Interaction of human tRNA-dihydouridine synthase-2 with interferon-induced protein kinase PKR

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ABSTRACT
PKR is an interferon (IFN)-induced protein kinase, which is involved in regulation of antiviral innate immunity, stress signaling, cell proliferation and programmed cell death. Although a low amount of PKR is expressed ubiquitously in all cell types in the absence of IFNs, PKR expression is induced at transcriptional level by IFN. PKR's enzymatic activity is activated by its binding to one of its activators. Double-stranded (ds) RNA, protein activator PACT and heparin are the three known activators of PKR. Activation of PKR in cells leads to a general block in protein synthesis due to phosphorylation of eIF2α on serine 51 by PKR. PKR activation is regulated very tightly in mammalian cells and a prolonged activation of PKR leads to apoptosis. Thus, positive and negative regulation of PKR activation is important for cell viability and function. The studies presented here describe human dihydouridine synthase-2 (hDUS2) as a novel regulator of PKR. We originally identified hDUS2 as a protein interacting with PACT in a yeast two-hybrid screen. Further characterization revealed that hDUS2 also interacts with PKR through its dsRNA binding/dimerization domain and inhibits its kinase activity. Our results suggest that hDUS2 may act as a novel inhibitor of PKR in cells.

INTRODUCTION
Interferons (IFNs) are cytokines with antiviral, antiproliferative and immunomodulatory properties, which they exert by inducing synthesis of several proteins (1,2). The IFN-induced, dsRNA-activated protein kinase PKR, a serine/threonine kinase, is a major mediator of the antiproliferative and antiviral actions of IFN (3–6). Although induced at the transcriptional level by IFNs, PKR is present at a low, basal level in most cell types (7). PKR’s kinase activity stays latent until it binds to one of its three known activators, double-stranded (ds) RNA, heparin and the protein activator PACT. In virally infected cells, dsRNA produced during viral replication or viral RNAs with extensive ds regions serve as PKR activators (8,9). Polyanionic agents such as heparin also activate PKR both in vitro (10) and in vivo (11). In addition, PACT is a cellular, protein activator of PKR, which heterodimerizes with PKR and activates it in the absence of dsRNA (12,13), thereby playing an important role in PKR activation in response to stress signals (14). The α-subunit of the eukaryotic protein synthesis initiation factor eIF-2 (eIF2α) is the most studied physiological substrate of PKR. Phosphorylation of eIF2α on Ser51 by PKR leads to an inhibition of protein synthesis (15,16). In addition to its central role in antiviral activity of IFNs, PKR is also involved in the regulation of apoptosis (17,18), cell proliferation (4,5), signal transduction (19–21) and differentiation (22,23).

The dsRNA-mediated activation of PKR has been characterized in detail (24–29). The dsRNA-binding domain (dsRBD) of PKR is composed of two copies of the dsRNA-binding motif (dsRBM), a sequence motif conserved in many RNA-binding proteins (30,31). Binding of dsRNA to PKR through these motifs causes a conformational change (32,33) that leads to an unmasking of the ATP-binding site in the kinase domain and results in autophosphorylation of PKR on several sites (34–36). The domains that are involved in dsRNA binding are also involved in mediating dimerization of PKR, which is essential for its kinase activity in the presence of dsRNA (37–40). Although the same domain mediates PKR’s dsRNA binding and dimerization, distinct residues have been identified that contribute to one or both of these properties (40). Heparin binds to PKR at a site that is nonoverlapping with PKR’s dsRBD and leads to PKR activation (41,42). Activation of PKR by heparin in vascular smooth muscle cells leads to an
arrest in cell cycle progression by causing elevation in p27kip1 protein levels, inhibition of Cdk2 activity and Rb phosphorylation (43). PACT interacts with PKR by binding to its dsRBD in a dsRNA-independent manner and activates PKR in response to cellular stress (14). Stress signals lead to phosphorylation of PACT at serine 287, which causes its higher affinity association with PKR leading to PKR activation, eIF2α phosphorylation and consequent inhibition of translation (44). PACT has three copies of dsRNA binding/dimerization motifs and the two amino-terminal copies are required for high-affinity binding to PKR (45). The third, carboxy-terminal motif is required for PKR activation presumably by making a direct contact with PKR’s catalytic domain (45,46).

