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RTP1, a Rat Homologue of Adenovirus E1A-associated Protein BS69, Interacts with DNA Topoisomerase II

Misook Oh, Geun Bae Rha, Jeong-Ho Yoon, Yang-Il Sunwoo[1], Seung Hwan Hong, and Sang Dai Park*

School of Biological Sciences, Seoul National University, Seoul 151-742, Korea;
[1]Department of Biology, College of Natural Sciences, Dong-A University, Busan 604-714, Korea

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Topoisomearse II is an essential enzyme in all organisms with several independent roles in DNA metabolism. Recently, it has been demonstrated that the C-terminal region of topoisomerases II is associated with heterologous protein-protein interactions in human and yeast. In this study, we identified that RTP1, a rat homologue of E1A binding protein BS69, is another topoisomerae II interacting protein by yeast two-hybrid screening. RTP1 has an E1A-binding domain and a MYND motif, which are known to be required for transcriptional regulation by binding to other proteins and interaction with the leucine zipper motif of topoisomerase II. The physical interaction between RTP1 and topoisomerase IIα was examined by GST pull-down assay *in vitro*. The expression level of RTP1 peaks in S phase as that of topoisomerase IIα. These results suggest that the interaction between topoisomerase IIα and RTP1 might play an important role in regulating the transcription of genes involved in DNA metabolism in higher eukaryotes.

Regulation of topological state of DNA is essential for a number of cellular processes such as replication, transcription, chromosome condensation, recombination, and DNA repair (Uemura et al., 1987; Yang et al., 1987; Hsiang et al., 1988; Wang et al., 1990; Adachi et al., 1991). This regulation is primarily executed by DNA topoisomerases that alter the topological state of DNA, but not its covalent structure. Topoisomerases fall into two classes, type I and type II, according to their action mechanism and sequence homology (Roca, 1995; Wang, 1996). The type II enzymes cleave both strands of double-stranded DNA, pass another helix through the break, and re-ligate the two strands, thereby catalyzing relaxation of negatively and positively supercoiled DNA molecules.

DNA topoisomerase II has been purified from a variety of organisms, including yeast (Goto and Wang, 1982), *Drosophila* (Hsieh and Brutlag, 1980), *Xenopus* (Baldi et al., 1980), human HaLa cells (Miller et al., 1981), and phage T4-infected *E. coli* (Liu et al., 1979). Generally, purified topoisomerase II requires ATP and Mg^{2+} for its enzymatic activities. It has been reported that there are two different isoforms of DNA topoisomerase II in human and rat. The newly identified isotype, named topoisomerase IIβ, is a 180 kD protein which is structurally similar but genetically and biochemically distinct from the previously reported topoisomerase II and exhibits differences in nuclear localization and cell cycle expression.

Based on the homology to the bacterial topoisomerase II (DNA gyrase), eukaryotic topoisomerase II can be divided into three distinct domains; N-terminal ATPase domain, central DNA breakage-reunion domain containing active tyrosine residue, and variable C-terminal domain (Shiozaki and Yanagida, 1991; Austin et al., 1995). The N-terminal and central domains are highly conserved between various species and are believed to constitute a catalytic core. The C-terminal domain is divergent and species-specific and is involved in non-catalytic roles such as regulation of enzyme activity or intracellular localization. Analysis of amino acid sequence of the topoisomerase II also revealed several phosphorylation sites and a potential leucine zipper motif at the C-terminal domain (Austin et al., 1993; Park et al., 1993). The leucine zipper motif is highly conserved among other eukaryotic topoisomerases II and may be important in protein-protein interaction with itself or other proteins (Kroll et al., 1993).

Interaction of eukaryotic topoisomerase II with other proteins has been reported in several studies. Casein kinase II was identified to interact with topoisomerase II (Cardenas et al., 1992; Bojanowski et al., 1993). In *S. cerevisiae*, two proteins, Sgs1 and Pat1 are known to interact with topoisomerase II and required for

* To whom correspondence should be addressed.
Tel: 82-2-880-6689, Fax: 82-2-887-6279
E-mail: sdpark@plaza.snu.ac.kr

faithful chromosome segregation (Watt et al., 1995; Wang, 1996). The association domain of topoisomerase II was localized at a putative leucine zipper motif. As mentioned earlier, the leucine zipper motif in mammalian enzyme could interact with other proteins containing this motif *in vitro* (Kroll et al., 1993). In parallel, it has been demonstrated that a novel chromosome associated *Drosophila* protein, barren product, binds to topoisomerase II and alters the activity of topoisomerase II (Bhat et al., 1996). This association was also required for proper chromosome segregation. In addition, elucidation of interactions between topoisomerase IIα and cellular proteins would be a key to answer to many questions on the action of topoisomerase II.

