SUMO Mediates Interaction of Ebp2p, the Yeast Homolog of Epstein-Barr Virus Nuclear Antigen 1-Binding Protein 2, with a RING Finger Protein Ris1p

Chiharu SHIRAI and Keiko MIYUTA

Department of Biofunctional Science and Technology, Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima 739-8528, Japan

Received March 3, 2008; Accepted April 16, 2008; Online Publication, July 7, 2008
[doi:10.1271/bbb.80131]

Ebp2p is essential for the assembly of 60S ribosomal subunits, and it interacts with other ribosome assembly factors in Saccharomyces cerevisiae. Two-hybrid screening exhibited that Ebp2p interacted with a small ubiquitin-related modifier (SUMO)-ligase Siz2p and SUMO-related proteins, Ris1p and Wss1p. Mutations of SUMO attachment sites of Ebp2p led to significantly weak interactions with Siz2p, Wss1p, and Ris1p, whereas they exhibited positive interactions with ribosome assembly factors. A SUMO-binding motif of Ris1p was required for interaction with Ebp2p. These results suggest that SUMO mediates the interaction between Ebp2p and SUMO related proteins and that Ebp2p switches its interaction partners via sumoylation.

Materials and Methods

Yeast strains and media. All yeast strains used were derivatives of W303-1B (MATa trp1 leu2 ade2 ura3 his3 can1-100), unless stated otherwise. Yeast cells were grown in synthetic complete medium containing 2% glucose (SC) or 2% galactose (SCGal), or dropout medium, depending on plasmid markers.

Plasmid construction. pRS313-EBP2-BV, a yeast low-copy plasmid, and pUC19-EBP2-BV, in which BamHI and EcoRI sites were introduced 23-bp 5' to the initiation codon and 8-bp 3' to the stop codon of EBP2, were constructed by PCR. ebp2 mutations were generated by site-directed mutagenesis using 5'-GAAATGGTGAAGTGATGAC-3' (K280R), 5'-CCAAGAATTGAGAGAAGAACCAAC-3' (K36,37R), 5'-GCCGATGTTAGAGAGAAGTTGCTG-3' (K61,62R), or 5'-AAAAGAAAGTTGAGAGAAGTTGAAA-3' (K47,48R) and pUC19-EBP2-BV as a template. To create pRS313-ebp2-myc, the 3' half of EBP2 ORF with the downstream region of pRS313-ebp2 was replaced with the corresponding region of pYCplac-EBP2-myc. The plasmid, GAL-HF-SMT3, encoding His6-FLAG-tagged Smt3 under the GAL promoter in pRS415, a yeast low-copy vector, was kindly provided by S. J. Elledge.

Two-hybrid screening and two-hybrid assay. pBTM116-EBP24) and the S. cerevisiae genomic DNA library7) (kindly provided by E. A. Craig) were intro-
duced into L40 cells, a his3 strain carrying lexA-HIS3 and lexA-lacZ constructs as reporter genes (kindly provided by R. Sternberg). Transformants in which both reporter genes were expressed were selected, and the plasmids in the cells were characterized. pACTII-RIS1 (336–599 ΔIII) was constructed by site-directed mutagenesis using a primer, 5’-CTCATCTGAAAGCGAGCTATTTTTC, and the DNA sequence was confirmed. Activation of the lacZ reporter gene was monitored by measuring β-galactosidase activity, and the activities were expressed in Miller’s units (9) as averages of three independent samples.

Indirect immunofluorescence, immunoprecipitation, and western analysis. Indirect immunofluorescence microscopy was done as previously described. Primary antibodies, rabbit anti-Ebp2p antibody (our unpublished data) and mouse anti-HA antibodies (12CA5, Roche Applied Science, Indianapolis, IN) were diluted 1:1000. Secondary antibodies (fluorescein isothiocyanate-conjugated goat-anti-rabbit IgG, ICN Pharmaceuticals, Aurora, OH, and rhodamine-conjugated goat-anti-mouse IgG, Jackson ImmunoResearch Laboratory, West Grove, PA) were diluted 1:300. Immunoprecipitation and western analysis were carried out as previously described.

