected HeLa cells.

FIGURE 4. Formation of a triple complex of TRBP, PACT and Dicer in mouse testicular tissue. *A*, fractionation analysis. Mouse testes were fractionated by Superdex 200 gel filtration and column fractions were analyzed by Western blotting with anti-TRBP (α -T), anti-PACT (α -P) and anti-Dicer (α -D) antibodies. Similar results were obtained when protein complex was first pulled down with anti-PACT or anti-TRBP (data not shown). *B*, co-immunoprecipitation. Extracts of mouse testes were co-immunoprecipitated with anti-TRBP. Input proteins, anti-TRBP serum and immunoprecipitates were analyzed by Western blotting with the indicated antibodies.

FIGURE 5. TRBP and PACT facilitate siRNA production by Dicer. *A*, *in vitro* Dicer cleavage assay. *In vitro* transcribed dsRNA of 566 bp in length (dsRL566; 5 μ g) was incubated with 2 units of recombinant Dicer for 16 hours at 37°C (lane 2). Increasing amounts of GST-TRBP (lanes 4-6; 0.2, 0.5 and 1 μ M), His-PACT (lanes 7-9; 0.2, 0.5 and 1 μ M) and GST-TRBP plus His-PACT (lanes 10-12; 0.2, 0.5 and 1 μ M) were added into the reactions. Cleaved RNA was analyzed by 15% urea polyacrylamide gel electrophoresis. An inverted gel image was shown and the arrow points to siRL product. Lane 1 contains 40 pmols of synthetic siRL as a positive control. *B*, quantitative analysis of results shown in *A*. Intensity of bands was quantified by Axo software, version 2.1. The readout from Dicer alone (lane 3) was taken as 100%. Values shown represent means \pm SD from three independent experiments.

FIGURE 6. TRBP and PACT are required for shRNA-mediated gene silencing. *A* and *B*, knockdown of TRBP and PACT expression by shRNA. HEK293T cells were transfected with Myc-TRBP or V5-PACT expression plasmid plus an empty vector (mock; lane 1) or plus expression vector for shTRBP, shPACT or shGFP (lanes 2-4). Expression of TRBP and PACT was determined by Western blotting with anti-Myc or anti-V5. *C*, Northern blot analysis of siRL production. HEK293T cells were transfected with shRL (lanes 2-5; 8 μ g) and shTRBP (lane 3; 8 μ g), shPACT (lane 4; 8 μ g), or shTRBP plus shPACT (lane 5, 4 μ g each). 32 P-end-labeled RNA oligos were used for hybridization with shRL precursor (~ 70 bp) and siRL cleavage product (~ 21 bp). RNA (20 μ g per lane) was analyzed by 12% denaturing polyacrylamide gel electrophoresis. Lane 1 contains 1 pmol of synthetic siRL as a positive control. *D*, influence of TRBP and PACT knockdown on shRNA activity. HEK293T cells were transfected with pRLCMV (20 ng), pGL3-control (20 ng), shRL (lanes 2-5, 7-10 and 12-15; 15 ng) and escalating amounts of shTRBP (lanes 3-5; 20, 50 and 100 ng), shPACT (lanes 8-10; 20, 50 and 100 ng) or shTRBP plus shPACT (lanes 13-15; 20, 50 and 100 ng). Lane 16 contains shRL (15 ng) and shGFP (100 ng) as a control for non-specific shRNA. Lane 17 is the same as lane 5 except that cells were co-transfected with pcDNA-TRBP (200 ng). Lane 18 is the same as lane 10 except that cells were co-transfected with pcDNA-PACT (200 ng). *Renilla* luciferase activity was normalized to firefly luciferase activity. Readouts from no shRNA controls (lanes 1, 6 and 11) were set to 100%. Results represent three independent experiments and error bars indicate SD. The difference between lane 13 and lane 3 (*) or between lane 13 and lane 8 (#) was statistically significant ($P < 0.005$ by *t*-test). *E*, influence of TRBP and PACT knockdown on siRNA activity. The experimental setting was the same as in *A*, except that 20 nM of siRL was added instead of shRL. Lane 16 contains siRL (20 nM) and siDicer (20 nM) as a positive control.

FIGURE 7. A working model for the interaction of TRBP, PACT and Dicer. TRBP (T) and PACT (P) directly interact with each other and associate with Dicer. This triple complex mediated the production of siRNA from either dsRNA or shRNA.

