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RPAP3 splicing variant isoform 1 interacts with PIH1D1 to compose R2TP complex for cell survival

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ABSTRACT

We previously characterized RNA polymerase II-associated protein 3 (RPAP3) as a cell death enhancer. Here we report the identification and characterization of splicing isoform of RPAP3, isoform 1 and 2. We investigated the interaction between RPAP3 and PIH1 domain containing protein 1 (PIH1D1), and found that RPAP3 isoform 1, but not isoform 2, interacted with PIH1D1. Furthermore, knockdown of RPAP3 isoform 1 by small interfering RNA down-regulated PIH1D1 protein level without affecting PIH1D1 mRNA. RPAP3 isoform 2 potentiated doxorubicin-induced cell death in human breast cancer T-47 cells although isoform 1 showed no effect. These results suggest that R2TP complex is composed of RPAP3 isoform 1 for its stabilization, and that RPAP3 isoform 2 may have a dominant negative effect on the survival potency of R2TP complex.

1. Introduction

We have previously reported that Monad (WDR92), a novel WD40 repeat protein, potentiates apoptosis induced by tumor necrosis factor-α and cycloheximide [1]. By affinity purification and mass spectrometry, RNA polymerase II-associated protein 3 (RPAP3), Pontin, Reptin, and PIH1 domain containing protein 1 (PIH1D1) were identified as Monad binding proteins [2–5]. Increasing evidence suggest that these four factors form a complex called R2TP complex [6–9].

Among the R2TP components, Pontin and Reptin are the most intensively studied. Pontin and Reptin are evolutionarily highly conserved AAA + ATPases, and components of multiprotein complexes like INO80 and SWR1 chromatin remodelling complexes [10] and the TIP60 histone acetyltransferese complex [11]. They perform a variety of functions, including regulation of transcription factor such as β-catenin and telomerase biogenesis [12,13]. Pontin and Reptin are also involved in the regulation of apoptosis. We have demonstrated that depletion of Reptin by small interfering RNA (siRNA) reduces cell survival and facilitates the phosphorylation of H2AX, a DNA damage marker [3]. Taniue et al. has shown that Pontin blocks p53-mediated apoptosis by repressing the expression of p53 and its target genes [14]. It has been reported that siRNA-mediated knockdown of Reptin in human hepatocellular carcinoma cells leads to apoptotic cell death [15], and *invivo* silencing of Reptin in xenografted tumors reduced tumor progression [16]. It has also been reported that Pontin and Reptin regulate phosphatidylinositol 3-kinase-related protein kinase such as mTOR [17,18].

Although we have reported that PIH1D1 possesses cell protective function against apoptosis [4], the mechanism has not been fully explored. It has been reported that PIH1D1 is important for the recovery of human cells from nutrient starvation [19]. The role of PIH1D1 in R2TP complex is gradually becoming clear. PIH1D1 binds directly to TEL2 and is required for the TEL2–R2TP complex interaction. Since PIH1D1 is labile [20,21], the stabilization of PIH1D1 may be rate-limiting step for the regulation of R2TP complex. So far, the involvement of RPAP3 in R2TP complex-regulated apoptosis is less clear. Since in yeast, Tah1 (the counterpart gene of RPAP3) functions to stabilize Pih1 and to promote the formation of an Rvb1-Rvb2-Tah1-Pih1 complex (yeast R2TP complex) [20,21], RPAP3 may also act as a chaperone for PIH1D1. We have previously reported that RPAP3 potentiates apoptosis [2,3,5]. This puzzling result prompted us to further investigate the effect of RPAP3 on apoptosis.

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In this manuscript, we report that the larger splicing variant of RPAP3 (isoform 1) and the smaller splicing variant (isoform 2) work as inhibitor and potentiator against doxorubicin-induced cell death, respectively. We further show the evidence that RPAP3 isoform 1, but not isoform 2, interacts with PIH1D1 for its stabilization.

2. Materials and methods

2.1. Reagents and antibodies

Anti-V5 antibody was from Invitrogen (Carlsbad, USA). Anti-RPAP3 antibody was from Sigma (St. Louis, USA). Anti-PIH1D1 antibody was from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-Reptin antibody, Anti-p70 S6 kinase, or Anti-Phospho-p70 S6 kinase (Thr389) was from Cell Signaling Technology (Beverly, USA). Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, 23040091) antibody was from Chemicon (Temecula, USA). Anti-Monad antibody was previously described [1].

