Molecular cloning of novel Monad binding protein containing tetratricopeptide repeat domains

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Abstract We have previously reported that Monad, a novel WD40 repeat protein, potentiates apoptosis induced by tumor necrosis factor-α(TNF-α) and cycloheximide (CHX). By affinity purification and mass spectrometry, we identified RNA polymerase II-associated protein 3 (RPAP3) as a binding protein of Monad. Overexpression of RPAP3 in HEK 293 potentiated caspase-3 activation and apoptosis induced by TNF-α and CHX. In addition, knockdown of RPAP3 by RNA interference resulted in a significant reduction of apoptosis induced by TNF-α and CHX in HEK293 and HeLa cells. These results raise the possibility that RPAP3, together with Monad, may function as a novel modulator of apoptosis pathway.

Structured summary:
MINT-6551090:
Monad (uniprotkb:Q96MX6) *physically interacts* (MI:0218) with *RPAP3* (uniprotkb:Q9H6T3) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-6551101, MINT-6551118:
Monad (uniprotkb:Q96MX6) *physically interacts* (MI:0218) with *RPAP3* (uniprotkb:Q9H6T3) by *pull down* (MI:0096)
MINT-6551132:
RPAP3 (uniprotkb:Q9H6T3) *physically interacts* (MI:0218) with *Monad* (uniprotkb:Q96MX6) by *anti bait coimmunoprecipitation* (MI:0006)

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1. Introduction

Apoptosis is an evolutionarily conserved and genetically regulated biological process that plays a fundamental role in the development [1,2]. Dysregulation of apoptosis has been linked to the pathogenesis of a variety of human diseases [3]. Apoptosis is mainly orchestrated by a family of aspartate-specific cysteine proteases, caspases [4,5]. There are two main path-

ways leading to the activation of caspases, the death receptor pathway and the mitochondrial pathway. The death receptor pathway is triggered by extracellular ligands, such as FasL and TRAIL. Binding of the ligands to the death receptor causes the formation of the death-inducing signaling complex. This leads to caspase-8 activation and subsequent caspase-3 activation [6]. In the mitochondrial pathway, stress signal from within the cell induces the release of many proteins, in particular cytochrome *c*, from mitochondria into the cytosol. Once released, cytochrome *c* stimulates the assembly of an apoptosome consisting of adapter protein Apaf-1 and procaspase-9 which triggers an activation of caspase-9. Then, caspase-9 activates caspase-3, cleaving a broad spectrum of proteins in the cells and leading ultimately to apoptosis [7–10].

In an effort to identify novel molecules involved in apoptosis, we identified WD40 repeat protein Monad/WD repeat domain 92 (WDR92) [11]. Overexpression of Monad in HEK293 cells potentiated apoptosis and caspase-3 activation induced by tumor necrosis factor-α (TNF-α) and cycloheximide (CHX) [11]. Here, we identify and characterize Monad-binding protein, FLJ21908, which has been named RNA polymerase II-associated protein 3 (RPAP3) [12]. RPAP3 contains tetratricopeptide repeat (TPR) domains. The function of these domains is unknown, but it has been postulated that they are necessary for protein–protein interactions. It has been also reported that TPR domain interacts with WD40 domain [13]. The TPR motif was first identified as a tandemly repeated 34 amino acid sequence in the cell division cycle genes *cdc16*, *cdc23*, and *cdc27* which encode subunits of the anaphase promoting complex [14,15]. In addition to cell cycle regulation, they are involved in processes such as transcriptional control, protein transport, and protein folding [16,17].

In an attempt to analyze the role of RPAP3 on apoptosis, we found that knockdown of RPAP3 by RNA interference resulted in a significant reduction of apoptosis induced by TNF-α and CHX, indicating that RPAP3 plays an important role in apoptosis.