Several cellular and viral inhibitors of PKR have been identified (47). P58IPK, the trans-activation response (TAR) RNA-binding protein (TRBP), nucleophosmin (NPM) and several virally encoded proteins inhibit PKR activity. Since PKR activation leading to eIF2α phosphorylation and inhibition of translation would be detrimental to viral replication, various viruses have developed efficient mechanisms to inhibit PKR activation (9,48,49). In this article, we report the identification of hDUS2 as a novel cellular PKR inhibitor. hDUS2 was identified as a PKR- and PACT-interacting protein using PACT as a bait protein in yeast two-hybrid screen. hDUS2 was reported recently to be the human homolog of Saccharomyces cerevisiae dihydrouridine synthase 2 (Dus2) enzyme (50). It was also shown to possess rRNA-dihydouridine synthase (DUS) activity. hDUS2 has one copy of the conserved dsRBM and our results indicate that hDUS2 interacts with PKR and PACT via its dsRBM. Binding of hDUS2 with PKR resulted in an inhibition of PKR activity both in vitro as well as in mammalian cells. Furthermore, hDUS2 overexpression inhibited stress-induced apoptosis in HT1080 cells indicating that it acts as an important negative regulator of PKR activity in cells.

**MATERIALS AND METHODS**

**Yeast two-hybrid screening for PACT-interacting proteins**

The PACT coding region expressed as a GAL DNA-binding domain fusion protein was used as bait. A total of 3 million transformants from a human placenta match."
At 24 h post-transfection, cell extracts were prepared. Cells were washed in ice-cold PBS and collected by centrifugation at 600 g for 5 min. They were lyzed by addition of an equal volume of lysis buffer (20 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 100 U/ml aprotinin, 0.2 mM phenylmethanesulfonyl fluoride, 20% glycerol, 1% Triton X-100). The lysates were centrifuged at 10 000 g for 5 min and the supernatants were collected as total cell extract. One hundred micrograms of total protein was used to immunoprecipitate flag-hDUS2 with anti-flag mAb–agarose in immunoprecipitation buffer (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 100 U/ml aprotinin, 0.2 mM phenylmethanesulfonyl fluoride, 20% glycerol, 1% Triton X-100). The agarose beads were washed four times with 500 μl of IP buffer each time. The proteins bound to the beads were then analyzed by western blot analysis with the anti-HA tag and anti-flag tag monoclonal antibodies (Sigma).

PKR kinase activity assay

PKR activity assays were performed using an anti-PKR monoclonal antibody (R & D systems; 71/10). HeLa M cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum. The cells were harvested when they were at 70% confluency. Cells were washed in ice-cold PBS and collected by centrifugation at 600 g for 5 min. They were lyzed by addition of an equal volume of lysis buffer (20 mM Tris–HCl pH 7.5, 5 mM MgCl₂, 50 mM KCl, 400 mM NaCl, 2 mM DTT, 1% Triton X-100, 100 U/ml aprotinin, 0.2 mM PMSF, 20% glycerol). The lysates were centrifuged at 10 000 g for 5 min and the supernatants were assayed for PKR activity. A 100 μg aliquot of total protein was immunoprecipitated using anti-PKR monoclonal antibody (71/10) in high-salt buffer (20 mM Tris–HCl pH 7.5, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 U/ml aprotinin, 0.2 mM PMSF, 20% glycerol, 1% Triton X-100) at 4°C for 30 min on a rotating wheel. Then 10 μl of Protein A-Sepharose slurry was added and incubation was carried out for a further 1 h. The Protein A-Sepharose beads were washed four times in 500 μl of high-salt buffer and twice in activity buffer (20 mM Tris–HCl pH 7.5, 50 mM KCl, 2 mM MgCl₂, 2 mM MnCl₂, 100 U/ml aprotinin, 0.1 mM PMSF, 5% glycerol). The PKR assay was performed with PKR still attached to the beads in activity buffer containing 0.1 mM ATP and 1 μCi of [γ-32P] ATP at 30°C for 10 min. The standard activator of the enzyme was 0.1 μg/ml poly(I)-poly(C) or 0.116 pmol of pure PACT protein. Purified hDUS2 protein in varying amounts as indicated was added to test its effect on PKR activity. Labeled proteins were analyzed by SDS–PAGE on a 12% gel followed by autoradiography.