In this study, to elucidate the unknown cellular function of topoisomerase II, yeast two-hybrid screening was used. From this screening, we identified the rat topoisomerase II-interacting protein, RTP1. The RTP1 shows striking sequence similarity with human BS69 protein, which binds to adenovirus E1A and regulates its transcription. The expression patterns of each transcript during cell cycle were also similar to each other. Therefore, our data suggest that topoisomerase IIα and RTP1 together may regulate the transcription of several genes engaged in DNA metabolism.

Materials and Methods

Strains and reagents

The yeast *Saccharomyces cerevisiae*, Y190 (*MAT-a ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3 gal4Δ gal80Δ*) was used for yeast two-hybrid screening. The plasmid pGBT9 for bait vector construction was purchased from Clontech. Rat lung Matchmaker cDNA library was also purchased from Clontech. Media and serum for growth of yeast and animal cell lines were purchased from Difco, Gibco-BRL, and Hyclone. All reagents and various enzymes were from Sigma Aldrich and Takara.

Yeast two-hybrid screening

Two-hybrid screening was performed as described in Clontech protocol manual (PT1020-1). Rat lung cDNA library was transformed into reporter strain Y190, which already had a bait vector pGBT9-PAD and then plated onto SD plates lacking amino acids tryptophan, leucine, and histidine but including 25 mM 3-amino triazole (3-AT). His^+ colonies were tested for β-galactosidase activity assay on X-gal plates. Plasmids were isolated from colonies that were blue on X-gal plates and back-transformed into Y190 cell harboring a bait plasmid to verify positive interactions. The bait plasmids were prepared as follows. The fragment of rat topoisomerase II cDNA (a.a 857-1042) was amplified by blunt-ended PCR with Vent Taq polymerase (New England Biolab.) using CT5 (5′-ATCGGGTGGTCCTGCAAA-3′) and CT3 (5′-TGCACCAAGCATTCCTAGAAGC-3′) primers. Following treatment with T4 polynucleotide kinase, the DNA was ligated to pGBT9 vector, which was blunt ended with Klenow enzyme after *Bam*HI digestion. The resulting construct was named as pGBT9-PAD. The other two plasmids, pGBT9-PADΔ Lzip and pGBT9-Lzip, were constructed as follows. The pGBT9-PAD was digested with restriction enzymes *Spe*I and *Sal*I to remove the leucine zipper motif and then self-ligated to generate pGBT9-PADΔLzip plasmid after treatment with Klenow fragment. The pGBT9-Lzip plasmid contains the leucine zipper motif from pCGSrT2 bearing wild type rat topoisomerase IIα. The exact orientation and sequences was verified by DNA sequencing.

Preparation of antibodies raised against RTP1 protein

Rabbit polyclonal antibodies were raised against RTP1 protein. The RTP1 cDNA was cloned into the pGEX4T-1 expression vector to make GST-tagged fusion protein and transformed into *E. coli* BL21. The induced protein was resolved by SDS-polyacrylamide gel electrophoresis and excised from the gel. The protein was eluted in PBS and injected into rabbits. The immune serum was used for Western blot analysis at 1 : 10000.

Preparation of nuclear extract

After washing with TD buffer (100 mM NaCl, 20 mM KCl, 0.5 mM Na_2HPO_4, and 20 mM Tris-HCl, pH 7.45), cells were scraped out and harvested. The cells were resuspended in Buffer A (100 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 20 mM Tris-HCl, pH 7.5, 0.1 mM PMSF, 10% glycerol, 0.2% NP-40, and 0.1% Triton X-100), incubated on ice for 10 min, and spun down. The pelleted nuclei were resuspended in Buffer A and a 1/10 sample volume of 10% SDS, and then sonicated.

Animal cell culture and cell cycle arrest

Mouse fibroblast NIH3T3 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (penicillin G and streptomycin). C6 rat glial tumor cells were maintained in monolayer culture in nutrient mixture F-10 supplemented with 15% horse serum, 2.5% fetal bovine serum and antibiotics. To synchronize the cell cycle, exponentially growing C6 cells were treated with 0.4 mM mimosine, 4 mM thymidine, or 25 mg/mL colcemide and further grown for 24 h.