2. Materials and methods

2.1. Cloning of RPAP3

Human RPAP3 cDNA, amplified by PCR using the IMAGE clone 5218249 as a template was cloned into pENTR/D TOPO vector using

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pENTR Directional TOPO cloning kit and subcloned into the gluta-thione S-transferase (GST)-tagged pDEST27 (pDEST27-RPAP3), using Gateway System according to the manufacture's instructions (Invitrogen, Carlsbad, USA). PCR products were also cloned into the pcDNA5/FRT/V5-His-TOPO TA cloning vector (pcDNA5-RPAP3). Sequences were confirmed by automated DNA sequencing.

2.2. Reagents

Recombinant human TNF-α was purchased from R&D systems (Minneapolis, USA). CHX was from Nacalai tesque (Kyoto, Japan). Anti-procaspase-3 antibody was from BD Transduction Laboratories (Lexington, USA). Anti-poly (ADP-ribose) polymerase (PARP) anti-body and staurosporine were from Calbiochem (La Jolla, USA). Anti-V5 antibody was from Invitrogen. Anti-glutathione-S-transferase (GST), anti-Bid and anti-cleaved caspase-8 antibody (18C8) were from Cell Signaling Technology (Beverly, USA).

2.3. Cell culture

Human embryonic kidney (HEK) 293 cells or HeLa cells were 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.

2.4. Production of antibodies

Rabbit Monad antibodies have been described previously [12]. Rabbit RPAP3 antibodies were produced by immunizing rabbits with synthetic peptides corresponding to human RPAP3 [amino acid 57–71].

2.5. Transfection and immunoblotting

HEK293 cells were seeded onto 60-mm Petri dishes and grown for 24 h. The indicated plasmid were transfected with Lipofectamine 2000 according to the manufacture's instructions (Invitrogen). After 4 h, transfected 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 (Boehringer Mannheim, Mannheim, Germany). 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 [18].

2.6. Establishment of stable cell line expressing RPAP3

Flp-In 293 cell line expressing V5-tagged RPAP3 was generated as described previously [11]. In brief, Flp-In 293 cells were transfected with either pcDNA5/FRT/V5-His-chloramphenicol acetyltransferase (CAT) or pcDNA5- RPAP3 using Lipofectamine 2000. Hygromycin (100 μg/ml)-resistant clones were examined for expression of RPAP3 by immunoblotting with anti-V5 antibody.

2.7. Isolation of the Monad complex

Flp-In 293 cells expressing Monad-V5 or CAT-V5 were harvested and lysed in extraction buffer, and the lysate was cleared. The lysate was incubated with anti-V5 resin (Sigma). After washing, complexes were eluted with Laemmli denaturing buffer and subjected to mass spectrometry after SDS–PAGE.

2.8. GST pull-down assay

HEK293 cells were transfected with either pcDNA5-Monad (V5-tagged Monad) or pDEST27- RPAP3 (GST-tagged RPAP3) using Lipofectamine 2000. After 48 h, cells were harvested and lysed in extraction buffer. The supernatant was incubated with Glutathione Se-pharose (Amersham Biosciences). The mixture was washed three times with the buffer described above, and eluted with 10 mM glutathione (in 50 mM Tris, pH 8.0). The eluted proteins were boiled in SDS-loading dye.

2.9. Immunoprecipitation

Equal protein concentrations of HEK293 lysates were incubated with 3 μg of anti-RPAP3 antibody for 16 h, followed by incubation with Protein G Sepharose (Amersham Biosciences) for 1 h. The se-pharose beads were washed five times with extraction buffer, associated proteins were recovered by boiling for 5 min in Laemmli buffer, sepa-rated by SDS–PAGE and subjected to immunoblotting with anti-Monad antibody.

2.10. Detection of apoptosis

Apoptosis was assayed using cell death detection ELISA (Roche Applied science, Indianapolis, USA) as described previously [19], according to the manufacturer's instructions.

2.11. RNA isolation and cDNA synthesis

Total RNA was extracted using TRIzol (Invitrogen), and reverse-transcribed with SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol.