Translation inhibition assay

The effect of co-transfection of pCDNA3.1(−) empty vector, TRBP/pCDNA3.1(−) and hDUS2/pCDNA3.1(−) on the reporter pGL2-Control (Promega) in HeLa cells was measured as described before (52). HeLa cells were transfected in six-well plates in triplicate with the indicated plasmids using Effectene transfection reagent. At 24 h after transfection, the cells were treated with 100 U/ml of IFN-β. Cells were harvested 48 h after transfection and assayed for luciferase activity after normalizing for the transfection efficiency by measuring the total protein.

Apoptosis assay

HT1080 cells were grown to 50% confluency on coverslips in six-well plates and co-transfected with 500 ng of the indicated effector and 100 ng of pEGFP-C1 (Clontech) plasmid using the Effectene reagent. The cells were observed for green fluorescent protein (GFP) fluorescence 24 h after transfection using an inverted fluorescence microscope, and were then treated with 0.1 μg/ml tunicamycin to induce ER stress and apoptosis. At 24 and 48 h after this treatment, the cells (coverslips) were washed twice with PBS and fixed in acetone:methanol (1:1, v/v) for 1 min, and were mounted in Vectashield (Vector Laboratories) mounting medium containing DAPI (4,6-diamidino-2-phenylindole). At least 300 fluorescent (GFP-positive) cells were counted as alive or dead, based on their morphology and nuclear DAPI staining. Intense DAPI staining indicates nuclear condensation, which is a hallmark of apoptosis. The experiment was repeated three times and the counting was done in a blinded manner to ascertain the validity of obtained numbers. The cells showing a normal flat morphology were scored as alive, and cells showing cell shrinkage, membrane blebbing, rounded morphology, partial detachment from the plate and chromatin condensation (intense DAPI stain) were counted as dead. Apoptosis (%) = (fluorescent cells with intense DAPI staining in the nucleus/total fluorescent cells) × 100.

RESULTS

hDus2 interacts with both PACT and PKR

Various stress signals induce cellular apoptosis via activation of PKR (18). PACT plays a central role in this pathway by functioning as an activator of PKR (13,14). In order to identify proteins that may regulate this stress-activated apoptotic pathway, we performed a yeast two-hybrid screen using PACT as a bait. Among the cDNA clones identified as the ones that encoded PACT-interacting proteins, there were 14 isolates of PACT, 3 isolates of JAZ and 1 isolate of hDUS2 (human dihydrouridine synthase-2). PACT is expected to interact with both PACT and PKR, and previous studies have shown homology with the yeast enzymes that have been previously shown to interact with dsRNA (53). hDUS2 interacts with both PACT and PKR in the yeast two-hybrid library was a partial cDNA clone and lacked the amino-terminal
sequences that encoded the amino-terminal 320 amino acids. We obtained the full-length cDNA clone for hDUS2 based on the Genbank sequence information (FLJ20399, accession no. NM_017803) by performing RT–PCR on total RNA isolated from HeLa cells. The full-length open reading frame for hDUS2 was sub-cloned into the yeast two hybrid vectors pGBK7T and pGADT7 to obtain GAL4 DNA-binding domain fusion and GAL4 activation domain fusion, respectively. In order to confirm that the full-length hDUS2 protein can interact with PACT and to test if hDUS2 interacts with PKR, we performed the yeast growth assay on triple drop-out medium lacking three amino acids: histidine, leucine and tryptophan. Growth on the triple drop-out medium indicates positive interaction between the two proteins that are being tested. As seen in Figure 1A, hDUS2 showed positive interaction in both combinations with PKR (sectors B and E), as well as PACT (sectors C and F). The negative controls with empty vectors did not show any growth on the plate (sectors A and D). These results confirmed that full-length hDUS2 interacts with both PACT and PKR. In order to further confirm the positive interaction between hDUS2, PKR and PACT we also tested activation of another reporter, β-galactosidase. Expression of β-galactosidase in yeast cells is indicated by blue color and occurs only if the proteins encoded by pGADT7 and pGBK7 constructs interact. As seen in Figure 1B, PKR/pGBK7T and PACT/pGBK7T both showed a positive interaction with hDUS2/pGADT7. The opposite combinations also showed blue color and thus confirmed the interactions. The negative controls with p53/pGBK7T and p53/pGADT7 all showed white color indicating no interaction with hDUS2, PKR and PACT. These results confirm the specificity of the interaction between hDUS2 and PKR or PACT. The interaction of hDUS2 with PACT and PKR was further confirmed by performing a biochemical interaction assay. In vitro translated, 35S-methionine labeled hDUS2 was allowed to interact with pure, recombinant hexahistidine tagged PKR or PACT proteins and was then pulled-down using Ni-agarose beads. As seen in Figure 1C, hDUS2 showed no interaction with DRIL1 protein bound to Ni-agarose beads (lane 2). However, it showed a strong interaction with both PKR (lane 3) and PACT (lane 4) bound to Ni-agarose beads. These results further confirm the in vivo interaction detected between these proteins in yeast two-hybrid assay.

