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Molecular cloning of novel Monad binding protein containing tetratricopeptide repeat domains

Yuki Itsuki[a,c], Makio Saeki[a,*], Hirokazu Nakahara[b], Hiroshi Egusa[c], Yasuyuki Irie[d], Yutaka Terao[e], Shigetada Kawabata[e], Hirofumi Yatani[c], Yoshinori Kamisaki[a,f]

[a] *Department of Pharmacology, Graduate School of Dentistry, Osaka University, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan*
[b] *The First Department of Oral and Maxillofacial Surgery, Osaka University, Japan*
[c] *Department of Fixed Prosthodontics, Graduate school of Dentistry, Osaka University, Japan*
[d] *Department of Pharmacology, Iwate Medical University, Morioka, Iwate, Japan*
[e] *Department of Oral and Molecular Microbiology, Graduate School of Dentistry, Osaka University, Japan*
[f] *E-Institute of Shanghai Universities, Division of Nitric Oxide and Inflammatory Medicine, Shanghai, China*

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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)

Keywords: Apoptosis; Cell death; Caspase; WD repeat; TPR; TNF-α

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 pathways 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

*Corresponding author. Fax: +81 6 6879 2914.
E-mail address: msaeki@dent.osaka-u.ac.jp (M. Saeki).

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pENTR Directional TOPO cloning kit and subcloned into the glutathione *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) antibody 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 Sepharose (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 sepharose beads were washed five times with extraction buffer, associated proteins were recovered by boiling for 5 min in Laemmli buffer, separated by SDS–PAGE and subjected to immunoblotting with anti-Monad antibody.

2.10. Detection of apoptosis

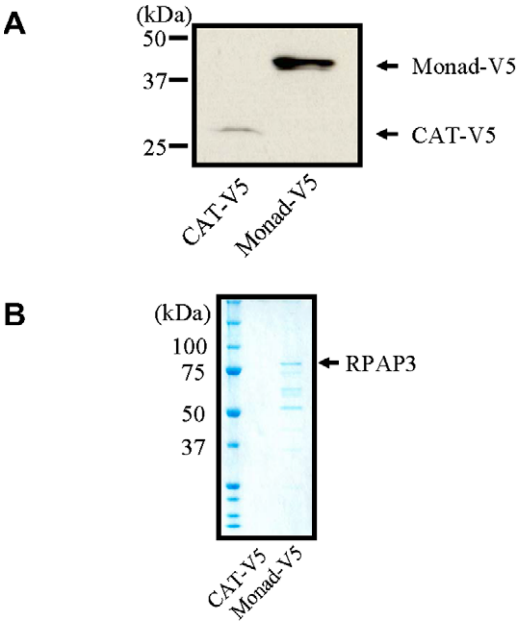
Apoptosis was assayed using cell death detection ELISA (Roche Applied science, Indianapolis, USA) as described previously [19], according to the manufacturer's instructions.

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



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1 MTSANKAIELQLQVKQNAEELQDFMRDLENWEKDIKQKDMELRRQNGVPEENLPPIRNGN
61 FRKKKKGKAKESSKKTREENTKNRIKSYDYEAWAKLDVDRILDELDKDDSTHESLSQESE
121 SEEDGIHVDSQKALVLKEKGNKYFKQGKYDEAIDCYTKGMDADPYNPVLPTNRASAYFRL
181 KKFAVAESDCNLAVALNRSYTKAYSRRGAARFALQKLEEAKKDYERVLELEPNNFEATNE
241 LRKISQALASKENSYPKEADIVIKSTEGERKQIEAQQNKQQAISEKDRGNGFFKEGKYER
301 AIECYTRGIAADGANALLPANRAMAYLKIQKYEEAEKDCTQAILLDGSYSKAFARRGTAR
361 TFLGKLNEAKQDFETVLLLEPGNKQAVTELSKIKKKPLKKVIIEETGNLIQTIDVPDSTT
421 AAAPENNPINLANVIAATGTTSKKNSSQDDLFPTSDTPRAKVLKIEEVSDTSSLQPQASL
481 KQDVCQSYSEKMPIEIEQKPAQFATTVLPPIPANSFQLESDFRQLKSSPDMLYQYLKQIE
541 PSLYPKLFQKNLDPDVFNQIVKILHDFYIEKEKPLLIFEILQRLSELKKRFDMAVMFMSET
601 EKKIARALFNHIDKSGLKDSSVEELKKRYGG 631

Fig. 1. Isolation of the Monad complex. (A) Cell extracts from Flp-In HEK293 cells stably expressing Monad-V5 or CAT-V5 were immunoblotted using anti-V5 antibody. (B) Monad interacting proteins were affinity-purified from Monad-expressing Flp-In HEH293 cells, separated 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.