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Email: tapchikhoahoc@hcmue.edu.vn; Website: http://tckh.hcmue.edu.vn

FUNCTIONAL INTERACTION BETWEEN MUS81-MMS4 AND RAD52 IN *SACCHAROMYCES CEREVISIAE*

Phung Thi Thu Huong[1*], Tran Hong Diem[1], Nguyen Luong Hieu Hoa[1], Vo Thanh Sang[1], Le Van Minh[2], Nguyen Hoang Dung[3]

[1]NTT Hi-Tech Institute - Nguyen Tat Thanh University

[2]Research Center of Ginseng and Materia Medica

[3]Institute of Tropical Biology, VAST

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ABSTRACT

Mus81-Mms4 is a well conserved DNA structure–specific endonuclease and efficiently cleaves different DNA structures that could arise during the repair of stalled/blocked replication forks and homologous recombination repair. Rad52 is an ezyme that stimulates main steps of DNA sequence-homology searching. In this study, we proved that Rad52 and Mus81-Mms4 possess a species-specific functional interaction, indicating that Rad52 and Mus81-Mms4 collaborate in processing of homologous recombination intermediates.

Keywords: functional interaction, homologous recombination, Mus81, Rad52.

TÓM TẮT

Tương tác chức năng giữa phức hợp Mus81-Mms4 và Rad52 ở Saccharomyces cerevisiae

Mus81-Mms4 là một endonuclease có tính bảo tồn và cắt cấu trúc ADN đặc trưng mà có thể hình thành khi tế bào sửa chữa chạc sao chép dừng/khóa và ADN lỗi bằng tái tổ hợp. Rad52 là enzyme xúc tác những bước chính trong quá trình tìm kiếm trình tự tương đồng. Chúng tôi chứng minh rằng Rad52 và Mus81-Mms4 tương tác về mặt chức năng mang tính đặc hiệu loài, qua đó chỉ ra rằng Rad52 và Mus81-Mms4 phối hợp hoạt động trong việc xử lí các phân tử ADN trung gian tái tổ hợp.

Từ khóa: Mus81, Rad52, tái tổ hợp tương đồng, tương tác chức năng.

1. Introduction

Homologous recombination (HR), which is critical in genome integrity maintenance, is required for the DNA repair of double-strand breaks (DSBs) as well as for the process of collapsed replication forks. In the budding yeast *Saccharomyces cerevisiae*, HR is mediated by RAD52 epistasis group that includes Rad52 protein which is the only enzyme required for virtually all HR events [1]. Rad52 can bind single-strand DNA in vitro, stimulate the annealing of complementary DNA, and elevate Rad51-catalyzed strand invasion by mediating the displacement of replication protein A from single-strand DNA to Rad51, which is one of the main steps of DNA sequence-homology searching in HR [2].

* Email: *ptthuong@ntt.edu.vn*

DNA recombination intermediates, such as the Holiday junctions (HJs)—one of the most frequent intermediates appearing in HR, which are generated downstream of HR—must be resolved by structure specific endonucleases, called resolvases. Mus81 is related to the structure-specific endonuclease XPF family and functions as a heterodimer with a partner protein, namely EME1 in humans and Mms4 in budding yeast [3], [4]. A role for Mus81 in HJs resolution was first investigated in works on *Schizosaccharomyces pombe* where all of the abnormal phenotypes of *S. pombe* mus81 deletion mutants were recovered by the expression of RusA, a bacteriophage resolvase which is specific for HJs [5]. However, the purification of the native or recombinant human or yeast Mus81 complexes expressed extremely low or no activity on intact HJs [6], [7], [8], [9], [10], [11], [12], [13]. Later report demonstrated that the recombinant fission or budding yeast Mus81 complexes showed strong activity on intact HJs based on the tetramer formation [14]. In contrast, more recent study proved that Mus81–Mms4 functions as a single heterodimer in recombinational DNA repair and poorly cleaves intact HJs [15]. Interestingly, the activity of Mus81–Mms4 is cell-cycle regulated by the phosphorylation of Mms4 by Cdc28 (CDK) and Cdc5 (Polo-like kinase) which enhance the activity of Mus81 complex on intact HJs in vivo [16], [17].