2.12. Quantitative real time PCR

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 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
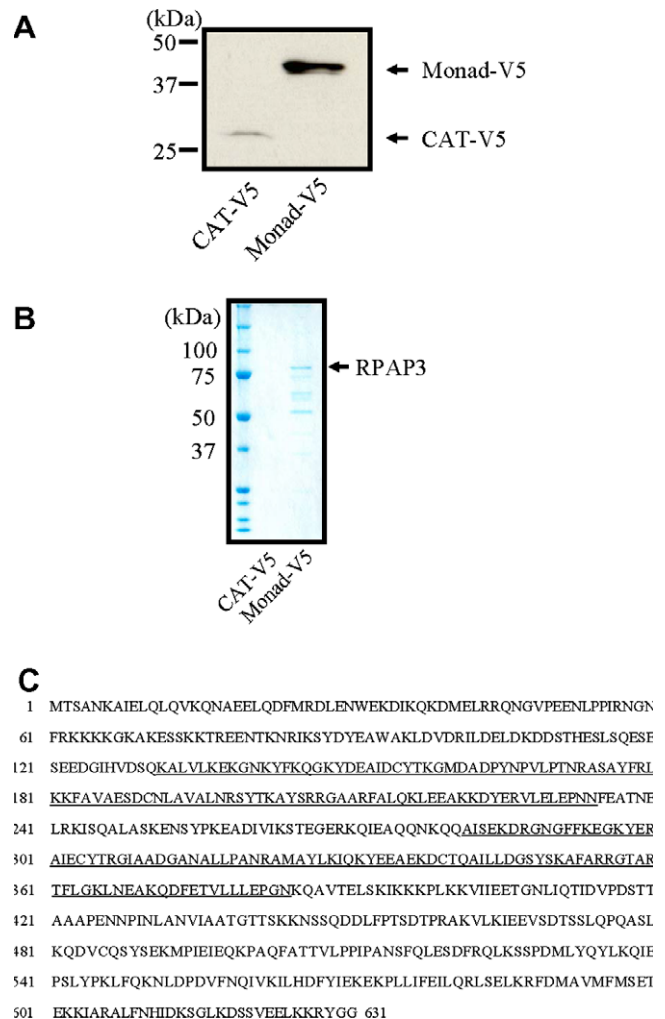
Fig. 1. Isolation of the Monad complex. (A) Cell extracts from Flp-In HEK293 cells stably expressing Monad-V5 or CAT-V5 were immu-noblotted using anti-V5 antibody. (B) Monad interacting proteins were affinity-purified from Monad-expressing Flp-In HEH293 cells, sepa-rated by SDS–PAGE, and stained with Coomassie Brilliant Blue. An equal amount of extract from CAT-expressing HEK293 cells was treated identically and used for control. Candidate polypeptides were excised and subjected to trypsin digestion. The proteins were identified by peptide mass fingerprinting. (C) Amino acid sequences of human RPAP3. The predicted TPR domains are underlined.