GST-pull down assay

E. coli cells harboring pGST-RTP1 was lysed in a buffer (50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM DTT and 100 mM NaCl), mixed with 10 mL bed volume of GST-sepharose beads, and incubated at 4℃ for 2 h with rotation. The beads were precipitated and washed 6 times with the same buffer. The beads were eluted with GST elution buffer and the eluents were analysed

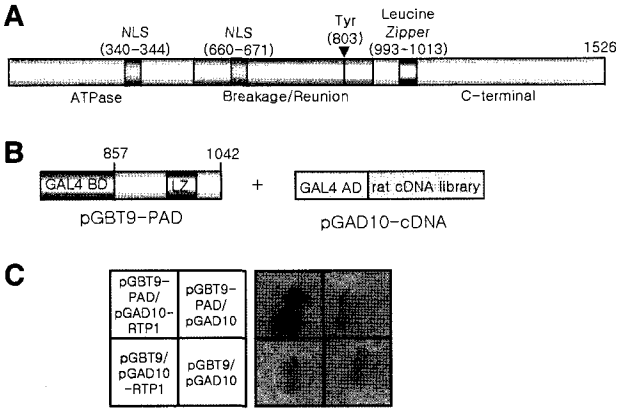

Fig. 1. Topoisomerase IIα interacts with RTP1 protein. A, Schematic representation of the rat topoisomerase IIα protein showing its functional domains. Nuclear localization signals (NLS), active tyrosine residue (▼), and leucine zipper motif (LZ) were also denoted. B, Schematic diagrams of two plasmids used in yeast two-hybrid screen. The bait vector pGBT9-PAD consisted of yeast GAL4 binding domain (BD) and partial rat topoisomerase II containing leucine zipper motif. The rat lung cDNA library was fused to GAL4 activation domain (AD) in pGAD10 vector. C, The expression of *lacZ* reporter gene between the bait and the prey harboring a putative topoisomerase II-interacting protein. While only the positive clone containing the partial topoisomerase IIα gene and prey cDNA named as RTP1 became blue, other cells transformed with one or both parental empty vector did not change their colony color.

by SDS-PAGE, followed by western blot with anti-topoisomerase II antibodies (TopoGen).

Results and Discussion

Yeast two-hybrid screening for proteins that interact with zinc-finger domain of rat topoisomerase IIα

The rat topoisomerase II protein contains several highly conserved domains (Fig. 1A). The active Tyr is at position 803 and two nuclear localization signals are found at amino acid positions 341-344 and 659-674, similarly to the human topoisomerase IIα. The enzyme also contains a putative leucine zipper motif at position 993-1014 and alternating charged residues clusters in its C-terminal one thirds (Park et al., 1993).

It is possible to postulate that the putative leucine zipper motif might be involved in protein-protein interaction. Along the same line, it has been demonstrated that Sgs1 and Pat1 interact with yeast topoisomerase II via the putative leucine zipper motif (Watt et al., 1995; Wang, 1996). To identify the proteins which interact with rat topoisomerase II, yeast two-hybrid screen was carried out. The pGBT9-PAD containing fragment of rat topoisomerase IIα (a.a 857-1042) and the DNA-binding domain of yeast GAL4 protein was used as a bait vector and rat lung cDNA library fused to the GAL4 activation domain was used as preys (Fig. 1B).

Forty eight His^+ and β-Gal^+ positive clones were isolated from about 5×10^6 independent transformants. The library DNA extracted from these positive clones was back-transformed into Y190 reporter strain harboring a bait vector pGBT9-PAD. Finally two clones

