DNA polymerase 5 acetylation by Eso1 is essential for Schizosaccharomyces pombe viability

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Abstract. Ecol/Eso1 protein plays an important role in chromosome segregation, DNA repair and gene regulation. Ecol mutation induces Roberts syndrome clinically and rDNA transcription disorders in vivo. In this study, we examined the role of Eso1 protein binding to polymerase 5 (Pol5) and the acetylation of Pol5 protein in the regulation of Schizosaccharomyces pombe (S. pombe) viability. Immunoprecipitation and mass spectrometry assays identified Eso1 protein binding to Cdc2, Pol5 and Cdc21, as well as other proteins. Pol5 protein specifically bound to Eso1 protein, but not to the Rad30 part or Rad30 part plus the additional zinc finger domain of Ecol protein. Mass spectrometry data further identified several acetylation or trimethylation modification sites in the lysine residues of the Pol5 protein. However, the mutation of the Pol5 K47 site to arginine was lethal to S. pombe. Eso1 protein was able to acetylate Pol5 protein and mediate S. pombe viability. On the whole, our data indicate that Eco1/Eso1 protein may play an important role in chromosome segregation, DNA repair and gene regulation.

Introduction

Ecol/Eso1 protein plays an important role in chromosome segregation, DNA repair and gene regulation (1-3). Ecol/Eso1 protein mediates the formation of the cohesion complex during DNA replication, chromosome separation and cell mitosis from the metaphase to the anaphase transition of the cell cycle. The cohesion complex is critical for the faithful chromosome segregation between two sister chromatids at the anaphase (4), consists of 4 highly conserved proteins (Smc3, Smc1, Scc3 and Scc1) (5,6), and functions to entrap the chromatids by forming a topological ring (7) during the cell cycle. Ecol/ Eso1 protein is an acetyltransferase and is able to acetylate the two conserved lysine sites in the Smc3 protein, enabling Smc3 to form the cohesion complex (8-11). A previous study revealed that mutations of ESCO2, the human ortholog of Eco1, were associated with a human developmental disorder known as Roberts syndrome (12). The role of Eco1/Eso1 in the contribution to chromosome structure and organization was then proposed (13). For example, our previous genetics screening data also demonstrated an intimate association between cohesion and chromatin separation (14). Recently, rRNA transcription and protein translation defects occurred in budding yeast that carry Roberts syndrome-related Eco1 mutations and in cells from patients with Roberts syndrome (15,16). These data indicate that Ecol/Eso1 protein may play an important role in Schizosaccharomyces pombe (S. pombe) viability. However, the molecular mechanisms through which Ecol protein functions in S. pombe, remain to be defined. For example, determining how and which protein Ecol/Eso1 protein binds to or interacts with, and any additional functions of Ecol/Eso1 protein may shed light into this matter.

In the present study, we first purified Eso1 protein using a tandem affinity purification (TAP) tag antibody and immunoprecipitated whole cell extracts from the wild-type S. pombe strain to identify Eso1 binding partners. We then focused on one of its interacting proteins, polymerase 5 (Pol5), as the Pol5 protein provides a conserved function in rDNA transcription in yeast (17,18). In addition, Myb-binding protein 1a (Mybbp1a), a human homologue of Pol5, has been shown to function in rDNA transcription and processing, and to be associated with early embryonic development and carcinogenesis (19,20).

Subsequently, we assessed how Eso1 interacts with Pol5 and and examined alterations in Pol5 protein acetylation, and the effects of these on S. pombe viability. This study aimed to provide insightful information regarding the interaction of Eso1 with Pol5 and the acetylation of Pol5 protein, and the effects of these on S. pombe viability. Future studies are also required in order to investigate whether and how Eso1 acetylates the Pol5 K47 site for S. pombe survival.

Materials and methods

Strains and culture media. The S. pombe strains used in this study are listed in Table I and were cultured in standard conditions, as previously described (21). For S. pombe transformation, the lithium acetate protocol was utilized, as described...
in a previous study (22). pDblet (acquired from Baumann's Lab, Stowers Institute, Kansas City, MO, USA) was used as a plasmid vector for the expression of Pol5 with or without K47 mutations, and 0.1%(w/v) 5-fluoroorotic acid (5FOA) was used to select against ura + S. pombe cells, which is supposed to contain transformed pDblet plasmids.