95 °C; 15 s at 95 °C, 1 min at 60 °C, 40 cycles). The TaqMan probe/primer sets for the endogenous control (glyceraldehyde-3-phosphte dehydrogenase, GAPDH) and target genes were as follows: GAPDH, Hs99999905_m1; Monad, Hs00399034_m1; RPAP3, Hs00226298_m1. The cycle threshold number (C_T), at which amplification entered expo-
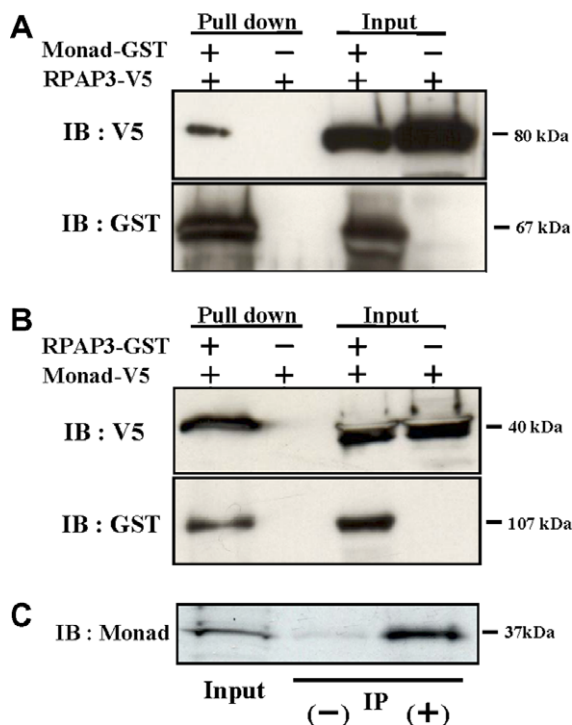
Fig. 2. Interaction of RPAP3 with Monad in HEK293 cells. HEK293 cells were transfected with expression vectors encoding RPAP3 and Monad. Lysates were prepared from transfected cells and were pulled-down with glutathione beads. Following separation by SDS–PAGE, immunoblotting was performed using anti-V5 antibody which recognizes the transfected RPAP3 (A) or Monad (B). Results using anti-GST antibody were also shown. (C) Immunoprecipitation (IP) of RPAP3 and Monad from HEK293 cells. HEK293 lysate was immunoprecipitated with control IgG (−) or RPAP3 antibody (+). Following separation by SDS–PAGE, immunoblotting was performed using anti-Monad antibody.

nential phase was used as an indicator of the relative amount of initial target RNA in each sample. Results are expressed as relative abundance of mRNA normalized to an internal control (GAPDH).

2.13. Knockdown experiments

RPAP3-specific siRNA was purchased from Qiagen and targeted the following sequences: 5′-GGACTATCTTTGAACATAA-3′ (RPAP3-siRNA1); 5′-GTATTTAAAGCAAATTGAA-3′ (RPAP3-siRNA2). AllStars Negative Control siRNA (Qiagen) was used as a control.

2.14. Statistical analysis

Data are expressed as means ± S.E.M. Statistical differences between groups were determined using Tukey test after ANOVA.