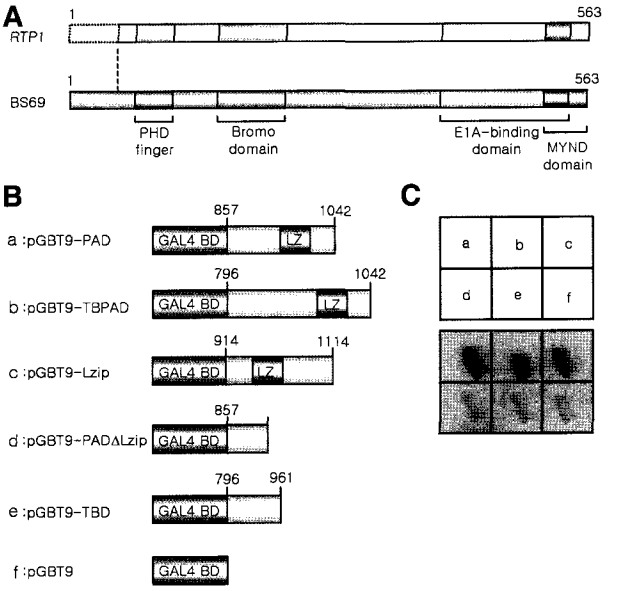

Fig. 2. The leucine zipper motif is necessary to the interaction between topoisomerase IIα and RTP1. A, Schematic alignment of RTP1 and BS69 protein. Their amino acid sequences are very similar to each other and their protein-protein interaction domains are evolutionarily conserved and consist of a PHD finger, bromodomain, E1A-binding domain, and MYND domain. Each domain was defined with the bracket. B, The leucine zipper motif of rat topoisomerase IIα may be needed to interact with RTP1. Various constructs were prepared to test which region of topoisomerse IIα was required for interaction with RTP1 by two-hybrid method. While the pGBT9-PAD, -TBPAD, and -Lzip contained leucine zipper motif, the pGBT9-PADΔLzip and TBD did not have leucine zipper motif. C, The blue signal was appeared only in the clones harboring a bait vector containing the leucine zipper motif.

showed positive signal in β-galactosidase assay (Fig. 1C). Two genes selected as a prey were designated as RTP1 (Rat Topoisomerase II associated Protein 1) and RTP2.

RTP1 is homologous to human BS69 protein, which has adenovirus E1A-binding motif

Comparison of their nucleotide sequences revealed that RTP1 and RTP2 are the same gene products and rat homologues of human BS69 protein. Hateboer et al. (1995) reported that BS69 directly binds to the adenovirus E1A protein and inhibits its transcriptional activation. The deduced partial amino acid sequences of RTP1 show a high degree of homology (96%) to that of human BS69 protein (Fig. 2A). BS69 protein has three conserved domains that regulate the transcription activity without binding directly to DNA. The N-terminus of BS69 contains a PHD finger domain (amino acids 63-110), which is found in many proteins involved in chromatin mediated transcriptional regulation. In addition, BS69 has a bromodomain (amino acids 145-215), which is present in chromatin remodeling protein SWI2/SNF2/BRM and histone acetyltransferases P/CAF and CBP/p300 proteins (Dhalluin et al., 1999). Finally, a MYND domain is near the C-terminus of BS69, which consists of two zinc finger domains,

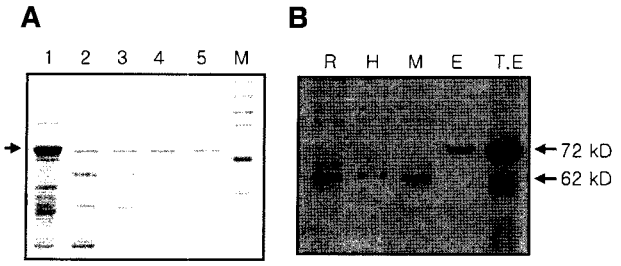

Fig. 3. The RTP1 protein was conserved in higher eukaryotes A, The SDS-polyacrylamide gel electrophoresis after the GST-RTP1 protein over-expression. 1; *E. coli* total cell extract after induction to over-express the GST-RTP1 protein, 2; Flow through of chromatography with glutathion sepharose beads, 3; The first washed solution, 4 and 5; The first and second eluents, respectively, M; protein markers. B, The raised anti-RTP1 antibodies could detect the mouse and human RTP1 homologue. About 62 kD bands corresponding to RTP1 were appeared in lanes of rat C6 (R), human HeLa (H), and mouse NIH3T3 (M) cell extracts by Western blot analysis using anti-RTP1 antibodies. The eluted (E) and over-expressed RTP1 proteins in *E. coli* total cell extract (T.E) were also detected at the size of 72 kD.

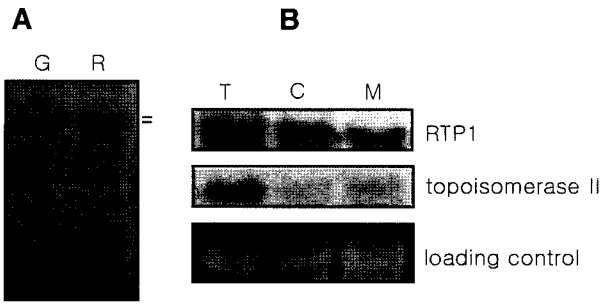

Fig. 4. The expression patterns of two proteins are similar to each other A, Direct association between topoisomerase IIα and RTP1 was analyzed by GST pull-down assay. *E. coli* extract harboring GST-RTP1 fusion protein or GST protein only was incubated with rat C6 nuclear extracts in the presence of glutathion sepharose beads and then the eluents from beads were analyzed by western blot with human topoisomerase II antibody. Two isoforms of topoisomerase II (α and β) were detected by topoisomerase II antibody due to their sequence homology. It was implied that RTP1 might interact with tiopoisomerase IIβ. B, Northern blot analysis showed RTP1 and topoisomerase II expression level peaks in S phase. The total RNA was extracted from C6 cells treated with thymidine (T), colcemide (C), or mimosine (M) and then subjected to northern blot with ^{32}P-labeled RTP1 probe or PAD probe to detect topoisomerase IIα.

corresponding to the binding region in RTP1 with rat topoisomerase IIα. It has been shown that the MYND domain can bind the transcriptional corepressor N-CoR, a component of histone deacetylase complex that causes transcriptional repression (Masselink and Bernards, 2000).