hDUS2 contains one copy of evolutionarily conserved dsRBM and interacts with dsRNA

Homology searches with Genbank sequences revealed that hDUS2 contains two domains, the DUS domain that functions in the enzymatic activity in modifying the uridines in tRNAs post-transcriptionally (50). The DUS domain in hDUS2 is located between residues 15 and 338 (Figure 1D). In addition to this, one copy of the previously characterized evolutionarily conserved dsRBM (30) is present in hDUS2 between residues 370 and 434. An alignment of the dsRBM of hDUS2 with the dsRBMs present in PKR, PACT and TRBP is shown in Figure 1E. The consensus residue lysine at position 58 of the dsRBM is absent in hDUS2 motif. This lysine has previously been shown to be important for conferring a high-affinity binding to dsRNA (52,54). It is noteworthy that hDUS2 has a glutamic acid at this position, thus raising a possibility that the dsRBM present in hDUS2...
may not mediate high-affinity binding to dsRNA. We examined this by comparing the binding of hDUS2, PACT and PKR to dsRNA covalently linked to agarose beads. As seen in Figure 2A, hDUS2 showed binding to dsRNA at 50 mM salt concentration but not at 300 mM salt. PACT and PKR both showed very strong binding to dsRNA at 50 and 300 mM salt concentrations. The binding of hDUS2 to dsRNA at 50 mM salt concentration is quite significant since firefly luciferase, a protein that is known not to interact with dsRNA does not show binding to dsRNA at this salt concentration. These results indicate that although hDUS2 interacts with dsRNA, its affinity for dsRNA is much lower than PACT and PKR. This could be due to the lack of the conserved lysine at position 58 of hDUS2 dsRBM, since this lysine is required for the high-affinity interaction with dsRNA (52,54).

hDUS2 interacts with PACT and PKR in mammalian cells

In order to test if hDUS2 interacts with PACT and PKR in mammalian cells, we tested the ability of flag-tagged hDUS2 to interact with HA-tagged PKR and PACT in HeLa cells. As shown in Figure 2B, when flag-hDUS2 was immunoprecipitated with anti-flag antibody, HA-tagged PKR and PACT both co-immunoprecipitated with it indicating that hDUS2 interacts with both PKR and PACT. The negative control HA-B56α, which is an unrelated protein, did not co-immunoprecipitate with flag-hDUS2, thus demonstrating the specificity of interaction. These results demonstrate that hDUS2 forms a complex with PKR and PACT in mammalian cells.

