Biomolecules & Therapeutics, **17(1)**, 12-16 (2009)
www.biomolther.org

ISSN: 1976-9148(print)/2005-4483(online)
DOI: 10.4062/biomolther.2009.17.1.12

Identification of Regulatory Role of KRAB Zinc Finger Protein ZNF 350 and Enolase-1 in RE-IIBP Mediated Transcriptional Repression

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(Received October 31, 2008; Revised January 6, 2009; Accepted January 16, 2009)

Abstract − One of the WHSC1/MMSET/NSD2 variant RE-IIBP is a histone H3-K27 methyltransferase with transcriptional repression activity. Overexpression of RE-IIBP in various types of leukemia suggests it's role in leukemogenesis. Here we identify two proteins, KRAB zinc finger protein ZNF 350 and enolase-1 as RE-IIBP interacting proteins by yeast two-hybrid screening and confirmed direct interaction *in vivo* and *in vitro*. Both proteins have been known for their role in transcriptional repression. Reporter assays using transient transfection demonstrated that both ZNF 350 and enolase-1 proteins synergistically repressed transcription with RE-IIBP, respectively. These results indicate both proteins have roles in RE-IIBP mediated transcriptional repression by involving co-repressor complex.

Keywords: RE-IIBP, Yeast two-hybrid screening, ZNF 350, Enolase-1

INTRODUCTION

In all eukaryotes, the histone modification plays an important role in the transcriptional regulation, development, and DNA repair (Kouzarides, 2007). The methylation of histone tails at specific residues are five lysines within K4, K9, K27, K36, K79 on H3 and one lysine K20 within histone H4 that have been methylated by specific histone methyltransferase (HMTase) (Sims *et al*., 2003). The different sites of methylation on histone result in different biological effect. Among various HMTases reported, the response element II binding protein (RE-IIBP) has been reported to express among the WHSC1/MMSET/NSD2 splice variants and was identified to bind to RE-II of human interleukin (IL-5) promoter and to repress its transcription (Garlisi *et al*., 2001). RE-IIBP contains SET-domain with histone H3-K27 HMTase activity and transcriptional repression through recruiting histone deacetylases (HDACs). Furthermore, we reported that RE-IIBP is overexpressed in blood cells of various types of leukemic patients, resulted in high H3-K27 methylation (Kim *et al*., 2008).

Protein-protein interactions are essential to cellular signal transduction mechanisms following biologically responsive signals. The yeast two-hybrid method is a powerful technique and flexible system for analyzing these protein-protein interactions (Ito *et al*., 2002). In the yeast two-hybrid system, the protein of interest, the "bait", is fused to a DNA-biding domain whereas the other protein, termed "prey" is expressed with the activation domain. When bait and prey proteins interact each other, it induces the expression of reporter genes in downstream of the DNA binding sites.

Transcription factors are well known to regulate important cellular processes, such as cell growth and differentiation. The common DNA binding motif of these transcription factors have been identified including the $(Cys)_2$-$(His)_2$-type zinc finger motif. It has been known that N-terminal domain of zinc finger genes, called the KRAB (Krüppel-associated box) domain containing protein, acts as a transcriptional repressor (Vissing *et al*., 1995). The KRAB domain homology consists of 75 amino acids and is predicted to fold into two charged amphipathic helices (Witzgall *et al*., 1994). Recently, co-repressor known as KAP1, TIF1, KRIP1 are required for transcriptional repression of KRAB-containing proteins. KAP1 binds to the KRAB domain of the KRAB-containing protein and serves as a scaffold for further recruitment of HP1, HDACs and SETDB1, to form heterochromatin (Schultz *et al*., 2002).

Enolase-1 (ENO-1), known as α-enolase, is the glycolytic enzyme that catalyzed the production of phosphoenolpyruvate from 2-phosphoglycerate. Also, ENO-1 cDNA

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encodes the Myc-binding protein-1 (MBP-1), which binds to the c-myc P2 promoter and down-regulates the expression of c-myc. The longer form (48 kDa) of ENO-1 localized in both cytoplasm and nuclei, while the shorter MBP-1 (37 kDa) is localized mostly in the nuclei (Ray and Miller, 1991; Subramanian and Miller, 2000). Recent reports suggest that ENO-1 as well as MBP-1 play an important role in the regulation of c-myc promoter activity. In addition, MBP-1 is involved in the regulation of cell growth and apoptosis (Feo *et al*., 2000).