In mitotic cells, the Mus81 heterodimeric complex has been shown to catalyze resolution of replication- and recombination-associated DNA structures formed during repair of stalled/collapsed replication forks or double-strand breaks [3], [5], [8], [11], [18], [19]. The Δ*mus81* mutants are hypersensitive to DNA damage agents such as ultra violet irradiation, MMS, hydroxide urea, 2-phenyl-3-nitroso-imidazo [1,2-α] pyrimidine, cisplatin, doxorubicin, tirapazamine, and camptothecin [3], [5], [8], [11], [18], [19]. Mus81-Mms4 complex in vitro can catalyze efficiently the cleavage of different DNA structures including nick HJs, D-loops, replication forks, and 3'-flaps that may form in vivo during many DNA transactions [5], [6], [9], [10], [11].

It was shown in vivo that Mus81 acted in parallel or redundant pathways with Sgs1/BLM, a member of the ubiquitous RecQ family of DNA helicases, to process the recombination intermediates [6], [9], [10], [11], [12], [19], [20], [21]. Moreover, the double deletion of Δ*mus81* or Δ*mms4* together with Δ*sgs1* induced synthetically lethal phenotype, which can be rescued by further deletion of recombination proteins, such as Rad51 or Rad52 [22], [23], [24], [25]. These evidences suggest that Mus81 functions downstream of HR repair redundantly with Sgs1, implying that Mus81 is the most important parallel pathway to Sgs1 during HR repair.

The physical and functional interactions between Mus81 complex and its partners are significantly important for cellular function of Mus81 [26], [27]. Rad54—one of the RAD52 epistasis groups—has been shown to be the stimulation factor of both Mus81-Eme1 and Mus81-Mms4 endonuclease activity [28], [29]. Besides, it has been reported

that Mus81 and Rad52 have a synergistic genetic interaction—synthetic growth defect—via diploid synthetic lethal analysis by microarray and through the yeast knockout heterozygous mutant collection [30]. Moreover, Mus81 was one of the genes found in the screening for increased spontaneous Rad52-YFP foci, which represent HR protein accumulation sites [31].

In this research, by using purified proteins, we examined the effect of Rad52 on the resolution of several kinds of DNA substrates resolved by Mus81-Mms4 complex endonuclease in vitro. Our data demonstrate that Rad52 stimulated Mus81-Mms4 endonuclease activity on a broad range of DNA substrates including nick HJs, while it inhibited human MUS81-EME1 endonuclease. We suggest that Mus81-Mms4 together with Rad52 effectively resolve DNA intermediates downstream of HR in DSB repair or stalled replication forks recovery to maintain genome stability.

2. Materials and method

2.1. Nucleotides, enzymes, and plasmids

The oligonucleotides used to construct different DNA substrates were synthesized commercially from Genotech (Daejeon, South Korea). T4 polynucleotide kinase was purchased from Enzynomics (Daejeon, Korea). Proteinase K was obtained from Duchefa Biochemie (Haarlem, Netherland). pET vectors used for protein expression in Escherichia coli were from Novagen (Darmstadt, Germany). [γ-32P] ATP (>3000 Ci/mmol) was purchased from IZOTOP (Budapest, Hungary).

2.2. Protein purification

2.2.1. Purification of Mus81-Mms4

pET28a-Mms4-Mus81 was expressed in *E. coli BL21-CodonPlus (DE3)-RIL* strain. Cells were pre-incubated at 37 °C and induced by 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) when the OD was between 0.5-0.7, followed by 4 hour (hr) incubation at 25 °C. Cells were harvested by centrifugation, washed with Tris-buffered saline, and stored at -80 °C. The cell pellet was resuspended in lysis buffer H100 (25 mM HEPES-NaOH/pH 7.5, 100 mM NaCl, 10% glycerol, 0.01% Nonidet P40 (NP40), and protease inhibitors). The number in H100 indicates the concentration of NaCl in mM. Following sonication, the crude lysate was clarified by centrifugation at 45000 rpm for 30 minutes (min). The supernatant was loaded on P-cell column pre-equilibrated with buffer H100. The column was then washed with 5-column volumes of buffer H150, and eluted with NaCl gradient from 150 to 1000 mM in buffer H. The eluate fractions were pooled, adjusted to 600 mM NaCl and 10 mM imidazole (IDZ, final concentration), and batch-incubated with His-Select nickel affinity (Ni-NTA) beads for 2 hr at 4 °C. After two steps of washing with buffers H600 plus 10 mM IDZ and H600 plus 50 mM IDZ, the bound proteins were eluted with buffer H600 plus 200 mM IDZ. Peak fraction was subjected to glycerol gradient sedimentation (GG) (5 mL, 15~35% glycerol in buffer H300) at 45000

rpm for 24 hr in a SW55 Ti rotor (Beckman). Fractions (250 µl each) were collected from the bottom of the GG tube. Peak fractions were then stored at -80 ℃.