2.2. Cell culture

U2OS, T-47, or HEK293 cells was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum containing 100 μg/ml streptomycin, 100 IU/ml penicillin and 1 μl/ml amphotericin B. Cells were treated with 3 μM doxorubicin (Sigma) and cell viability was measured by MTT (Sigma, M2128) assay. Data are expressed as mean ± SEM. Statistical differences between groups were determined using Tukey test after ANOVA.

2.3. Cloning of RPAP3

Human RPAP3 cDNA amplified by PCR was cloned into pENTR/D TOPO vector using pENTR Directional TOPO cloning kit and subcloned into pcDNA3.1/nV5-DEST using Gateway System according to the manufacturer's instructions (Invitrogen). Sequences were confirmed by automated DNA sequencing.

2.4. Transfection and Immunoblotting

HEK293 cells were seeded onto 60 mm Petri dishes and grown for 24 h. The indicated plasmids were transfected with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). After 4 h-transfection, the cells were returned to growth medium and incubated for 48 h. Cells were lysed in extraction buffer (1% Triton X-100, 120 mM NaCl, 5 mM EDTA, 10% glycerol and 20 mM Tris, pH 7.4) including protease inhibitor cocktail (Roche, Indianapolis, USA). Total protein was mixed with Laemmli denaturing buffer, separated by SDS–PAGE and transferred to PVDF membranes (Millipore Corporation, Bedford, USA). Immunoblotting was carried out as described previously [22].

2.5. Immunoprecipitation

Immunoprecipitaion was carried out as described previously [23]. Briefly, equal protein concentrations of lysates were incubated with 3 μg of anti-V5 antibody for 16 h, followed by incubation with Protein G Sepharose (Amersham Biosciences) for 1 h. The Sepharose beads were washed five times with the buffer described above, associated proteins were recovered by boiling in Laemmli buffer.

2.6. RNA isolation, cDNA synthesis and quantitative real time PCR

Total RNA was extracted using TRIzol (Invitrogen), and reverse-transcribed with SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. TaqMan Gene Expression Assay based quantitative real time PCR was performed with an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, USA). Each TaqMan assay was conducted in four replicates for each RNA sample. They were assayed with Universal PCR Master Mix using universal cycling conditions (10 min at 95 °C; 15 s at 95 °C, 1 min at 60 °C, 40 cycles). The TaqMan probe/primer sets for the endogenous control and target genes were as follows: GAPDH, Hs99999905_m1; RPAP3, Hs00226298_m1; PIH1D1, Hs00215579_m1. Results are expressed as relative abundance of mRNA normalized to an internal control (GAPDH).

2.7. Knockdown experiments

RPAP3 or PIH1D1-specific siRNA was purchased from Qiagen and targeted the following sequences: 5′-TTGAAGGATAGTTCTGTC-GAA-3′ (target-1, used for rescue experiment); 5′-AAGGACTAT CTTTGAACATAA-3′ (target-2) or 5′-CCTACGACGTAGCTGTCAATT-3′ (target-1); 5′-CCCGCTGCAGATCAACTCTCA-3′ (target-2). AllStars Negative Control siRNA (Qiagen) was used as a control.

2.8. Lentiviral transduction and establishment of stable cell line

The PCR-amplified coding sequence for human PIH1D1 or Reptin was cloned into pENTR/D TOPO and subcloned into pLenti6.3/V5-DEST using Gateway System. This vector was cotransfected into 293FT cells with ViraPower packaging mix (Invitrogen) to generate the lentivirus according to the manufacturer's protocol. Cells were transduced with the lentivirus and stable cell lines were generated by selecting with blasticidin.

3. Results

3.1. Cloning of RPAP3

To obtain the entire open reading frame of RPAP3, we designed primers specific for the RPAP3 gene and performed RT-PCR using total RNA prepared from HEK293 cells as template. Two transcripts were amplified (data not shown). To verify the identity of the PCR products, the bands were subjected to DNA sequencing. Comparison of the sequence of each transcript revealed that the smaller transcript is a new splicing variant of RPAP3 with exon 12 deleted (Fig. 1A). This splicing event results in the deletion of 34 amino acids (Fig. 1B). To avoid confusion, we refer to them hereafter as RPAP3 isoform 1 and 2, respectively. Retrospectively, we noticed that we previously used RPAP3 isoform 2 in the experiment [2,3,5]. In the present study, we compared the effect of RPAP isoforms 1 and 2 on cell death.