In this study, using yeast two-hybrid screening, we have identified various RE-IIBP interacting proteins including KRAB zinc finger protein ZNF 350 and ENO-1. We have demonstrated that ZNF 350 and ENO-1 can interact with RE-IIBP both *in vitro* and *in vivo*. Furthermore, we suggest that this interaction might play important role in the previously reported RE-IIBP mediated transcriptional repression.

MATERIALS AND METHODS

Plasmid constructs

For construction of mammalian expression vectors, pcDNA3.1-RE-IIBP was described previously (Kim *et al*., 2008). pcDNA6-RE-IIBP-myc and pGBKT7-RE-IIBP were prepared by subcloning PCR amplication of coding region or carboxyl-terminal region (pGBKT7-RE-IIBP, amino acid 160-560) from pcDNA3.1-RE-IIBP. Sequences of all constructs were confirmed by DNA sequencing. ENO-1 was amplified from pCMV-ENO-1 and subcloned into pcDNA6-myc-HisA vector (Invitrogen). pcDNA3.1-ZNF 350 was kindly provide by Dr. Olivia M. Pereira-Smith at Baylor College of Medicine.

Yeast two-hybrid screening

All assays were carried out according to the protocols of MATCHMAKER GAL4 Two-Hybrid System 3 (BD Biosciences). AH109 yeast strain of *Saccharomyces cerevisiae* was transfected with the pGBKT7-RE-IIBP plasmid "bait" construct and bone marrow cDNA library. In this library, cDNAs are fused to the GAL4 activation domain in pGADT7. Independent clones were found to grow on a minimal medium lacking leucine, tryptophan, and histidine with 2.5 mM 3-amino-1,2,4-triazole. In order to confirm the positive reactions, colony-lift filter assay was used to check the activity of β-galactosidase. Plasmid DNA from positive yeast clones were further characterized by sequencing and analyzed for gene homology by the BLAST database.

Cell culture and transfections

Human embryonic kidney HEK293 cells were cultured in DMEM containing 10% fetal bovine serum (FBS), penicillin-streptomycin (50 units/ml). Transient transfection was performed by 1×PEI solution with different plasmid DNA according to manifucturer's instructions.

Immunoprecipitation and western blot

Protocols for immunoprecipitation and western blot were described previously (Kim *et al*., 2008). HEK293 cells were transfected with pcDNA3.1-RE-IIBP, pcDNA6-ENO-1 and pcDNA3.1-ZNF350 by 1×PEI solution. Transfected cells were lysed with RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% Sodium Deoxychloate, 1×Protease inhibitor cocktail). Cell lysates were incubated with anti-myc or anti-His antibodies for overnignt at 4^{o}C with a gentle rotation, followed by incubation with protein G-agarose (Amersham Biosciences) for 2 hrs. After washing with PBS buffer for three times, the immunoprecipitates were separated on 12% SDS-PAGE, transferred to nitrocellulose membrane. The proteins were analyzed in immunoblots with anti-His (Qiagen), anti-HA and anti-ENO-1 antibodies (Santacruz).

Transfection assay

The transfection assay was conducted using CMX-Gal4-SV40 reporter and pcDNA6-RE-IIBP as internal controls where indicated. The quantity of DNA in each transfection was kept constant via the addition of pcDNA6. HEK293 cells were transfected with CMX-Gal4-SV40 reporter (100 ng) and pcDNA6-RE-IIBP (200 ng), pcDNA6-ENO-1 (100, 200, 300 ng), pcDNA3-ZNF350 (100, 200, 300 ng). Cells were harvested and assayed for luciferase activity using a luciferase assay system (Promega). Each value is expressed as the mean of four replicates from a single assay, and the results were confirmed by at least three repetitions.

RESULTS AND DISCUSSION

Isolation of ZNF 350 and ENO-1 in a yeast two hybrid screen

To further understand the function of HMTase RE-IIBP, we sought to identify interacting proteins using a yeast two-hybrid screening. We sought to determine whether GAL4 (DB)-RE-IIBP could be used as a bait to screen for mammalian cDNAs in a yeast cDNA expression library. Especially, we used the part of the RE-IIBP (amino acids residues 160-560) that includes the PHD, PWWP, SET, and post-SET domains (Fig. 1A and B). For this screen, we

used a bone marrow cDNA library in which cDNA was fused to GAL4 (AD) in pGADT7 vector. More than 10^6 transformants were screened for their ability to grow on His-plates and to produce β-galactosidase. Then the positive candidates were confirmed using restriction enzyme digestion and sequencing (Fig. 1C). We isolated the 12 true positives and represented in Table I. Among the isolated cDNAs from this screen, we focused two cDNAs; KRAB zinc finger protein ZNF 350 and Homo sapiens ENO-1.