2.2.2. Purification of Rad52

pET28b-Rad52 was expressed in *E. coli BL21-CodonPlus (DE3)-RIL* strain. Cells were pre-incubated at 37 ℃ and induced by 0.1 mM IPTG when the OD was 0.8, followed by 4 hr incubation at 25 ℃. Cells were harvested by centrifugation, washed with Tris-buffered saline, and stored at -80 ℃. The cell pellet was resuspended in lysis buffer T200 (50 mM Tris-HCl/pH 8.0, 200 mM NaCl, 10% glycerol, 0.01% NP40, and protease inhibitors). Following sonication, the crude lysate was clarified by centrifugation at 45000 rpm for 30 min. The supernatant was applied sequentially onto pre-equilibrated Q Sepharose and SP Sepharose column. Elution with NaCl gradient from 200 to 1000 mM in buffer T followed the washing step of 5-column volumes of buffer T200. The peak fractions were pooled and adjusted to 500 mM NaCl and 10 mM IDZ, followed by loading on Ni-NTA column. After four steps of washing with buffers T500 plus 50 mM IDZ, T2000 plus 40% ethylene glycol and 50mM IDZ, T500 plus 50 mM IDZ and T500 plus 100 mM IDZ, sequentially, the bound proteins were eluted with buffer T500 plus 500 mM IDZ. Peak fraction was subjected to GG (5 mL, 15~35% glycerol in buffer T500) at 45000 rpm for 24 hr in a SW55 Ti rotor (Beckman). Fractions (12 drops each) were collected from the bottom of the GG tube. Peak fractions were then stored at -80 ℃.

2.3. ***Substrate preparation and nuclease assay***

2.3.1. Substrate preparation

The preparation of DNA substrates and their labeling at the 5’ end are as described previously [32]. Briefly, the first oligonucleotide is labeled at its 5’-end by incorporating [γ-32P] ATP by T4 polynucleotide kinase, and then annealed with the other oligonucleotides. The annealing reaction is performed by using PCR machine (95 ℃, 5 min; 65 ℃, 30 min; cycle: 65 ℃, 8 min, -0.5 ℃/cycle, 80 cycles). The annealed substrate is purified by 10% polyacrylamide gel electrophoresis prior to use. The oligonucleotides used to construct different DNA substrates were synthesized commercially from Genotech (Daejeon, South Korea). [γ-32P] ATP (>5000 Ci/mmol) were purchased from IZOTOP (Budapest, Hungary). The location of radioisotopic label is indicated in each substrate.

2.3.2. Nuclease assay

The nuclease assays with Mus81-Mms4 were performed in reaction mixture (20 µl) containing indicated amount of enzymes, 10 fmol of substrate, 25 mM Tris-HCl/pH 8.0, 100 mM NaCl (final concentration), 5 mM MgCl2, 5 % glycerol, 0.1 mg/mL BSA, 0.1% NP40, and 0.2 mM DTT. Reactions were incubated at 30 ℃ for 30 min, followed by the deproteinization by incubating with 0.1 % SDS and 10 µg of proteinase K at 37 ℃ for 15 min. 1/6 reaction volume of 6X stop solution (60 mM EDTA/ pH 8.0, 40% sucrose, 0.6% SDS, 0.25% BPB, 0.25% xylene cyanol) was added to stop reactions. The products were

subjected to electrophoresis for 40 min at 150 V in 0.5X TBE (45 mM Tris, 45 mM boric acid, 1mM EDTA). The gels were dried on a DEAE-cellulose paper and autoradiographed. Labeled DNA products were quantified with the use of a phosphor-imager (BAS-1500, FUJIFILM).