3.2. RPAP3 isoform 1 interacted with PIH1D1

HEK293 cells were transfected with V5-RPAP3 isoform 1 or isoforms 2 and immnoprecipitaed with anti-V5 antibody. Immunoblotting showed that V5-RPAP3 isoform 1 alone interacted with PIH1D1 (Fig. 1C). Monad, another binding protein of RPAP3, interacted with RPAP3 isoform 1 and 2 (Fig. 1C).

3.3. RPAP3 isoform 1 stabilized PIH1D1

We confirmed the presence of RPAP3 isoform 1 by immnoblotting in human osteosarcoma U2OS cells (Fig. 2, lane 1). We evaluated the amount of PIH1D1 after transfection of RPAP3 siRNA. PIH1D1 was markedly reduced after transfection of RPAP3 siRNA (Fig. 2, lane 2). This result suggests thatPIH1D1 exists depending

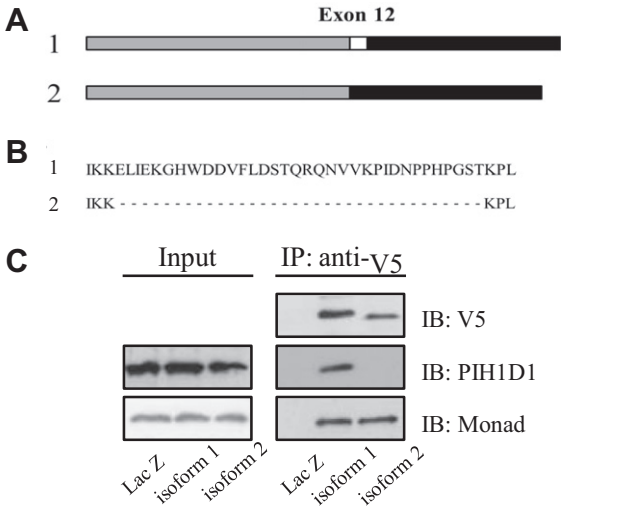

Fig. 1. Interaction of PIH1D1 with RPAP3. (A) Schematic representation of the two splicing variants of RPAP3, (B) Deleted part of amino acid alignment of RPAP3 and (C) HEK293 cells were transfected with expression vectors encoding V5-RPAP3 or V5-LacZ (control). Lysates were immunoprecipitaed (IP) with anti-V5 antibody and immunoblotted (IB) using anti-V5, PIH1D1, or Monad antibody.

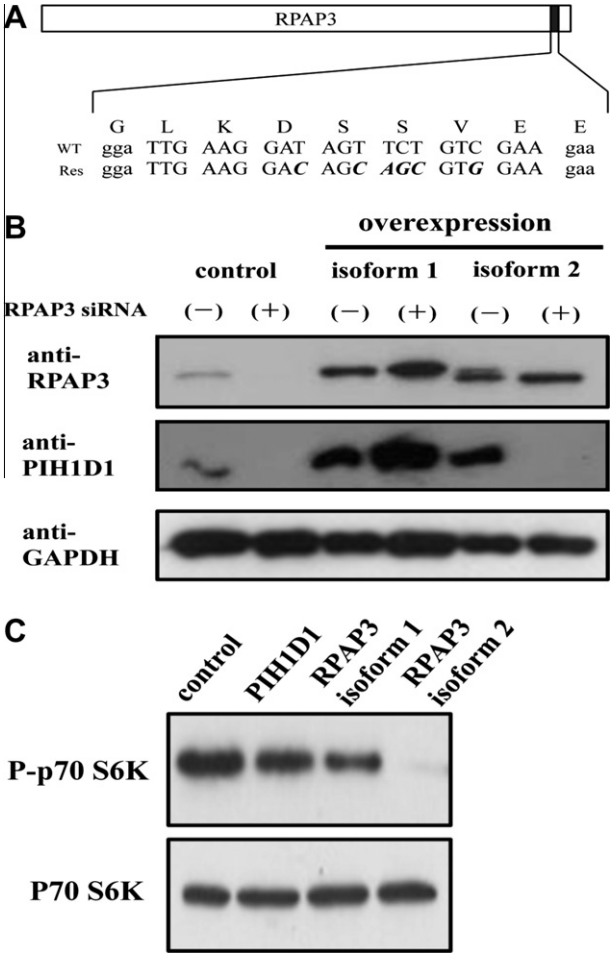

Fig. 2. Effect of RPAP3 siRNA on PIH1D1 protein level. (A) Schematic representation of the RPAP3 cDNA sequences. The RPAP3 siRNA targeted sequence is indicated by capital letters. Silent mutations that were introduced are indicated by bold/italic letters (WT: wild type, Res: resistant), (B) U2OS cells overexpressing siRNA-resistant RPAP3 isoform 1 or isoform 2 was transfected with RPAP3 siRNA. Lysates were prepared from transfected cells and immunoblotting was performed using anti-RPAP3, PIH1D1, or GAPDH antibody and (C) LacZ (control), RPAP3 isoform 1, 2, or PIH1D1-overexpressing T-47 cells were analyzed by immunoblotting with anti-p70 S6 kinase or anti-Phospho-p70 S6 kinase (Thr389) antibody.