Yeast two-hybrid assays were applied to further confirm the interaction between RE-IIBP and ZNF 350 and ENO-1. AH109 yeast cells transfected with GAL4 (DB) fused to RE-IIBP alone could not grow on both Trp and Leu negative plate. Instead, yeast cells expressing RE-IIBP together with ZNF 350 or ENO-1 could grow on those plates suggesting the true interaction between RE-IIBP and ZNF 350 or ENO-1 (Fig. 2A). We also confirmed the interaction of RE-IIBP with the ZNF 350 and ENO-1 in yeast by measuring β-galactosidase activity (Fig. 2B).

ZNF 350 has been known to interact between BRCA1, HDAC and ZNF 350 through KRAB domain and is involved

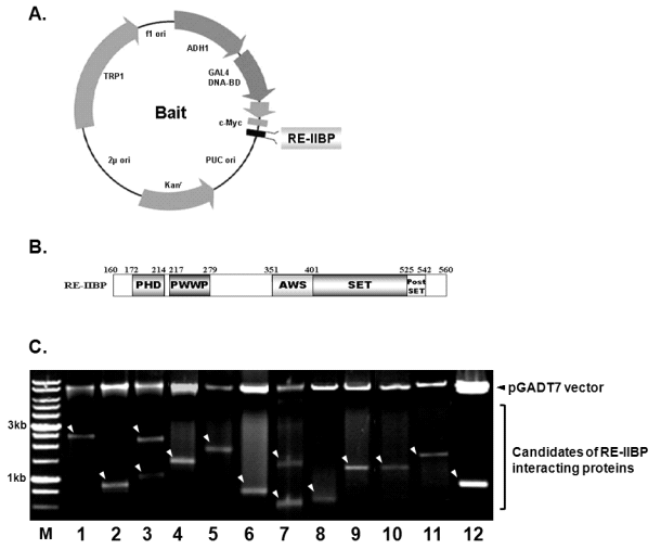

Fig. 1. Structural features of the pGBKT7-RE-IIBP protein. (A) RE-IIBP was subcloned into pGBKT7 vector contains GAL4-DNA binding domain. (B) Schematic view of RE-IIBP domains: amino acids 172-214 were predicted to be the PHD domain; amino acids 217-279 were predicted to be the PWWP domain; amino acids 402-525 were predicted to be the SET-domain. amino acids 525-542 were predicted to be the PostSET domain. (C) Positive candidates of RE-IIBP interacting proteins were digested with BamHI and EcoRI restriction enzymes. M represents 1 kb ladder as a size marker.

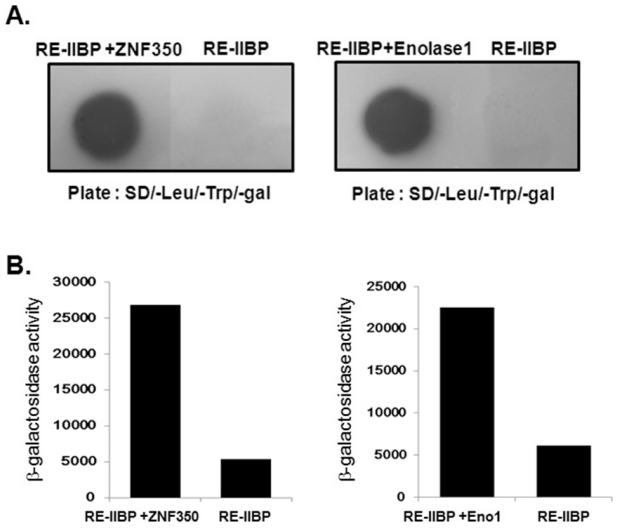

Fig. 2. RE-IIBP interacts with ZNF 350 and ENO-1 in yeast two-hybrid system. The pGBKT-7-RE-IIBP was co-transfected with pGADT7-ZNF 350 or pGADT7-ENO-1 into AH109 yeast cells, and the interaction was seen by β-galactosidase staining (A) and its quantifications are shown (B).