3. Results

3.1. Purification of recombinant Mus81-Mms4 and Rad52

We purified Mus81–Mms4 complex using the procedure as described in Materials and Methods. The quality of purified recombinant protein was examined by polyacrylamide sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) (Fig. 1A). Protein concentration was quantitated by Bradford Protein Assay and Bovine Serum Albumin standard-line method. Next, endonuclease activity of Mus81-Mms4 complex was examined by nuclease assay using 3'-flap as a substrate and the purified recombinant protein exhibited significant catalytic activity on this substrate as expected (Fig. 1B and C). Recombinant Rad52 was also expressed in E. coli and highly purified after 3 steps of purification. Representative fractions of purified Rad52 after glycerol gradient sedimentation showed a sharp peak and the purity of recombinant protein (Fig. 1D). Taken together, we have succeeded to purify recombinant Mus81-Mms4 complex which is markedly active and Rad52 to near homogeneity, satisfying the quality requirement of next biochemical assays.

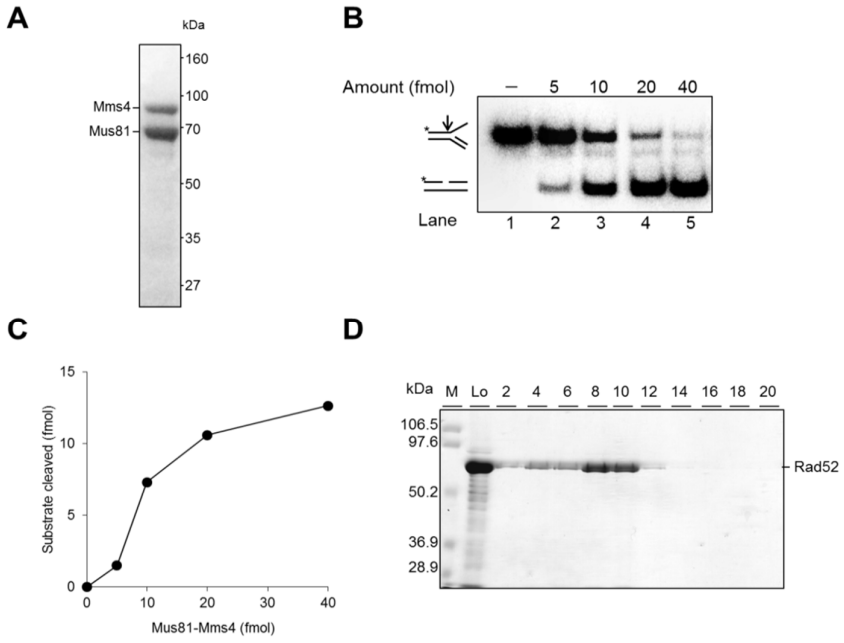

Figure 1. *Purification of Mus81-Mms4 complex and Rad52*

A) Purity of recombinant Mus81-Mms4 complex after purification. Protein ladder is indicated on the right in kilo Dalton. B) Endonuclease activity of recombinant Mus81-Mms4 complex on 3'-flap substrate. Indicated amount of protein complex were used to resolve 10 fmol of substrate in 30 min at 30 °C. Cleaved products (gap DNA duplex) were analyzed by 10% native polyacrylamide gel electrophoresis. C) Substrate cleaved (fmol) formed in B were plotted against amount of Mus81-Mms4 used (fmol). D) 10% SDS-PAGE gel of fractions from glycerol gradient sedimentation step of Rad52 purification shows the homogeneity of purified Rad52.

3.3. Stimulation of Mus81-Mms4 endonuclease activity by Rad52

Strong elevation of Mus81-Mms4 DNA cleavage activity by Rad52 was observed in a concentration-dependent manner. Figure 2 shows that Rad52 stimulated cleavage of 3'-flap substrate by Mus81-Mms4. 2.5 fmol of Mus81-Mms4 only resolved around 0.5 fmol of 3'-flap substrate, while Rad52 did not cleave this substrate (Fig. 2A, lanes 3 and 4). Interestingly, flap cleavage was enhanced about 3-fold when 500 fmol of Rad52 was added (Fig. 2A, lane 7). Particularly, the pre-incubation of 500 fmol of Rad52 with substrate led to the increase of the stimulation effect to approximately 4-fold (Fig. 2A, lane 8). The time course experiment provided more details on the effect of Rad52 on the cleavage of Mus81-Mms4 (Fig. 2B). The stimulation of Rad52 to Mus81-Mms4 endonuclease activity was significant through the whole time course analysis, upto 70 min (Fig. 2B, compare lanes 4 to 7 with lanes 7 to 11; Fig. 2C).