on RPAP3. To rule out off-target effects of siRNA, we used a rescue strategy. We established U2OS cells overexpressing isoform 1 or isoform 2 and introduced silence mutations in the target sequence of siRNA and made the mRNA insensitive to siRNA. As expected, transfection with RPAP3 siRNA affected only endogenous RPAP3 isoform 1 (Fig. 2, lane 5, upper band), but did not decrease the levels of siRNA-resistant, overxpressed RPAP3 isoform 2 (Fig. 2, lane 5, lower band). In isoform 1-overexpressing cells, PIH1D1 protein level was no more decreased by the siRNA treatment, whereas it almost completely diminished in isoform 2-overexpressing cells. These results suggest that RPAP3 isoform 1 can stabilize PIH1D1, while RPAP3 isoform 2 cannot. We also evaluated RPAP3 level after transfection of PIH1D1 siRNA, and found that RPAP3 protein levels did not change (data not shown), although PIH1D1 was efficiently knocked down. We also investigated the effect of RPAP3 isoforms on downstream signaling in breast cancer T-47D cells. RPAP3 isoform 2 decreased phosphorylation of direct downstream effectors of mTOR, p70 S6 Kinase at residue Thr389, without affecting the amount of p70 S6 Kinase (Fig. 2C). On the other hand, RPAP3 isoform 1 or PIH1D1 did not affect the mTOR signaling pathway (Fig. 2C). These results suggest that RPAP3 isoform 2 destabilizes mTOR complex and subsequently decreases phosphorylation of p70 S6 kinase.

3.4. RPAP3 silencing does not decrease PIH1D1 mRNA level

We considered the possibility that RPAP3 could regulate PIH1D1 expression at the transcription. However, transfection of U2OS cells with RPAP3 siRNAs (target-1,2) did not alter PIH1D1 mRNA levels, although RPAP3 mRNA decreased (Fig. 3). Reciprocally, RPAP3 mRNA levels were not silenced with PIH1D1 siRNAs (target-1,2), although PIH1D1 mRNA was effectively silenced (Fig. 3).

3.5. Effect of RPAP3 isoform 1 or 2 on doxorubicin-induced cytotoxicity

We investigated the effect of RPAP3 on doxorubicin-induced cell death in T-47D cells. As reported previously [2,3,5], overexpression of RPAP3 isoform 2 significantly promoted doxorubicin-induced cell death (Fig. 4), while isoform 1 had no effect on cell death. In addition, PIH1D1 and Reptin also showed no effect on doxorubicin-induced cell death.

4. Discussion

During the search of database, it came to our attention that there were two mRNA splicing isoforms of RPAP3. We cloned the two splicing variants from HEK293 cells. RPAP3 isoform 1 harbors 34 more amino acids than does RPAP3 isoform 2. The remaining parts of the two proteins are identical amino acid sequences. In this study, we indicated that these 34 amino acids are important for binding and stabilization with PIH1D1. These results suggest that R2TP complex is composed of RPAP3 isoform 1, but not isoform 2. Rosenbaum's group demonstrated that the expression of Pontin and Reptin is strictly co-regulated by a post-translational mechanism [24]. Considering that PIH1D1 is reported to be labile in yeast [20,21], it is possible that RPAP3 isoform 1 might work as a chaperon for PIH1D1, and that in the absence of RPAP3 isoform 1, newly translated PIH1D1 might be targeted by proteasomal degradation like in the case of Pontin and Reptin [24].