Results and Discussion

Ebp2p interacted with a SUMO ligase and SUMO-related proteins

To identify additional proteins that physically interact with Ebp2p, we performed a yeast two-hybrid screen on a yeast genomic library. We isolated SIZ2/NFI1, RIS1, and WSS1. SIZ2 encodes a SUMO ligase. Two isolated clones had an identical DNA region encoding from amino acid (a.a.) residue 349 to 726 of Siz2p containing part of the Siz/PIAS RING domain, which is characteristic of SUMO ligases. One clone contained the region encoding from 336 to 599 a.a. of Ris1p, including a potential SUMO-binding motif. Ris1p has conserved motifs that are present in a family of DNA-dependent ATPases, SWI2/SNF2-like proteins. It has been found that the ris1 null mutant has significantly lower mating-type switching rates, and that RIS1 interferes with silencing when overexpressed. Two isolated clones containing a region encoding 231–269 a.a. of Wss1p. WSS1 has been isolated as a weak, high-copy suppressor of a temperature-sensitive smt3 mutant. It has been suggested that Wss1p has a role in stabilizing or processing stalled or collapsed replication forks.

Ebp2p was modified with SUMO

Physical interaction of Ebp2p with Siz2p was confirmed by co-immunoprecipitation using cells expressing Ebp2p-yc and HA-Siz2p (Fig. 1). Since Siz2p is a SUMO-ligase, we next examined whether Ebp2p was sumoylated. In a western blot of Ebp2p with anti-my antibodies, we detected two faint bands with reduced electrophoretic mobility besides an intense band of Ebp2p-myc (Fig. 2A). In order to learn whether the slower bands corresponded to SUMO-conjugated Ebp2p, we constructed an Ebp2p-yc strain in which His6-tagged SUMO was expressed from the GAL1 promoter. Nickel affinity chromatography followed by western blotting with anti-myc antibodies revealed that Ebp2p was sumoylated (Fig. 2B).

It has been proposed that (I/V/L) KX (E/D) is a consensus sequence in which SUMO is conjugated to the lysine residue. Ebp2p contains four matches to this consensus sequence caused a significant decrease in the intensity of the bands corresponding to SUMO-conjugated forms of Ebp2p in subsequent experiments.

Interaction of Ebp2p with Ris1p

We performed a two-hybrid assay to determine whether the physical interactions of Ebp2p were affected by the mutations in the sumoylation sites. Ebp2p-yc, K36,37,61,62R and Ebp2p-SNM led to significantly
weak interactions with Siz2p (349–726 a.a.), Wss1p (231–269 a.a.), and Ris1p (336–599 a.a.), whereas they exhibited positive interactions with Nop12p and Loc1p, which are factors in 60S ribosome assembly.\(^5\) (Fig. 4).

These results suggest that Ebp2p switches its interaction partners via sumoylation.

It has been proposed that the region encoding 367–379 a.a. (KNSSIIIISDEDE) of Ris1p is a SUMO-binding motif.\(^7\) The domain of Ris1p that we identified in this screening contained the SUMO-binding motif (Fig. 5A). To determine whether this motif is required for interaction with Ebp2p, we constructed a plasmid for two-hybrid analysis in which three isoleucines were removed within the SUMO-binding motif of Ris1p.
(Ris1ΔIII). Two-hybrid analysis revealed that Ris1ΔIII did not interact with Ebp2p (Fig. 5B). This result suggests that the SUMO-binding motif of Ris1p (367–379 a.a.) is necessary for the interaction of Ris1p with Ebp2p, although we cannot deny that conformational change in Ris1/C1 leads to a defect in the interaction with Ebp2p.