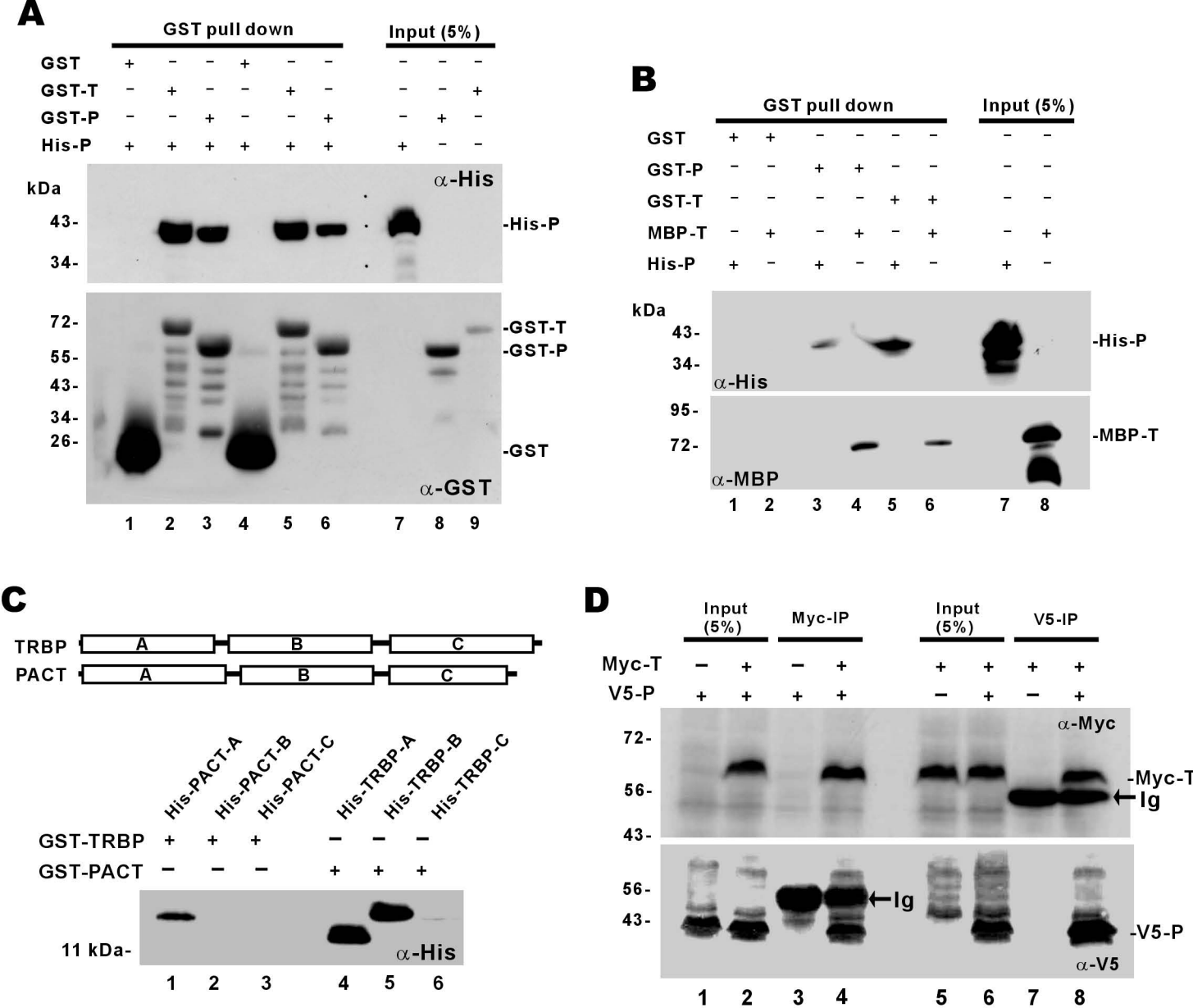


Figure 1

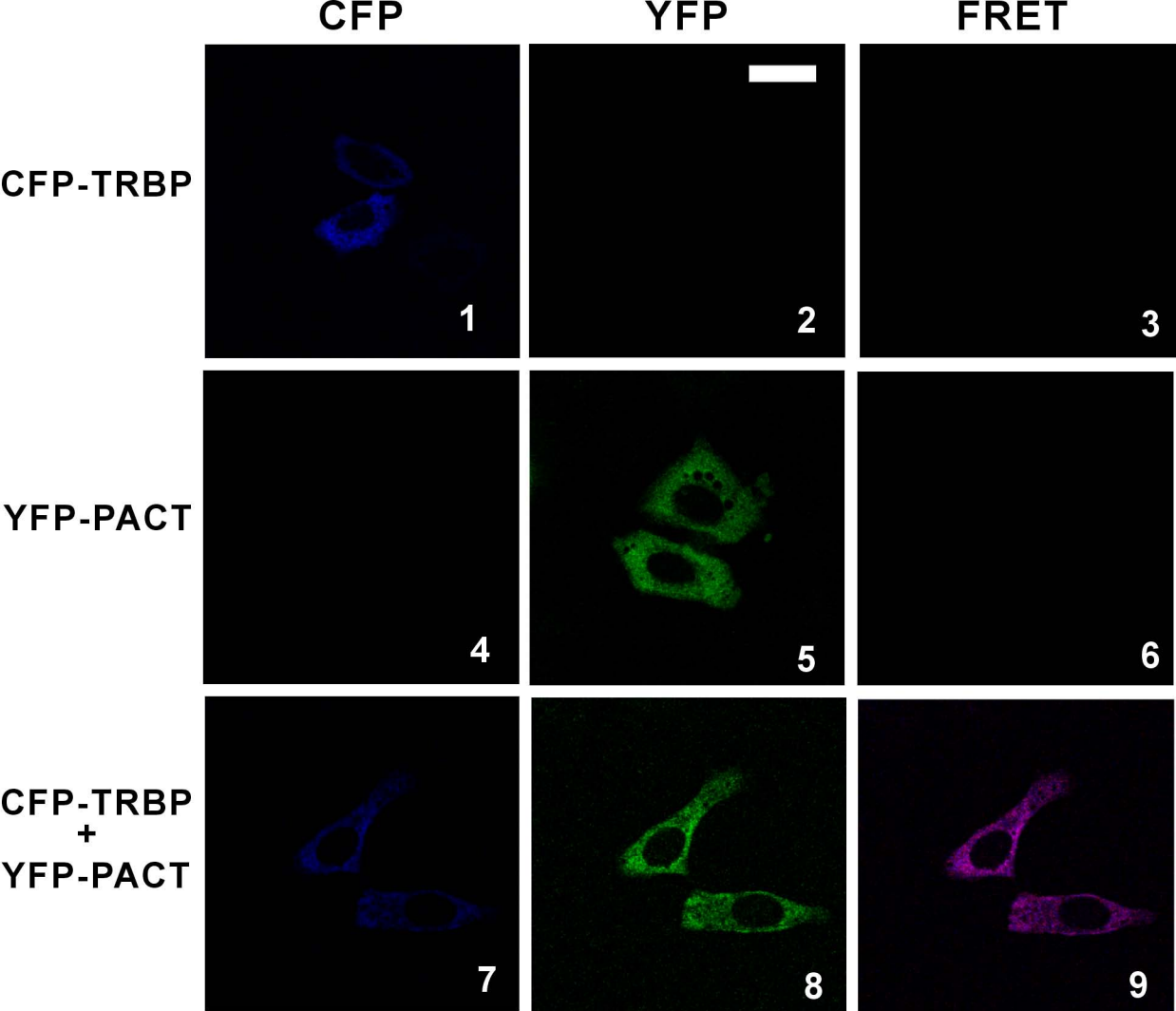