Figure 2. hDUS2 interaction with dsRNA, PACT, and PKR. (A) dsRNA-binding characteristics of hDUS2. Binding of hDUS2 to dsRNA was analyzed by polyI:polyC-agarose-binding assay. The binding was assayed at 50 and 300-mM salt concentration as indicated. The T lanes represent total proteins from reticulocyte lysates. The positions of hDUS2, PACT, PKR and Luciferase (Luc) are indicated by arrows. (B) hDUS2 interaction with PACT and PKR in mammalian cells. HeLa cells were transfected with (i) HA-B56α/pCDNA3.(-), (ii) HA-PACT/pCDNA3.1(-), (iii) HA-PKR/pCDNA3.1(-) and (iv) Flag-hDUS2/pCDNA3.1(-). Twenty-four hours after transfection the Flag-tagged hDUS2 protein was immunoprecipitated using anti-Flag monoclonal antibody conjugated to agarose. The immunoprecipitates were analyzed by western blot analysis with anti-HA and anti-Flag monoclonal antibodies. The positions of HA-PKR, HA-PACT and Flag-hDUS2 are as indicated by arrows.

Mapping the interaction domains in PKR and hDus2

To map the domains within PKR that mediate its interaction with hDUS2, we tested the interaction of hDUS2 with two different deletion mutants of PKR using the in vitro translated. Hexahistidine-tagged, pure recombinant hDUS2 protein was incubated with 5μl of the reticulocyte lysates before pull-down with Ni-charged affinity resin. As a negative control, his-tagged DRIL1 protein that does not interact with PKR was immobilized on Ni-agarose beads in lanes 7–9. The proteins that associate with recombinant hDUS2 are also pulled down with the beads and were analyzed by SDS-PAGE followed by phosphorimager analysis. Lanes 1–3 represent total proteins from the translation mix (20% of input in lanes 4–9), lanes 4–6 represent proteins pulled down with hDUS2 bound to Ni-charged beads, and lanes 7–9 represent negative controls with proteins pulled down with DRIL1 bound to Ni-charged beads. (B) Mapping the PKR interaction domain within hDUS2. hDUS2 and its deletion mutant Δ307 were in vitro translated. Hexahistidine-tagged, pure recombinant PKR protein was incubated with 5μl of the reticulocyte lysates before pull-down with Ni-charged affinity resin. As a negative control, his-tagged DRIL1 protein that does not interact with hDUS2 was used in lanes 5 and 6. Lanes 1 and 2 represent total proteins from the translation mix (20% of input in lanes 4–9), lanes 3 and 4 represent proteins pulled down with hexahistidine-tagged pure recombinant PKR bound to Ni-charged beads, lanes 5 and 6 represent negative controls with proteins pulled down with his-tagged pure DRIL1 bound to Ni-charged beads.

Figure 3. Mapping the hDUS2 interaction domain within PKR. (A) PKR and its deletion mutants DRBD and Δ170 were in vitro translated. Hexahistidine-tagged, pure recombinant hDUS2 protein was incubated with 5μl of the reticulocyte lysates before pull-down with Ni-charged affinity resin. As a negative control, his-tagged DRIL1 protein that does not interact with PKR was immobilized on Ni-agarose beads in lanes 7–9. The proteins that associate with recombinant hDUS2 are also pulled down with the beads and were analyzed by SDS-PAGE followed by phosphorimager analysis. Lanes 1–3 represent total proteins from the translation mix (20% of input in lanes 4–9), lanes 4–6 represent proteins pulled down with hDUS2 bound to Ni-charged beads, and lanes 7–9 represent negative controls with proteins pulled down with DRIL1 bound to Ni-charged beads. (B) Mapping the PKR interaction domain within hDUS2. hDUS2 and its deletion mutant Δ307 were in vitro translated. Hexahistidine-tagged, pure recombinant PKR protein was incubated with 5μl of the reticulocyte lysates before pull-down with Ni-charged affinity resin. As a negative control, his-tagged DRIL1 protein that does not interact with hDUS2 was used in lanes 5 and 6. Lanes 1 and 2 represent total proteins from the translation mix (20% of input in lanes 4–9), lanes 3 and 4 represent proteins pulled down with hexahistidine-tagged pure recombinant PKR bound to Ni-charged beads, lanes 5 and 6 represent negative controls with proteins pulled down with his-tagged pure DRIL1 bound to Ni-charged beads.