Table I. The candidates of RE-IIBP interacting proteins identified by DNA sequencing

No.	Gene name	Accession	Description
1	MCM7	NM_005916	MCM7 minichromosome maintenance deficient 7
2	POLR2E	NM_002695.2	DNA directed RNA polymerase II polypeptide E
3	E1B-AP5	AJ007509	E1B-55 kDa-associated protein5 isoform d
4	ENO1	NM_001428.2	Homo sapiens enolase 1, (alpha)
5	SFXN5	NM_114579.2	Homo sapiens sideroflexin5, mRNA
6	ZNF 350	AF309561	KRAB zinc finger protein ZFQR
7	ATG3	NM_022488	ATG3 autophagy related 3 homolog
8	BSDC1	NM_018045.5	Homo sapiens BSD domain containing1
9	PLOD3	NM_001084.4	Procollagen-lysine, 2-oxoglutarate5-dioxygenase 3 precursor
10	EEF1A1	NM_001402	Eukaryotic translation elongation factor1 alpha1 variant
11	ATP5J	NM_001003696.1	ATP synthase F0 subunit 6
12	POLR3A	NM_007055	RNA polymerase III subunit RPC155-D

in the cellular DNA damage response (Peng *et al*., 2002; Tan *et al*., 2004). ENO-1 plays an important role in cell growth regulation in neuroblastoma and suppression of prostate cancer cell growth (Ejeskar *et al*., 2005; Ghosh *et al*., 2005).

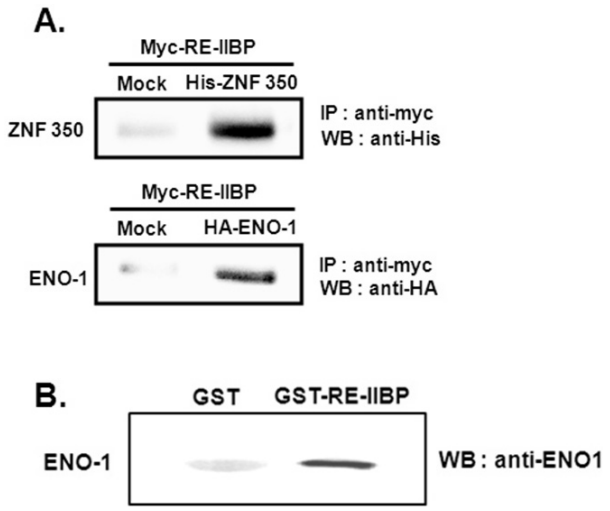

Fig. 3. *In vivo* and *in vitro* interaction of RE-IIBP with ZNF 350 or ENO-1. (A) Immunoprecipitation analysis to show the physical interaction of RE-IIBP and ZNF 350 and ENO-1. The HEK293 cells were transfected with myc-RE-IIBP together with either His-tagged ZNF 350 or HA-tagged ENO-1 and immunoprecipitated with anti-myc antibodies. After separation on SDS-PAGE gel, the RE-IIBP was detected with anti-His or anti-HA antibodies. (B) GST-RE-IIBP and GST-ENO1 were purified with glutathione sepharose 4B as *in vitro* GST pull-down assay and the bound ENO-1 were detected by immunoblotting with anti-ENO-1 antibodies.

ZNF 350 and Enolase-1 interaction with RE-IIBP

To test the interaction between RE-IIBP and ZNF 350, we performed mammalian immunoprecipitation assay. Myc-tagged RE-IIBP construct was transiently transfected and proteins were co-expressed with pcDNA 3.1-His empty vector or His-tagged ZNF 350 in HEK293 cells. Cell lysates were immunoprecipitated with Myc antibodies and immunoblotted with His antibodies. ZNF 350 proteins were co-immunoprecipitated with RE-IIBP, suggesting their interaction *in vivo* (Fig. 3A). The same immunoprecipitation assay was performed with ENO-1 to confirm the interaction between RE-IIBP and ENO-1. Fig. 3A indicated that Myc-RE-IIBP was immunoprecipitated by Myc antibodies and interacting HA-ENO-1 was immunoblotted by HA antibodies. Again, we pulled down purified ENO-1 using GST-RE-IIBP beads and then immunoblotted with anti-ENO-1 antibodies (Fig. 3B). Taken together, these results indicated both ZNF 350 and ENO-1 interacted with RE-IIBP *in vivo* and *in vitro*.