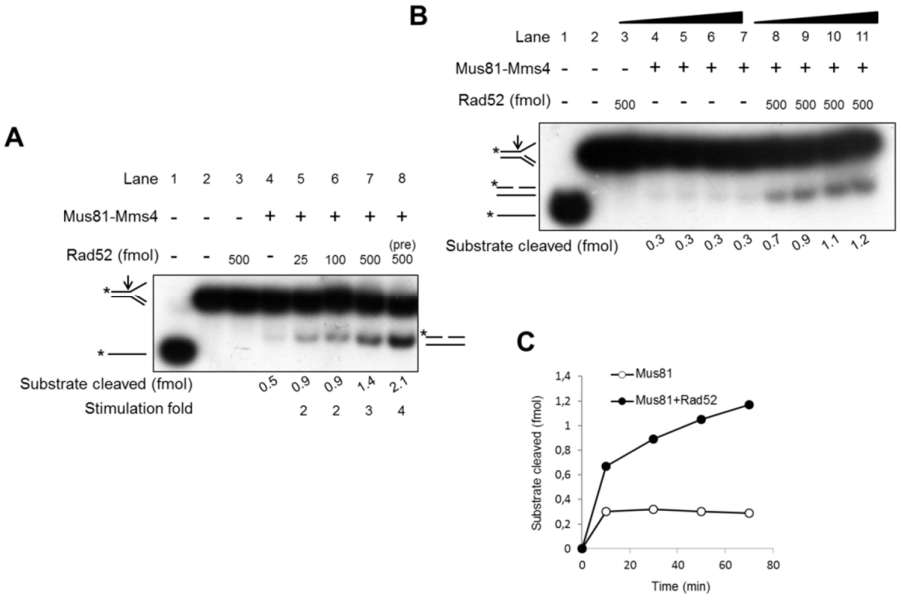

Figure 2. *Rad52 stimulates Mus81-Mms4 endonuclease activity*

A) Rad52 concentration-dependent elevation of Mus81-Mms4-mediated 3'-flap cleavage. The reaction mixtures containing Mus81-Mms4 (2.5 fmol) and indicated amount of Rad52 (fmol) were incubated with 3'-flap substrate (10 fmol) at 30 °C for 30 min, then analyzed in a 10% denaturing-polyacrylamide gel. Abbreviation: pre, pre-incubation of Rad52 (500 fmol) with substrate at 30 °C for 5 min, followed by addition of Mus81-Mms4 to the mixture to initiate reaction; Arrow indicates the cleavage site of Mus81-Mms4. B) Time course of enhancement of Mus81-Mms4-mediated 3'-flap cleavage by Rad52. Mus81-Mms4 (2.5 fmol) was incubated with 3'-flap substrate (10 fmol) in the absence or presence of Rad52 (500 fmol) for 30 min at 30 °C. Aliquots of the reactions were taken at different time points (10, 30, 50 and 70 min) and analyzed. C) Cleaved product (fmol) in B was plotted against time points (min).

Then we investigated the effect of Rad52 on the Mus81 cleavage efficiency of different DNA substrates. We found that Rad52 stimulated Mus81 resolution activity on all tested DNA substrates, up to 3-fold in case of PX junctions (Fig. 3, lanes 21 and 22). Noticeably, as for replication fork and nick HJs, the stimulation was at least 10-fold when 500 fmol of Rad52 was added (Fig. 3, lanes 7 and 14). The significant enhancement in case of replication fork and nick HJs substrates may be due to the preference of Mus81-Mms4 for replication fork and nick HJs to 3'-flap and PX junctions in vitro. We concluded that the stimulation of Mus81-Mms4 endonuclease activity by Rad52 is intrinsic and varies depending on structure of DNA substrates cleaved by Mus81-Mms4.