The T lanes represent total proteins from reticulocyte lysates. The positions of hDUS2, PACT, PKR and Luciferase (Luc) are indicated by arrows. The positions of hDUS2, PACT, PKR and Luciferase (Luc) are indicated by arrows.
PKR, which are known to mediate protein–protein interactions. When an unrelated protein DRIL1 was immobilized on the Ni-agarose beads, none of the \textit{in vitro} translated protein showed any interaction with it, thereby demonstrating specificity of the interactions between hDUS2 and PKR. In order to map the domain within hDUS2 that mediates the interaction with PKR, similar experiments were done with a deletion mutant of hDUS2. A307 lacks the amino-terminal residues 1–307. \textit{In vitro}-translated \textsuperscript{35}S-methionine-labeled hDUS2 proteins were mixed with pure, hexahistidine-tagged PKR recombinant protein and Ni-agarose beads and the proteins that bound to PKR were analyzed by SDS-PAGE. As seen in Figure 3B, full-length hDUS2 and A307 both showed interaction with PKR (lanes 3 and 4). The A307 mutant of hDUS2 lacks the amino-terminal DUS domain but retains the single dsRBM present in hDUS2, thus indicating that its interaction with PACT and PKR is mediated via its dsRBM. The negative control protein DRIL1 did not show any binding to hDUS2, thus demonstrating that the interaction between PKR and hDUS2 is specific.

To further test the contribution of the two dsRBMs within DRBD of PKR in mediating interaction with hDUS2, we tested the ability of several PKR point mutants within the two motifs (52) to interact with pure recombinant hDUS2 using the \textit{in vitro} interaction assay. The results are shown in Figure 4. As seen in the bar graph, F41A, K60A and A68D mutants within dsRBM1 showed severely reduced interaction with hDUS2. A mutation of the same residues in dsRBM2 was less detrimental to the interaction with hDUS2. These results strongly indicate that the interaction of PKR with hDUS2 is primarily mediated by its dsRBM1 of PKR and that the residues F41, K60, and A68 serve an important function.

**hDus2 inhibits PKR's kinase activity both \textit{in vitro} and \textit{in vivo}**

In order to understand the functional consequence of hDUS2's interaction with PKR, we tested the effect of hDUS2 on PKR's kinase activity using the \textit{in vitro} kinase activity assay. As seen in Figure 5A, recombinant hDUS2 inhibited the PKR kinase activity in a dose-dependent manner. hDUS2 was capable of inhibiting PKR activity when it was activated by dsRNA as well as PACT. These results demonstrate that hDUS2 acts as a PKR inhibitor. To test this further \textit{in vivo} in mammalian cells, we performed the translation inhibition assay to check for PKR activation. The effect of a protein encoded by an expression construct on activation of PKR in mammalian cells can be measured by assaying the expression of a co-transfected reporter gene such as luciferase (12). Using the translation inhibition assay (46,55–58), we next determined the ability of the hDUS2 expression constructs to inhibit PKR in HeLa cells. As seen in Figure 5B, co-transfection with wild-type PKR reduced luciferase activity dramatically, whereas co-transfection with TRBP expression construct increased the reporter activity, due to inhibition of endogenous PKR activation. Co-transfection with hDUS2 expression construct also increased luciferase activity in a dose-dependent manner, indicating that hDUS2 inhibits the activation of PKR. These results further indicate that hDUS2 is not a very strong inhibitor of PKR since it did not increase the reporter activity to the same extent as the TRBP expression construct. At 100 ng DNA concentration, hDUS2 construct showed only about half as much increase in activity of the reporter than the TRBP construct. Thus, although hDUS2 does inhibit PKR activity, it does not act as efficiently as TRBP. These results indicate that to achieve an effective inhibition of PKR activity, hDUS2 may need to be expressed at high levels relative to PKR levels in mammalian cells. In order to test if hDUS2 overexpression can effectively inhibit activated PKR, we tested if co-transfected hDUS2 expression construct can relieve the translational repression of luciferase expression caused by active PKR. As seen in Figure 5C, co-transfection of the reporter plasmid with wild-type PKR expression construct reduced luciferase activity dramatically, whereas co-transfection with hDUS2 expression construct along with wt PKR increased the reporter activity in a dose-dependent manner. Co-transfection of PKR expression construct with an
expression plasmid encoding DRIL1 protein, which has no effect on PKR activation showed no effect on the reporter gene activity thus demonstrating that the inhibitory effect of hDUS2 on PKR activation is specific. These results strongly indicate that hDUS2 is able to inhibit the PKR activity in mammalian cells.