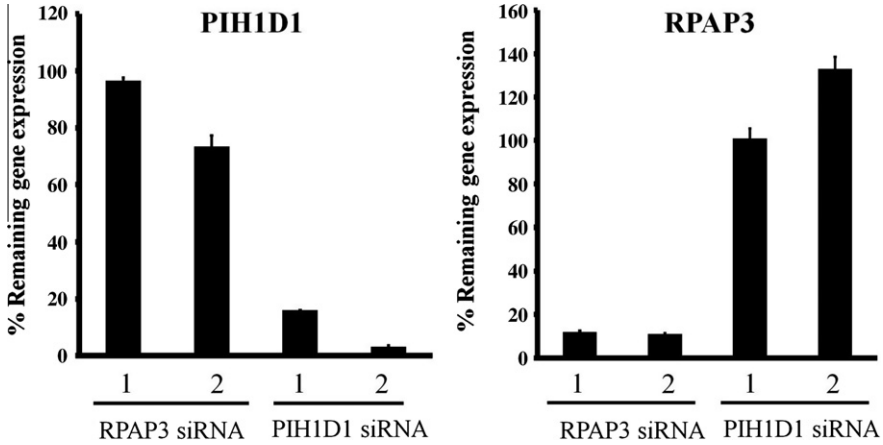

Fig. 3. No cross effect of siRNA on mRNA expression of RPAP3 and PIH1D1. Relative mRNA levels were analyzed at 48 h after the treatment of U2OS cells with siRNA for RPAP3 (target-1, 2) and PIH1D1 (targrt-1, 2) and expressed as percentage to that of control siRNA-treated cells from four independent experiments (mean ± SEM). Data were normalized based on GAPDH mRNA copy numbers.

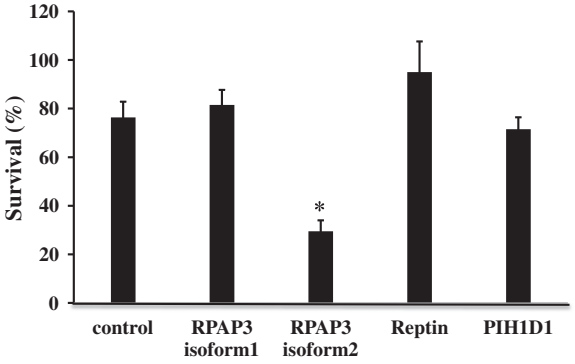

Fig. 4. Potentiation of doxorubicin-induced cell death by RPAP3 isoform 2. T-47 cells were seeded 4000 cells per well in 96-well plate. Cell viability of RPAP3, PIH1D1, Reptin, or LacZ (control)-overexpressing cells was analyzed by MTT assay 48 h after doxorubicin treatment (3 μM). Cell viability (mean ± SEM) was expressed as percentage to that of doxorubicin-untreated cells from four independent experiments.*$P < 0.05$ compared with those of control cells.

From our findings, one would expect that the depletion of RPAP3 or PIH1D1 would result in the similar cellular phenotype. Retrospectively, we noticed that we used RPAP3 isoform 2 in the previous experiment [2,3,5]. In the present study, we compared the effect of RPAP3 isoforms 1 and 2, and confirmed that RPAP3 isoform 2 alone potentiated cell death. In RPAP3 isoform 2-overexpressing cells, the depletion of endogenous RPAP3 failed to stabilize PIH1D1, presumably due to no interaction of RPAP3 isoform 1 with PIH1D1. In addition, overexpression of RPAP3 isoform 2 potentiated doxorubicin-induced cell death. Therefore, these results suggest that RPAP3 isoform 2 may reduce the interaction of endogenous RPAP3 isoform 1 with PIH1D1 with a dominant negative effect on the R2TP complex.

Increasing evidence suggests that R2TP complex is essential for cell survival. Studies in yeast have demonstrated that both Reptin and Pontin are required for cell viability [25]. In addition, Pontin has reported to be essential for the function of both embryonic pluripotent cells and adult hematopoietic stem cells [26]. Moreover, it has been reported that Reptin and Pontin are overexpressed in several cancers including hepatocellular carcinoma, colon and bladder cancer, and melanoma [27,28]. We also reported that Reptin and PIH1D1 inhibited cell death [3,4]. Therefore, R2TP complex components may represent a potential target for cancer therapy. Inhibition of the components of R2TP complex expression or its ATPase activity represents a plausible way to impede tumor growth and induce apoptosis in cancer cells. Several compounds which inhibit enzymatic activity of Pontin and show inhibitory effect on tumor proliferation are discovered [29].

In this study, we showed that RPAP3 splicing variants, isoform 1 and 2, work differently against doxorubicin-induced cell death and mTOR signaling. We further showed the evidence that RPAP3 isoform 1, but not isoform 2, interacts with PIH1D1 for its stabilization probably due to the protection from its degradation. Elucidation of the mechanism by which RPAP3 isoform 2 reduces the cell survival pathway may provide approaches for the treatment of cancer.

Acknowledgments

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