**Mutagenesis and protein tagging.** DNA sequences corresponding to Pol5 CDNAs were amplified using standard PCR from the S. pombe genomic DNA. The primers used are listed in Table II. The resulting PCR product was then cloned into a pCloneNat1 vector [kind gift from Gregan's Lab, Max F. Perutz Laboratories, Vienna Biocenter (VBC), Vienna, Austria]. To create the Pol5 K47N and Pol5 K47R mutant strains, a site directed mutagenesis kit (QuickChange II; Agilent Technologies, Santa Clara, CA, USA) was employed at the corresponding residues using the primers listed in Table II. The resulting plasmids, pCloneNat1-pol5 K47N and pCloneNat1-pol5 K47R, were linearized with the restriction enzyme, BstBI, by digesting nucleotides at the start codon, and then transferred into the diploid S. pombe strains. The tetrads analysis of the expected mutation strains was then confirmed by DNA sequencing and used in this study. These wild-type and mutated Pol5 with TAP, HA or FLAG tag were then tagged according to a previously study (23) and online protocol (http://mendel.imp.ac.at/Pomab_tagging/).

**Cloning and expression of GST-Eso1 protein.** The full-length Eso1 CDNAs and its different truncated forms, including Rad30 homologue fragment (1-519 or M519r), Rad30 fragment plus the additional second zinc-finger domain (1-568 or R568e) and Ecol homologue fragment (520-871) were amplified from the genomic DNA of S. pombe using PCR primers (Table II). The PCR product containing the BamHI and Xmal restriction sites were cloned into the pGEX-4T-1 vector (kind gift from Gerton's Lab, Stowers Institute) in a manner that produces an N-terminally GST tagged Eso1 protein and its different truncated forms, respectively. The resulting plasmids were transferred into E. coli B21 (DE3) cells (# 200131; Agilent Technologies), and were amplified and DNA sequence-confirmed. To express the N-terminally GST tagged Eso1 protein and its different truncated forms, plasmids were transferred into E. coli B21 (DE3) cells and 0.3 mM of IPTG was added to induce the expression of Eso1 protein in DE3 cells at 25°C for 3 h. The glutathione resin was then utilized to pull-down and purify these GST fused proteins, as previously described (24).

**TAP purification and liquid chromatography-mass spectrometry (LC-MS/MS) analysis.** Trichloroacetic acid (TCA)-precipitated proteins from cultured S. pombe strains were resuspended in 30 µl of buffer containing 100 mM of Tris-HCl pH 8.5, 8.5 M of urea and 5 mM of Tris(2-carboxyethyl)-phosphine hydrochloride (Pierce, Rockford, IL, USA), and alkylated with 10 mM of iodoacetamide (Sigma, St. Louis, MO, USA). Subsequently, a two-step digestion procedure was applied. In brief, 0.5 µg of endoproteaseinase Lys-C (Roche Applied Science, Indianapolis, IN, USA) was added to each sample and incubated for at least 6 h at 37°C. The samples were then diluted with 2 M of urea in 100 mM of Tris-HCl pH 8.5 and 2 mM of calcium chloride. Subsequently, the samples were digested with 0.5 µg of trypsin (Promega, Madison, WI, USA) at 37°C overnight with shaking. The following, the reaction was quenched by the addition of 5% formic acid, and the peptide mixtures were then loaded onto a 100-µm fused silica microcapillary column packed with 8 cm of reverse-phase material (Aqua; Phenomenex, Torrance, CA, USA) for LC-MS/MS analysis in a Deca-XP ion trap mass spectrometer equipped with a nano-LC electrospray ionization source (Thermo Fisher Scientific, Waltham, MA, USA). The full MS spectra were recorded on the peptides over a 400-1,600 m/z range. Mass spectrometer scan functions and HPLC solvent gradients were controlled by the XCalibur data system (Thermo Fisher Scientific). RAW files were extracted into mzs file format using RAW Xtract v.1.0. The protein spectra were searched against the Swiss-Prot database using the Mascot software program (http://www.matrixscience.com/).