3. Results and discussion

3.1. Identification of RPAP3

To identify the proteins that interact with Monad, we have generated a stable cell line that expresses V5-tagged Monad (Fig. 1A). Cell extracts were prepared from this line and subjected to affinity purification using V5 antibodies. After washing, binding proteins were eluted from beads and fractionated by SDS–PAGE. As shown in Fig. 1B, a number of Monad-associated proteins were observed compared with control samples. By mass spectrometry, we identified RPAP3 as a specific interactor of Monad. Fig. 1C shows the amino acid sequence of human RPAP3 (GenBank™ Accession No. BC056415). RPAP3 is a protein of 631 amino acids with a deduced molecular mass of 72 kDa and contains tetratricopeptide repeat (TPR) domains (residues 133–234 and 282–383). TPR domains consist of three or more TPR motifs, which are highly degenerate 34 amino acid repeats. RPAP3 contains six TPR motifs, forming two TPR domains. The exact function of these repeats has not been elucidated, but, it is believed that these domains participate in protein–protein interactions [16,17]. The open reading frame of human RPAP3 was amplified by PCR and cloned into the expression vector with C-terminal V5-tag. V5-tagged RPAP3 was transiently transfected into HEK293 cells and cell lysates were immunoblotted with anti-V5 antibody. RPAP3 was expressed as an approximately 80 kDa protein (Fig. 4B).
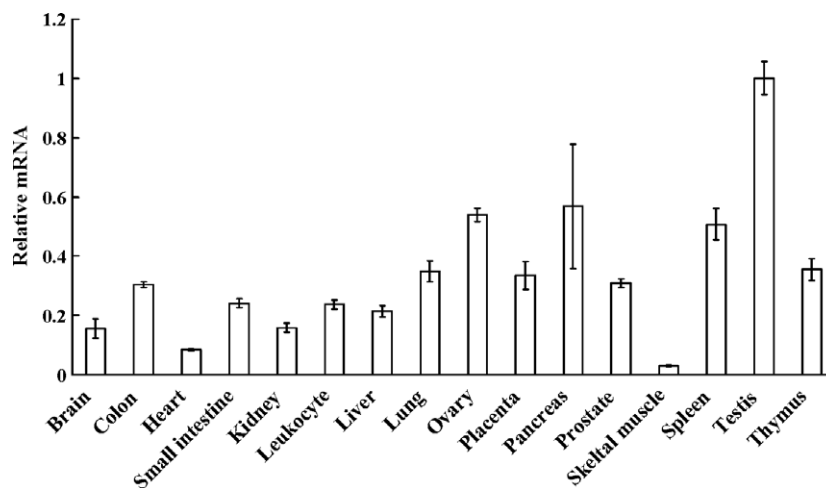
Fig. 3. Quantitative real time PCR analysis of RPAP3. Relative mRNA expression levels of RPAP3 were shown. After normalizing against endogenous GAPDH, the amounts of transcripts were compared to those found in testis, which was arbitrarily defined as 1.0. The results represent the average values with S.E.M. of triplicates.