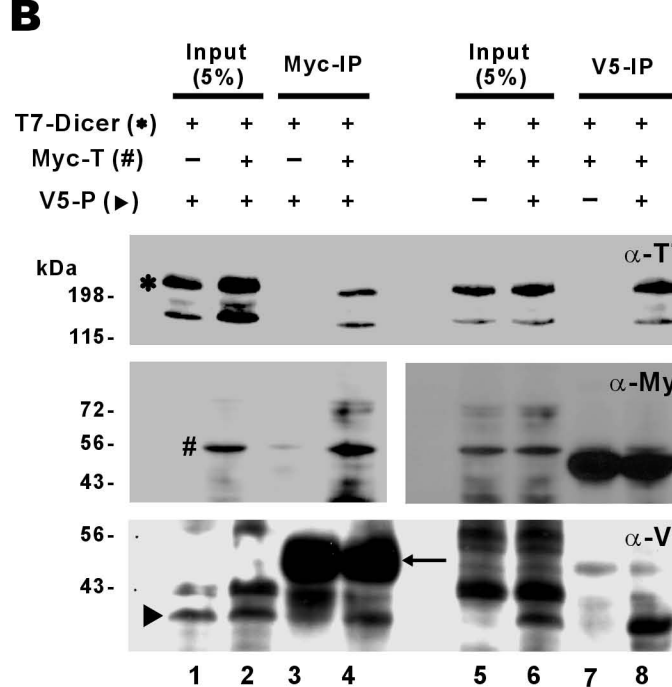
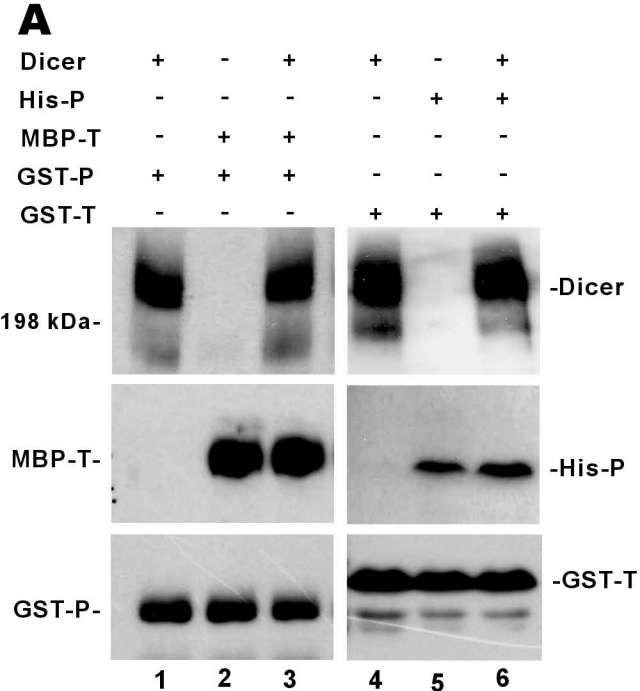