**Immunoprecipitation and western blot analysis.** S. pombe cells from a 100-ml culture grown into a 0.8 OD-595 were pelleted and immediately frozen in liquid nitrogen. The S. pombe cell pellets were then suspended in 1 ml of lysis buffer containing 50 mM of Tris pH 7.5, 150 mM of NaCl, 0.1% NP-40, 1 mM of DTT, 10% glycerol and a protase inhibitor tablet. The cells were lysed by adding glass beads followed by bead-beating for 60 sec 5 times with 1 min intervals on ice. The supernatant was then separated by centrifugation at 14,000 rpm at 4°C for 20 min, and subjected to co-immunoprecipitation assay with an anti-FLAG antibody for Eso1-FLAG protein and an anti-HA antibody for Pol5-HA protein, respectively. Specifically, the supernatant was added with 30 µl of anti-FLAG or anti-HA affinity gel (#E6779; EZview™ Red Anti-HA Affinity Gel; Sigma-Aldrich, Shanghai, China) and incubated at 4°C overnight. The following day, the mixtures were washed 5 times with wash buffer containing 50 mM of Tris pH 7.5, 150 mM of NaCl and 1% Triton X-100; they were then eluted in 2X SDS buffer containing 10 mM of Tris pH 7.5, 1 mM of EDTA and 1% SDS. The elutes were loaded onto a 4-12% Bis-Tris gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred onto a nitrocellulose membrane (Whatman, Piscatway, NJ, USA). For western blot analysis, the membrane was blocked with 5% skimmed milk for 1 h at room temperature and blotted with primary antibodies [anti-FLAG antibody (1:3,000; #F3165; Sigma), GST antibody (1:3,000; Abmart, Berkeley Heights, NJ, USA), a-HA antibody (1:10,000; #sc-7392; Santa Cruz Biotechnology, Santa Cruz, CA, USA)] against HA and FLAG tags (Sigma-Aldrich) respectively, as previously described (24). The glutathione resin bound with variant forms of Eso1 was incubated with whole cell extracts of S. pombe expressing C-terminally HA-tagged Pol5 at 4°C overnight, and the resulting samples were subjected to western blot analysis with the anti-HA tag antibody (Sigma-Aldrich).

**In vitro acetylation assay.** Purified GST-tagged Ecolp and HA affinity gel bound with Pol5-HA was added with a HAT buffer containing 50 mM of Tris pH 8.0, 5% glycerol, 0.1 mM of EDTA, 50 mM of KCl, 1 mM of DTT, 1 mM of PMSF and 10 µM of Acetyl-CoA. This was followed by incubation at 30°C for 60 min, as previously described (24). The samples were then subjected to western blot analysis with anti-acetylysine antibody for acetylation levels and HA antibody as a loading control.
Tables

Table I. Yeast strains used in this study.

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Table II. Primers used in this study.

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<td>Pol5 K47N</td>
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<td>Pol5</td>
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Results

Esol protein interaction with Pol5 protein in S. pombe.

To identify proteins that can physically interact with Esol protein, Esol cDNA was tagged with a TAP tag at the C-terminus and this fusion protein was expressed under its endogenous promoter (25) using a protocol we have previously described (25). The proteins binding to Esol-TAP protein were then purified using immunoprecipitation and mass spectrometry followed by TAP. Our data revealed that Esol protein was able to bind to Cdc2, Pol5 and Cdc21, as well as other proteins (Fig. 1A). The following experiments focused on Pol5 protein due to its function in rRNA synthesis and cell proliferation. The specificity of their binding was confirmed by immunoprecipitating the whole cell extracts of strains (Fig. 1B). Specifically, Pol5-HA protein could only be detected from whole cell extracts of strains that express both HA-tagged Pol5 and FLAG-tagged Esol, indicating the specificity of their interaction.