**Functional consequence of hDus2-mediated inhibition of PKR activity**

The inhibitory effect of hDUS2 being weaker than TRBP (Figure 5B), we tested the biological significance of it. Since PKR is known to be involved in stress signal-induced apoptosis, we tested if overexpression of hDUS2 can inhibit apoptosis induced by tunicamycin, an antibiotic that causes endoplasmic reticulum (ER) stress due to accumulation of unfolded proteins in the ER (59). PKR is involved in inducing apoptosis in response to ER stress (60,61). As seen in Figure 6, treatment of HT1080 cells with tunicamycin induced cell death in about 38% of cells transfected with the empty vector at 24 h, which further increased to about 52% at 48 h. In cells transfected with hDUS2 expression construct, tunicamycin induced cell death in about 30% cells at 24 h, which showed only an extremely marginal increase to about 34% at 48 h. The transfected samples that were not treated with tunicamycin did not show significant cell death. Thus, at 48 h after treatment with tunicamycin, there is a significant inhibition of cell death by overexpression of hDUS2 indicating that hDUS2 is able to inhibit PKR activation and the consequent apoptosis. These results establish that inhibition of PKR activity by hDUS2 may play an important role in regulating stress-induced apoptosis.

**DISCUSSION**

PKR activity is regulated very tightly in cells and several cellular proteins have been described as inhibitors of PKR (47). One of the best-studied proteins is p58IPK, which is a member of the tetratricopeptide repeat family (62–64). P58IPK inhibits PKR activity by interacting directly with it and by preventing its dimerization (65). Influenza virus evades the host antiviral response partially by recruiting p58IPK to repress PKR-induced eIF2α phosphorylation (66). TRBP is a cellular protein that was identified by its ability to bind human immunodeficiency virus type 1 (HIV-1) TAR RNA (67). TRBP inhibits PKR activity by sequestration of its activator dsRNA, and also by direct interaction with PKR (38,58). Recent data suggests that in astrocytes, low TRBP levels support and innate HIV-1...
resistance via PKR activation (68). Thus, TRBP may play a major role in viral expression by regulating PKR activation. NPM is an abundant nucleolar phosphoprotein implicated in ribosome biogenesis (69). NPM interacts with PKR and inhibits eIF2α phosphorylation and PKR-mediated apoptosis (70). NPM has also been implicated in mediating antiviral activity of the tumor suppressor ARF thus linking the Arf/mdm2/p53 pathway to PKR (71). The heat shock proteins Hsp70 and Hsp90 have also been shown to bind to PKR and inhibit its activation (48,72). In addition to these cellular inhibitors of PKR, numerous viral proteins and RNAs have been established to act as PKR inhibitors during viral infection thereby evading the host cell’s innate antiviral response (8,49).

In this article, we describe hDUS2 as a novel inhibitor of PKR activity. hDUS2 was reported recently to have 39% homology to Dus2 enzyme of *Saccharomyces cerevisiae* and to possess tRNA–DUS activity (50). It was also shown to exist in a complex with EPRS, a glutamy-aryl-tRNA synthetase and to enhance general translational efficiency in mammalian cells. hDUS2 was identified as a PACT-interacting protein in our yeast two-hybrid screen using PACT as a bait. Further characterization of the interaction between PACT and hDUS2 (data not shown) indicated that this interaction was mediated by the single copy of dsRBM present in hDUS2. Our work on PKR and PACT has demonstrated that PKR and PACT interact with each other via the dsRBM motifs (45). The amino-terminal two copies of dsRBM present in PACT are essential for mediating the high-affinity interaction with PKR via the two dsRBMs present in PKR. We reasoned that hDUS2 may also show interaction with PKR via the two dsRBMs. Originally identified as a PACT-interacting protein, hDUS2 did indeed interact with PKR equally well, both in *in vivo* (in mammalian and yeast cells) and in *in vitro*. Although there are two copies of dsRBM present in PKR, our results indicate that hDUS2 interacts with PKR mainly via the amino-terminal copy of the motif. Thus, the two dsRBMs do not contribute equally to PKR’s interaction with hDUS2 and the first or the amino-terminal motif is responsible for mediating the interaction. Within this motif, mutations at position 41 (phenylalanine), 60 (lysine) and 68 (alanine) affected the interaction between PKR and hDUS2 adversely, thereby identifying these residues as essential for the interaction. Mutations of the same residues in motif 2 did not show much effect on the PKR–hDUS2 interaction. In addition to this, the interaction between hDUS2 and PKR is by direct protein–protein interaction and not likely to be mediated via dsRNA since mutations F131A, K150A, K154E and A158D that are known to destroy PKR’s dsRNA binding (26,52) have very little adverse effect on PKR–hDUS2 interaction. Although hDUS2 carries one copy of the conserved dsRBM, it does not interact with dsRNA with high affinity. This may be attributed to the lack of a crucial conserved lysine at position 58 of the conserved motif (Figure 1E). This lysine has been shown to be essential for the high-affinity interaction with dsRNA (52). hDUS2 carries an aspartic acid residue at this position, which may contribute to its lack of high-affinity dsRNA binding.