Figure 2

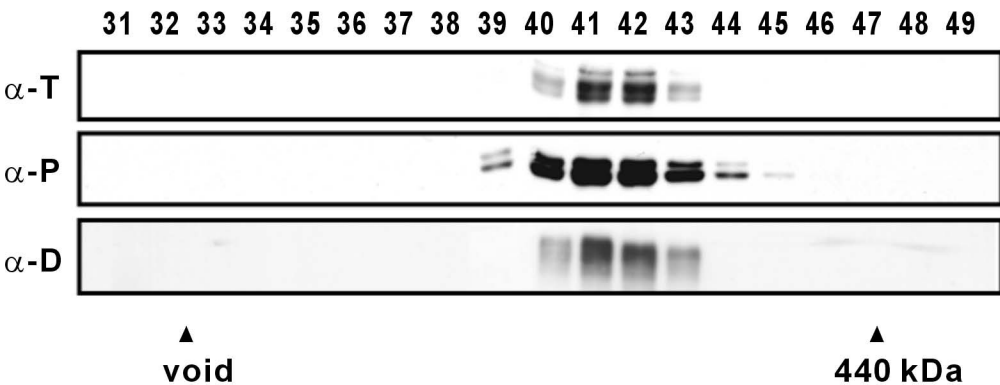
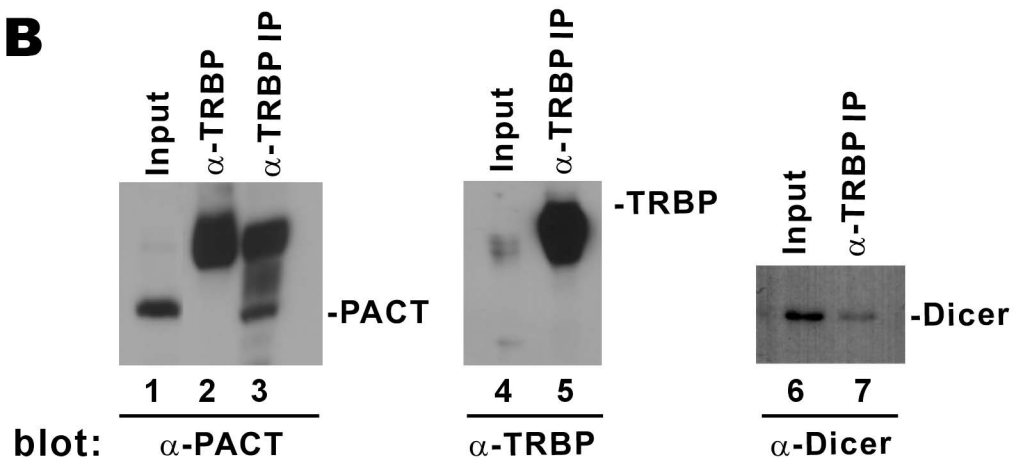