However, given the characteristics of the fusion Esol protein in S. pombe, which expresses both budding yeast Eco1 and Rad30 homologue (26), our present data was unable to distinguish the binding of Pol5 protein to Eso1 protein from the binding of Pol5 to the Rad30 homologue of Esol protein. Thus, we amplified DNA sequences of Rad30 homologue fragment (1-568 or R568*), Rad30 fragment plus the additional second zinc-finger domain (1-519 or M519*), Ecol homologue fragment (520-871) and the full length Esol cDNA using genomic DNA (Fig. 1C). We then inserted these amplicants into pGEX-4T-1 to produce the expected form of Esol protein with the N-terminal GST-tag (Fig. 1D). Following GST pull-down assays and western blot analysis with anti-GST antibody, the correct expression of Esol proteins were confirmed (Fig. 1D). These proteins were then utilized to pull-down Pol5 in the whole cell lysis of S. pombe expressing C-terminally HA-tagged Pol5 for western blot analysis with anti-α-HA antibody. We found that the results were consistent with our first set of data, which showed that Pol5 protein binds to Esol protein from either Esol-FLAG and Pol5-HA co-immunoprecipitation or mass spectrometry analysis following the purification of Esol by TAP (Fig. 1); Pol5-HA was able to pull-down the full-length recombinant GST-Esol protein. Strikingly, Pol5-HA could have also pulled-down the Ecol homology part of Esol, but not the Rad30 part or Rad30 part plus additional zinc finger domain of Ecol protein (Fig. 1E), suggesting that the Ecol homology part of Esol mediated binding to the Pol5 protein.

Mass spectrometry identification of Pol5 protein acetylation sites.

C-terminal TAP tagging and Pol5 protein expression in vitro was established according to a protocol described in a previous study (23). Proteins were purified using an anti-TAP tag antibody and separated using SDS-PAGE. A prominent protein band of approximately 120 kDa was found after silver staining (Fig. 2A), which was in agreement with the predicted molecular weight of Pol5 protein plus TAP tag. Possible post-translational modifications of this purified Pol5 protein were then analyzed using mass spectrometry, and several acetylation or trimethylation modification sites in the lysine residues of Pol5 protein were identified (Fig. 2B). The Pol5 K47 residue attracted our attention, because there was a 100% modification ratio (although the number of the total spectrum count was low); the DNA sequence alignment of S. pombe
and *S. cerevisiae* indicate that K47 residue is well conserved in budding yeast (Fig. 2C).

**Acetylation of Pol5 protein K47 residue mediated *S. pombe* viability.** The effects of Pol5 K47 acetylation on *S. pombe* cells were assessed, and pol5-K47R/pol5+ heterozygous diploids were prepared. Following sporulation, only two spores in each diploid grew (Fig. 3A). DNA sequencing analysis was then performed. The results revealed that all viable spores contained a wild-type (non-mutated) allele of the *Pol5* gene, indicating that *S. pombe* expressing mutant Pol5 K47R was unable to survive. Moreover, since arginine (R) is structurally similar to lysine (K), which does not undergo acetylation change, we found that arginine substitution of lysine led to the absence of the acetylation of Pol5 K47; this resulted in the lethality to *S. pombe*. In addition, our data also revealed that Pol5 K47N acetyl-mimetic mutant *S. pombe* cells grew normally (Fig. 3A).

Furthermore, we constructed the shuffle plasmid by integrating the *Pol5* coding region cDNA plus the 200-bp upstream and downstream sequences into the pDblet vector. The transformation of this plasmid into *S. pombe* fully rescued the growth of Pol5 K47R mutants (Fig. 3). Subsequently, 5FOA was added into *S. pombe* to select against the expression of *Ura4+* in pDblet-Pol5 plasmid-transferred *S. pombe*. Consistent with the data on Pol5 K47R mutation without pDblet-Pol5 plasmid, our data revealed that *S. pombe* growth was limited and lethal (Fig. 3B).

**Eso1 acetylation of Pol5 protein K47 residue mediates *S. pombe* viability.** We explored whether Eso1 protein is able...
Figure 2. Tandem affinity purification (TAP) and characterization of Pol5 protein from *Schizosaccharomyces pombe* (*S. pombe*). (A) TAP-tagged Pol5 cDNA was transferred into *S. pombe*, and whole cell lysis was extracted from *S. pombe*. Pol5 protein was then purified by the TAP system. Purified proteins were separated by SDS-PAGE and visualized by silver staining. (B) Illustration of potential post-translational modification sites in Pol5 protein. (C) Mass spectrometry identification of the Pol5 K47 site. The acetylated lysine residue of Pol5 protein in *S. pombe* is conserved to K53 of Pol5 in budding yeast (a red box). In the alignment, asterisks indicate identity, while the colons and dots indicate similarity between amino acids.