Recombinant hDUS2 protein showed a marked inhibition of PKR’s kinase activity *in vitro*. The inhibition was effective when PKR was activated either by addition of dsRNA or recombinant PACT. However, hDUS2 was effective in inhibiting PACT-induced PKR activation more efficiently than dsRNA-induced PKR activation since even the lowest amount of hDUS2 (80 ng) showed a marked reduction in PKR autophosphorylation when activated by PACT. This may be due to the ability of hDUS2 to interact both with PKR and PACT. hDUS2’s interaction with PACT may inhibit PACT’s interaction with PKR in addition to inhibition by directly interacting with PKR, thus resulting in a more efficient inhibition. The biological significance of this if any is currently unknown. hDUS2 also showed inhibition of PKR in mammalian cells as seen in the translation inhibition assay. However, hDUS2 did not inhibit PKR activation as efficiently as PKR’s well-studied inhibitor TRBP (Figure 5B). Since this is not due to lower level of expression of hDUS2 as compared to TRBP (data not shown), we conclude that hDUS2 is not a strong inhibitor of PKR *in vivo*.

However, since the results shown in Figure 5C indicate that when overexpressed, hDUS2 can inhibit activated PKR it can be speculated that hDUS2 may regulate PKR activity under conditions (or in cells) where its expression...
Inhibition of PKR activity in NIH 3T3 cells by hDUS2 overexpression in human nonsmall cell lung carcinomas (NSCLC). It was observed that upregulation of hDUS2 expression is upregulated in mammalian cells. Overexpression of PKR inhibitors p58\(^{\text{PK}}\) and TRBP also results in tumorigenesis. It remains to be tested if overexpression of hDUS2 also can lead to cell transformation. In addition, it would also be interesting to examine if hDUS2-mediated inhibition of PKR is involved in tumorigenesis in cases of NSCLC. Since our results demonstrate that hDUS2 can inhibit apoptosis, overexpression of hDUS2 in NSCLC may offer a growth advantage to cancer cells.

hDUS2 has been shown to enhance the translation rate in mammalian cells possibly by enhancing the 40S ribosomal fraction activity of EPRS complex. In view of the results presented here, it is also possible that hDUS2 may enhance the rate of translation by inhibition of elf2\(^{\alpha}\) phosphorylation brought about by activated PKR. This may be of biological significance in cancer progression since enhanced PKR expression is reported in some types of cancers. In melanomas and colorectal cancers, PKR overexpression can be correlated with enhanced PKR activity. However, in breast cancer cells, PKR overexpression does not correlate with increased PKR activity. It is thought that an inhibitor of PKR may be expressed at high levels in such cancers, leading to an inhibition of PKR activity. In future, it would be of interest to examine if hDUS2 may be involved in PKR inhibition in these types of cancers.

Acknowledgements

Funding has been provided by National Institutes of Health (HL63359 to R.C.P.) and American Heart Association (0555503U to R.C.P.). The Open Access publication charges for this article were waived.

Conflict of interest statement. None declared.

References