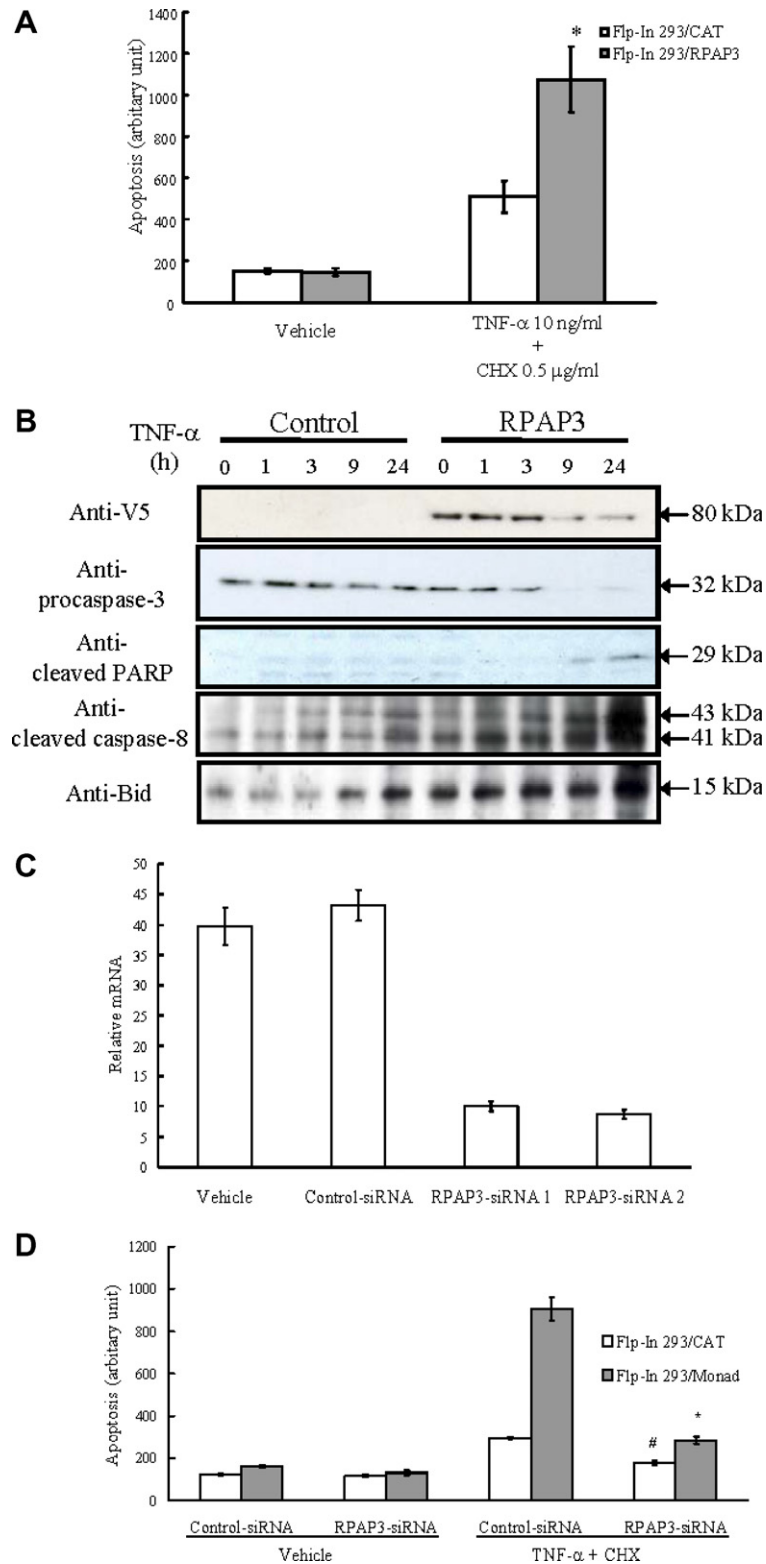
Fig. 4. (A) RPAP3 potentiates apoptosis induced by TNF-α and CHX. Flp-In HEK293 cells stably overexpressing RPAP3 were treated with TNF-α (10 ng/ml) and CHX (0.5 µg/ml) for 24 h. Relative DNA fragmentation was determined by cell death detection ELISA. The results represent the average values with S.E.M. from three independent experiments. $^{*}P < 0.01$ compared with vehicle-treated CAT-expressing cells. (B) RPAP3 activates procaspase-3 and PARP processing induced by TNF-α and CHX. Cell lysates were analyzed by immunoblotting using anti-V5, anti-procaspase-3, anti-PARP, anti-cleaved caspase-8 or anti-Bid antibody. One of the representative results from three independent experiments is shown. (C) RNAi-mediated knockdown of RPAP3 in HEK293 cells. Transcriptional level of RPAP3 was determined by real time PCR. The relative expression of RPAP3 was normalized to GAPDH. Average values of three independent experiments are shown. Bars represent S.E.M. (D) Monad-expressing Flp-In HEK293 cells transfected with control siRNA or RPAP3 siRNA1 were treated with TNF-α (10 ng/ml) and CHX (0.5 µg/ml) for 24 h. Relative DNA fragmentation was determined by *cell death detection ELISA*. The results represent the average values with S.E.M. from three independent experiments. $^{\#}P < 0.01$ compared with control-siRNA-treated CAT-expressing cells. $^{*}P < 0.01$ compared with control-siRNA-treated Monad-expressing cells.