Synergistic effects on RE-IIBP mediated transcriptional repression by ZNF 350 and ENO-1

Previously, we have demonstrated that histone H3 lysine 27 methyltransferase RE-IIBP represses basal transcription through HDAC1 recruiting (Kim *et al*., 2008). We have shown further that RE-IIBP not only promotes H3-K27 methylation of IL-5 promoter but also maintains hypo-acetylation states via recruiting HDAC1 to the region. Since both ZNF 350 and ENO-1 proteins were interacting with RE-IIBP, we next asked whether theses proteins can affect transcriptional activities of RE-IIBP.

First, we tested whether both ZNF 350 and ENO-1 have any intrinsic regulatory role by themselves. In order to determine the transcriptional activities of both proteins, we

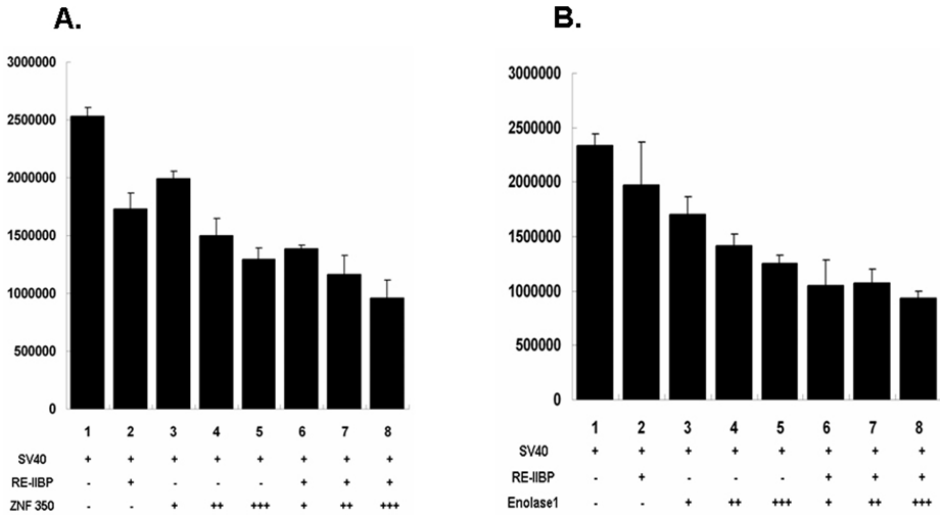

Fig. 4. ZNF 350 and ENO-1 acts transcriptional repressor mediated RE-IIBP. HEK293 cells were co-transfected with SV40 reporter vector and with or without RE-IIBP together with increasing concentrations of ZNF 350 (A) or ENO-1 (B) as indicated. Following transfection, cells were grown for 48 hours, and cell extracts were prepared and assayed for luciferase activity.

conducted transient transfection assay using CMX-Gal4-SV40 reporter system. The transient transfection of the SV40 promoter driven reporter itself elicited certain levels of basal transcriptional activities (Fig. 4A and B lanes 1). Transfection of increasing amount of ZNF 350 and ENO-1 slightly repressed basal transcription level, respectively, indicating both proteins have intrinsic transcriptional repressive activities (Fig. 4A and B lanes 3-5). This is consistent with previous reports that both ZNF 350 and ENO-1 have previously reported to show transcriptional repression activities (Vissing *et al*., 1995, Feo *et al*., 2000). As expected, when we co-transfected either ZNF 350 or ENO-1 constructs with RE-IIBP, they further decreased transcriptional activity of RE-IIBP in dose dependent manners (Fig. 4A and B lanes 6-8). The synergistic effects of transcriptional repression by RE-IIBP and both ZNF 350 and ENO-1 raise an interesting question regarding the RE-IIBP co-repressor complex formed by both proteins. Since both proteins have known for their transcriptional repression, maybe they form different co-repressor complex according to specific physiological circumstances they encounter. It is still not known whether both ZNF 350 and ENO-1 proteins forms the same complex and contribute to the RE-IIBP mediated repression activity. Further investigation will elucidate the components of each co-repressor complex and the physiological meaning of those interactions between the subunits.

ACKNOWLEDGMENTS

This research was supported by the Chung-Ang University Research Scholarship Grant in 2008.

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