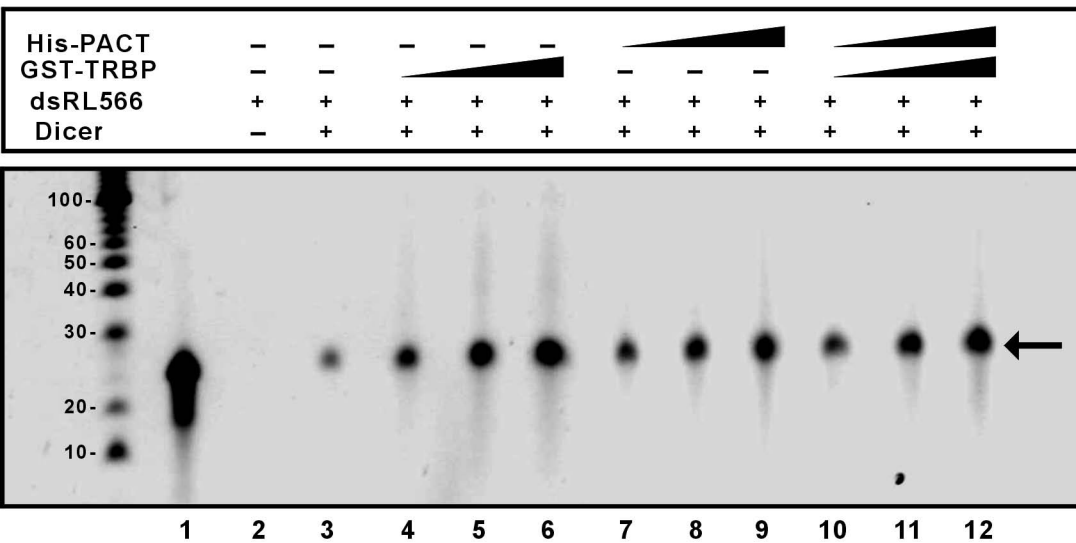
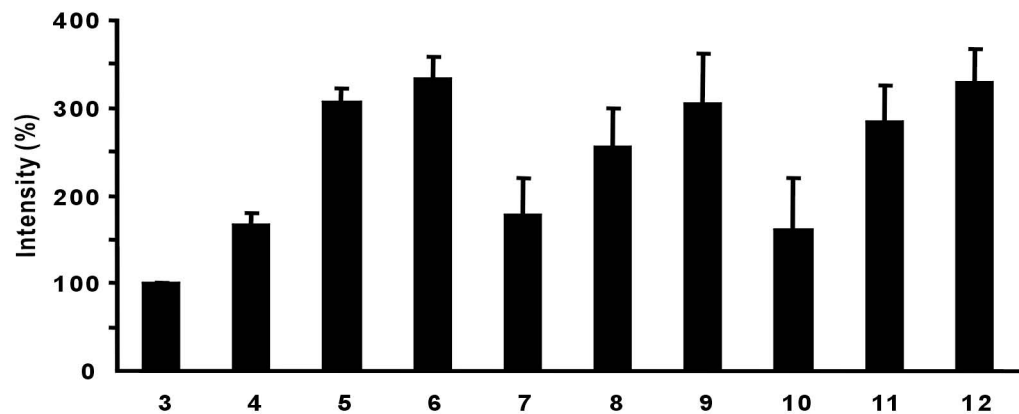
C

	TRBP	PACT	Dicer (<i>in vitro</i>)	Dicer (<i>in cells</i>)
TRBP	+	+	+	+
TRBP-A	+	+	-	+
TRBP-B	+	+	-	+
TRBP-C	-	-	+	+

	TRBP	PACT	Dicer (<i>in vitro</i>)	Dicer (<i>in cells</i>)
PACT	+	+	+	+
PACT-A	+	+	-	+
PACT-B	-	-	+	+
PACT-C	-	-	+	+