In order to learn if Ris1p binds to any sumoylated proteins, we used three septins previously reported to be sumoylated. Ris1p interacted with Cdc3p and Cdc11p, but not with Shs1p (Fig. 5C). This result suggests that Ris1p recognizes the SUMO of specific proteins.

**Subcellular localization of Ris1p**

The subcellular localization of Ris1p-HA was analyzed by indirect immunofluorescence microscopy (Fig. 6). Compared to the localization of Ebp2p in the nucleolus, the region adjacent to the DAPI-stained nucleoplasm, Ris1p-HA was localized in both the nucleolus and the nucleoplasm, suggesting that Ris1p interacts with Ebp2p in the nucleolus. It remains to be elucidated if sumoylation of Ebp2p affects the localization of Ris1p.

The mutants with the sumoylation-defective form of Ebp2p grew as fast as the wild-type cells (data not shown). Disruption of RIS1 did not affect the growth

---

**Fig. 4.** SUMO Attachment Sites of Ebp2p Required for Interaction with Siz2p, Wss1p, and Ris1p.

β-Galactosidase activities were measured in yeast transformants harboring pBTM116-EBP2 (dark gray bars), pBTM116-ebp2-K36,37,61,62R (light gray bars), or pBTM116-ebp2-SNM (white bars) and pACTII-SIZ2 (349–726), pACTII-WSS1 (231–269), pACTII-RIS1 (336–599), pACTII-NOP12, or pACTII-LOC1.

**Fig. 5.** SUMO-Binding Motif of Ris1p Required for Interaction with Ebp2p.

A, Schematic structure of Ris1p. Motifs and the domain obtained in two-hybrid screening are shown. B, A SUMO-binding motif of Ris1p was required for its interaction with Ebp2p. β-Galactosidase activities were measured in yeast transformants harboring pBTM116-EBP2 and pACTII-RIS1 (336–599) (Ris1) or pACTII-RIS1 (336–599 ΔIII) (Ris1ΔIII). C, Ris1p did not interact with all proteins that are known to be sumoylated. β-Galactosidase activities were measured in yeast transformants harboring pACTII-RIS1 (336–599). Each of the plasmids for the LexA binding domain fused Cdc3p, Cdc11p, and Shs1p (kindly provided by A. Toh-e).

**Fig. 6.** Ris1p in the Nucleolus and Nucleoplasm.

CS168 (RIS1-HA) cells were grown in SC medium at 27 °C to early log phase. Ebp2p, Ris1p-HA, and DNA were detected by indirect immunofluorescence microscopy using anti-Ebp2p antibodies, anti-HA antibodies, and 4′, 6-diamidino-2-phenylindole (DAPI, 1 μg/ml) respectively. The morphology of the cells was observed by differential-interference-contrast (DIC). Arrows indicate the boundary between the chromatin region and the nucleolus.
rate, whereas overexpression of *RIS1* led to a growth defect (data not shown). Although the biological meaning of the sumoylation of Ebp2p remains unclear, we speculate that the sumoylation of Ebp2p is required for fine-tuning of ribosome synthesis and other systems.

Recent proteomic studies have determined that several hundred proteins can be sumoylated in *S. cerevisiae*. Sumoylation and desumoylation regulate many biological processes, including transcription, cell cycle progression, DNA damage response, and signal transduction. In this study, we determined the SUMO attachment sites of Ebp2p. We suggest that Ebp2p switches its interaction partners via sumoylation-desumoylation. It has been reported that some proteins, including Ris1p and Wss1p, contain putative motifs required for non-covalent interaction with SUMO. It is possible that proteins that have a SUMO-binding motif receive signals “sumoylated” and regulate intracellular various events.

**Acknowledgments**

We thank Y. Kikuchi for helpful discussion, and R. Sternglanz, S. J. Elledge, E. A. Craig, K. Tanaka, and A. To-e for yeast strains and/or plasmids. C.S. was supported by JSPS Research Fellowships for Young Scientists.

**References**

for gaining insights into protein sumoylation in yeast. 