As shown in Fig. 2A, the E1A-binding motif is found in the C-terminal part of RTP1 (Fig. 2A). The isolated RTP1 protein has all three domains like BS69, and this result suggests that the protein might be involved in chromatin remodeling and transcriptional regulation.

RTP1 interacts with the putative leucine-zipper motif of rat topoisomerase IIα

To determine whether the putative leucine zipper motif of rat topoisomerase IIα interact with RTP1, the two-hybrid interaction assay was repeated using several constructs containing variable regions of rat topoisomerase IIα cDNA (Fig. 2B). The pGBT9-TBPAD containing the active tyrosine and a leucine zipper and another construct pGBT9-Lzip including a leucine zipper motif could also activate the transcription of reporter gene. However, the pGBT9-PADΔLzip having deleted leucine zipper and pGBT9-TBD containing active tyrosine without the leucine zipper motif failed to activate the transcription of reporter gene (Fig. 2C). This result strongly indicates that rat topoisomerase IIα specifically interacts with RTP1 via its leucine zipper motif.

RTP1 is a conserved protein in higher eukaryotes

To examine the expression level of RTP1 and its binding activity to topoisomerase IIα *in vitro,* polyclonal antibodies against RTP1 were prepared as described in Materials and Methods. The GST-RTP1 protein purified from *E. coli* was used to raise anti-RTP1 antibodies (Fig. 3A). The antibodies could specifically detect RTP1 homologues in mouse and human (BS69) as well as RTP1 in rat cell extract (Fig. 3B). This observation implies that the RTP1 homologues exist in the mouse tissue as well as in the human, suggesting that RTP1 is conserved in higher eukaryotes.

The expression level of topoisomerase IIα and RTP1 peaks in S phase

Based on the fact that BS69 acts as a transcriptional co-repressor and a potential tumor suppressor via interaction with other proteins, we further examined the interaction of topoisomerae IIα with RTP1 *in vitro* and expression level of the two genes. GST pull-down assay by GST-RTP1 protein revealed that rat topoisomerase IIα interacts with RTP1 *in vitro* (Fig. 4A). If these two proteins work together in transcriptional regulation, their expression patterns would also be similar to each other. Northern blot analysis was carried out to measure the transcriptional level of topoisomerase IIα and RTP1 in rat C6 cell after treatment with agents for specific cell cycle arrest (Fig. 4B). The expression of topoisomerase IIα and RTP1 peaks in cells treated with thymidine, a specific drug for cell cycle arrest at S phase. The patterns of their expression level were similar to each other. It was known that topoisomerase IIα shows a peak of mRNA expression in late S phase and the pattern of its expression is also maintained upon tumor virus transformation (Woessner et al., 1991; Goswami et al., 1996; Isaacs et al., 1998). The similar expression pattern of RTP1 and topoisomerase IIα suggests that topoisomerase IIα and RTP1 might be subject to common transcriptional regulation.

Conclusion

It has been reported that topoisomerase IIα contains a

potential leucine zipper motif which is required for protein-protein interactions. The DNA binding and dimerization regions of cAMP responsive element binding protein (CREB), the activating transcription factor-2 (ATF-2), or GCN4 could interact with a C-terminal fragment of human topoisomerase IIα and also modify the activity of topoisomerase II via protein-protein interaction (Kroll et al., 1993).

Another result shows that human topoisomerase IIα region (857-1447) also interacts with GH factor-1 and E1A (unpublished data of Kroll et al.). Furthermore, E1A interacts with various chromatin modifying proteins, transcriptional regulators, and BS69, a RTP1 homologue in human (Lee et al., 1991; Geisberg et al., 1994; Hateboer et al., 1995). BS69 also interacts with c-Myb, N-CoR, and B-myb through its MYND domain in C-terminus and regulates their transcription (Masselink and Bernards, 2000; Ladendorff et al., 2001; Masselink et al., 2001). Collectively, adenovirus E1A protein interacts with BS69 and BS69 and topoisomerase IIα interact with each other. Therefore, it is postulated that RTP1 might regulate the transcription of genes involved in DNA metabolism exerted by topoisomerase II through interaction with rat topoisomerase IIα.

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