Figure 3

A**B****Figure 4**

A**B****Figure 5**

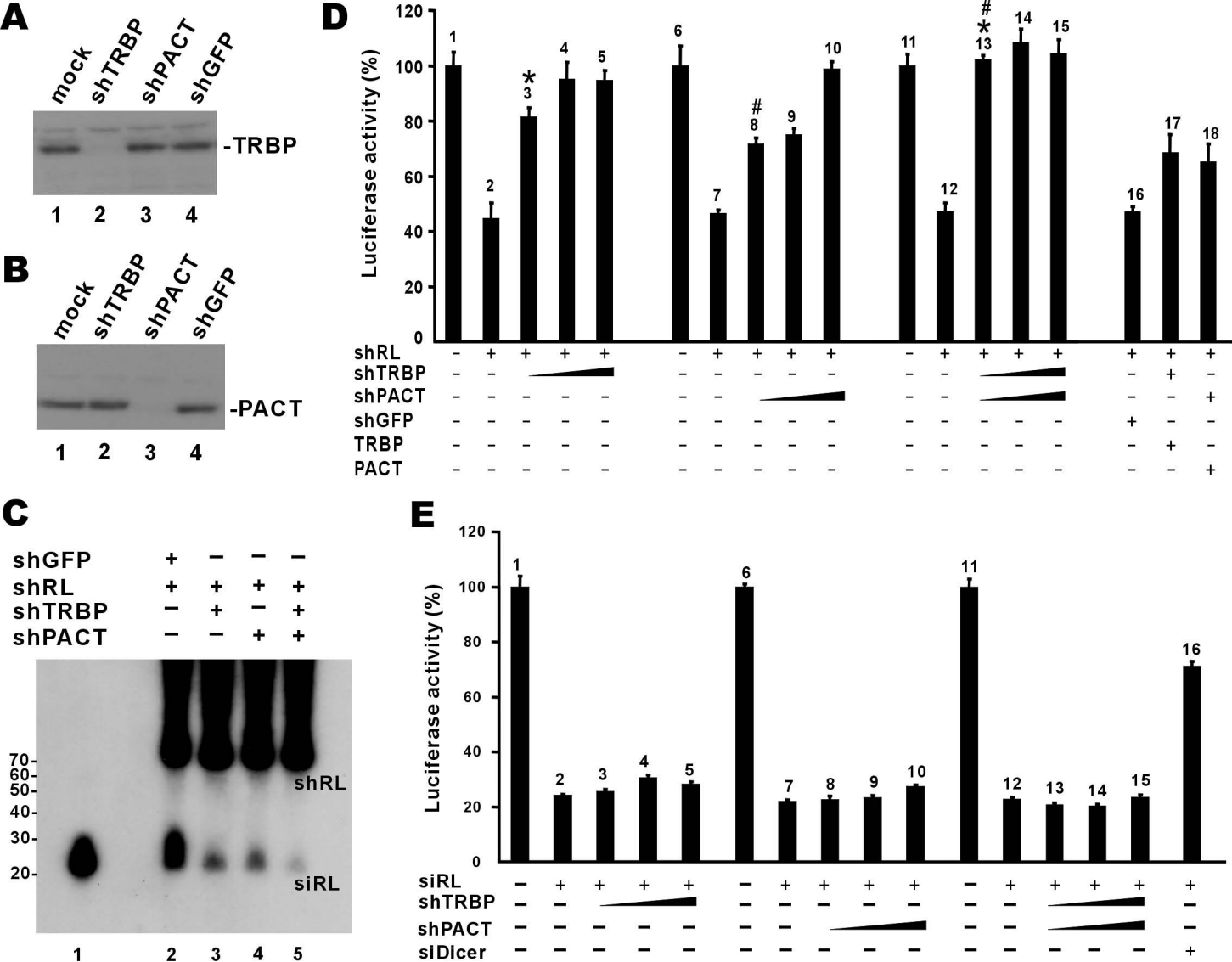


Figure 6

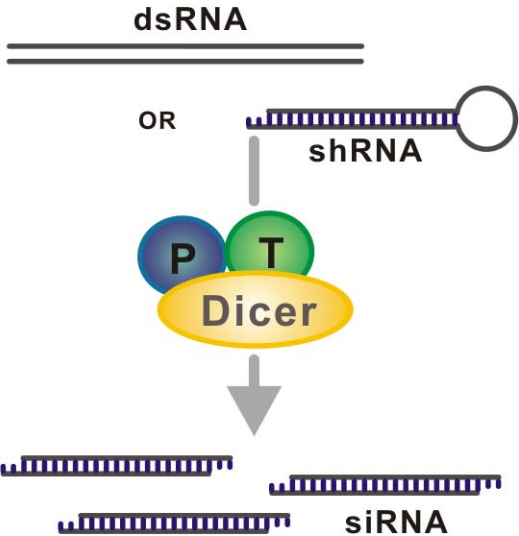


Figure 7