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Figure 3. Lethality of non-acetylated K47 residue mutation in Pol5 protein in *Schizosaccharomyces pombe* (*S. pombe*). (A) POL5K47R/POL5 and POL5 K47N/POL5 heterozygous diploids were sporulated, and the viability of each tetrad was analyzed. POL5 K47R mutant spores are labeled with red circles, and POL5 K47N mutant spores are labeled with green circles. (B) Wild-type (WT) and Pol5 K47R cells containing pDblet-pol5 plasmids were plated on EMM-URA and YES 5'FOA plates. (C) Pol5 was acetylated by Eco1 *in vitro*. Pol5-HA was immunoprecipitated on an HA affinity gel from whole cell extracts of *S. pombe* cells expressing C-terminally HA tagged Pol5. The beads were then incubated with or without Eco1 in HAT buffer present with acetyl coenzyme A.
to acetylate Pol5 using acetylation assay following a protocol described in a previous study (27). We found that Pol5-HA protein was immunoprecipitated with EZview™ Red Anti-HA Affinity Gel from *S. pombe*. This was then incubated with the recombinant Eco1 protein purified from *E. coli* via the GST tag. Pol5 protein acetylation was detected by western blot analysis with an anti-acetyl-lysine antibody following the addition of Ecol protein (Fig. 3C).

**Discussion**

In the present study, we first performed immunoprecipitation and mass spectrometry assays to identify several candidate proteins that bind to Esol protein (i.e., Cdc2, Cdc21, Cdc15, Rad24, Rad25 and Pol5). Functionally, Cdc2, as a serine/threonine kinase, is a highly conserved protein and a key player in the regulation of cell cycle progression (22,28). Cdc21 is a member of the MCM family of nuclear proteins, which can regulate DNA replication and cell cycle progression (29). Cdc15 is also involved in the formation of the cytokinetic contractile ring (28). Moreover, Rad24 and Rad25 are cell cycle checkpoint proteins that belong to the highly conserved 14-3-3 protein family (29-31), which regulates cell cycle arrest and DNA damage repair in response to UV light-induced DNA damage (24-27). Pol5 protein functions in yeast rDNA transcription (17,18) and the human homologue of Pol5, Mybbp1a, demonstrating functions in rDNA transcription, early embryonic development and tumorigenesis (19,20). Thus, given the important functions of Esol protein in cells, including the sister chromatin cohesion complex and mediation of DNA repair (2), it seems reasonable to hypothesize that the binding of Esol to these proteins illustrates the functions of Esol protein.

Furthermore, we focused on the interaction of Pol5 protein with Esol protein to assess their regulation of *S. pombe* viability. We performed a co-immunoprecipitation assay to further confirm the binding of Eco1 to Pol5 protein and found that Pol5 protein only bound to Esol protein, but not to the Rad30 part or Rad30 part, plus the additional zinc finger domain of Ecol protein, indicating that their binding was specific. Upon their binding, Esol protein was able to acetylate Pol5 protein. Our confirmative evidence indicated that there were several acetylation sites in Pol5 protein lysine residues (Fig. 2B) and that arginine substitution of lysine led to the lack of Pol5 K47 acetylation, which resulted in the lethality of *S. pombe*; whereas for Pol5 K47N, the acetyl-mimetic mutant had normal *S. pombe* proliferation; thus, the Esol acetylation site in Pol5 protein could be at the Pol5 K47. However, at present, there are no Pol5 K47 acetylation antibodies available; thus, a definitive proof would require the involvement of a mass-spec analysis of the acetylation on Pol5 before and after incubation with Esol protein. Future studies are required to further verify the Esol acetylating Pol5 K47 site, although our present data suggest that Pol5 protein acetylation was essential in the regulation of *S. pombe* viability.

In conclusion, previous studies have revealed the essential role of Eco1/Esol protein in the formation of the cohesion complex between the two sister chromatids, and that the acetyl-mimetic mutation, Smc3 K47N, made Ecol protein dispensable. In the present study, we found that the *S. pombe* carrying non-acetylatable mutations of Pol5 protein (i.e., Pol5 K47R) was unable to survive, indicating the essential role of Pol5 protein in *S. pombe*. However, this study was not able to rule out whether other acetyltransferases, apart from Esol, acetylate Pol5 protein. Future studies are warranted to verify Esol, particularly the acetylate Pol5 K47 site; although the present study suggested so.

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**References**