To demonstrate that Monad and RPAP3 indeed interact, GST pull-down assay was performed. The HEK293 cells were transfected with RPAP3 (V5-tagged) in the presence or absence of Monad (GST-tagged), and the cell lysates were pulled-down with glutathione beads. Western blot analysis with anti-V5 antibody following pull-down showed that RPAP3 interacted with Monad in the cells (Fig. 2A). As shown in Fig. 2B, we confirmed the result by transfecting with Monad (V5-tagged) in the presence or absence of RPAP3 (GST-tagged). We next examined whether RPAP3 and Monad associate physiologically. Using HEK293 lysate, we confirmed that RPAP3 interacted with Monad (Fig. 2C).

3.2. Tissue distribution of RPAP3 mRNA

Using quantitative real time PCR, we examined the mRNA distribution of RPAP3 in various human tissues. The human RPAP3 transcript was expressed in most tissues examined. As shown in Fig. 3, RPAP3 transcripts were most abundant in testis, and also present at lower levels in numerous other tissues including ovary, pancreas, and spleen when we used GAPDH as a control. It should be noted that Monad transcripts were also most abundant in testis, as we reported previously [11].

3.3. Effect of RPAP3 on apoptosis

We generated HEK293 cells stably expressing V5-tagged RPAP3. To examine apoptosis in the stable cell line, HEK293 cells were treated with TNF-α and CHX for 24 h. Apoptosis was assessed using *Cell death Detection ELISA*. As shown in Fig. 4A, RPAP3 enhanced apoptosis induced by TNF-α and CHX. Expression of RPAP3 in stable cell line was confirmed by immunoblotting with anti-V5 antibody. As shown in Fig. 4B, the band of RPAP3 disappeared during the apoptotic process. In addition, RPAP3 induced the activation of caspase-3, as demonstrated by the processing of procaspase-3 and the cleavage of the caspase-3 substrate PARP (Fig. 4B). In the death receptor pathway, pro-apoptotic Bcl-2 family member Bid needs to be cleaved by caspase-8 and the cleaved Bid conveys the apoptotic signal to mitochondria [20]. To determine the molecular basis underlying RPAP3-enhanced apoptosis, we checked this critical step in TNF-α-induced apoptosis. As shown in Fig. 4B, cleaved products of caspase-8 (p41 and p43) and Bid (p15) were observed in RPAP3-over-expressing lysate. These results indicate that RPAP3 promotes TNF-α-induced apoptosis through death receptor-mediated caspase-3 activation pathway.

To test whether endogenous RPAP3 regulates apoptosis, we knocked down endogenous RPAP3 by small interfering RNA (siRNA). We generated two unique siRNAs and both resulted in a four-fold reduction in RPAP3 mRNA in HEK293 cells (Fig. 4C). We examined the effect of siRNA knockdown of RPAP3 (RPAP3-siRNA1) on apoptosis in Monad-expressing HEK293 cells. Treatment with siRNA against RPAP3 resulted in a significant reduction of apoptosis induced by TNF-α and CHX (Fig. 4D). Similar results were obtained when we used RPAP3-siRNA2 (data not shown). Protective effect of RPAP3 siRNA was also observed when we treated HeLa cells, with TNF-α/CHX or staurosporine (Fig. S1). These results suggest that RPAP3 plays an important role in apoptosis.

TPR domains are composed of loosely conserved 34-amino acid sequence motifs that are repeated 1–16 times per domain.

Originally identified in components of the anaphase promoting complex [14,15], TPR domains are now known to mediate specific protein interactions in numerous cellular contexts. TPR-containing proteins include the anaphase promoting complex (APC) subunits cdc16, cdc23, and cdc27, the NADPH oxidase subunit p67 phox, hsp90-binding immunophilins, transcription factors, the PKR protein kinase inhibitor, and peroxisomal and mitochondrial import proteins [16,17]. We previously reported that Monad has WD40 repeat domain [11]. It has been reported that TPR domain binds to WD40 domain [13]. Thus, it is postulated that the TPR domain of RPAP3 acts as a binding site for Monad.

WD40 repeat domain, which was originally identified in the β-subunit of heterotrimeric G-proteins [21] is involved in diverse biological functions including signal transduction [22,23], RNA splicing [24], and transcription [25–29]. It has been reported that the CUL4-DDB1 ubiquitin E3 ligase associates with multiple WD40 repeats proteins and that each WD40 repeats protein functions as a substrate-specific adaptors for CUL4-DDB1 ubiquitin E3 ligase [30–33]. Therefore, Monad or RPAP3 might participate in the event involved in ubiquitin-proteasome pathway. Identification of substrate protein(s) recruited for ubiquitination by Monad or RPAP3 should clarify the role of Monad and RPAP3 in apoptosis.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.05.041.

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