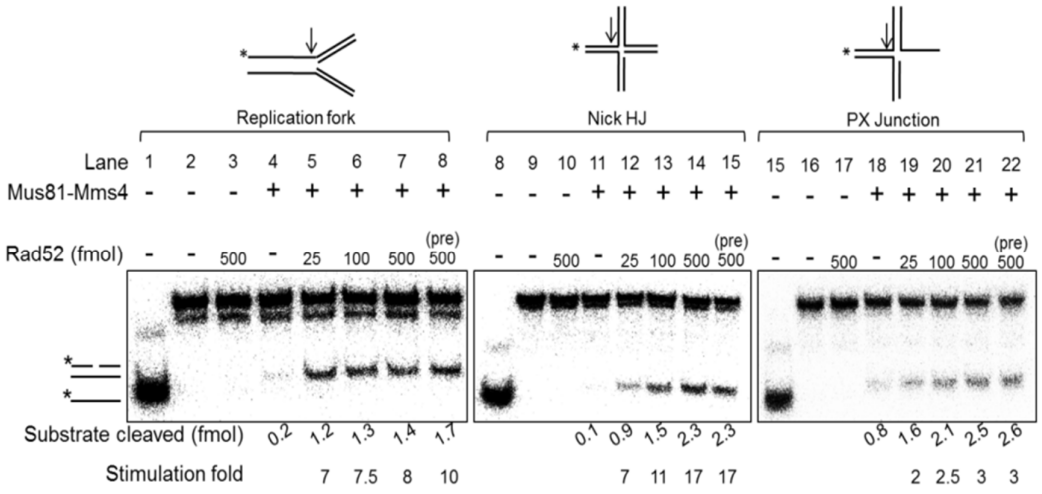

Figure 3. *Stimulation of Rad52 on Mus81-Mms4 cleavage activity on various DNA substrates.*

Effect of Rad52 on Mus81-Mms4 endonuclease activity on different substrates: replication fork, nick HJ, and PX junction, was investigated by the same procedure in Fig. 2A.

3.3. The species-specific stimulation of Rad52 to Mus81-Mms4

The specificity of stimulation of Mus81-Mms4 endonuclease activtity by Rad52 was then investigated to identify whether this enhancement effect is universal or species-specific to yeast proteins. Therefore, human MUS81-EME1 was purified and examined the endonuclease activity in the absence and presence of yeast Rad52 with 3'-flap as a substrate. It was very clear that yeast Rad52 failed to elevate the catalytic activity of human MUS81-EME1 in vitro (Fig. 4, lanes 4 to 6). In contrast, yeast Rad52 strongly inhibited human MUS81 endonuclease (Fig. 4, lanes 8 to 10). Accordingly, human Rad52 was unable to enhance the endonuclease activity of yeast Mus81-Mms4 (data not shown). These results proved that the stimulation of Mus81-Mms4 enzymatic activity by Rad52 is species-specific in budding yeast.

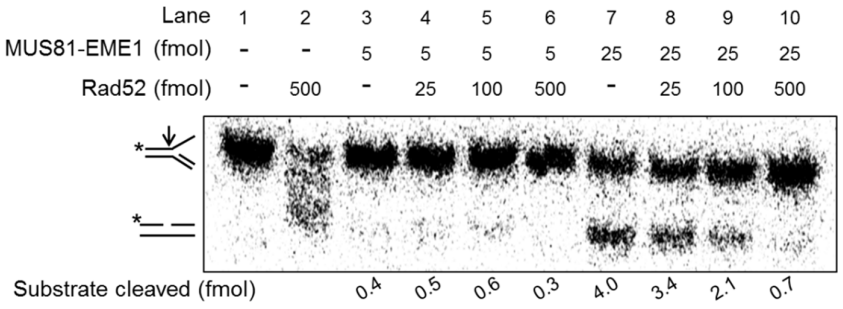

Figure 4. *Yeast Rad52 inhibits endonuclease activity of human MUS81-EME1 complex.*

The reactions were performed in the presence of indicated amount of human MUS81-EME1 and yeast Rad52 in buffer containing 50 mM Tris-HCl/pH 8.0, 1 mM DTT, 0.25 mg/mL BSA, 2 mM MgCl2, 50 mM NaCl (final concentration) and 10 fmol 3'-flap substrate. Reactions were incubated at 37 ℃ for 30 min and stopped by addition of stop buffer. Cleaved products (gap DNA duplex) were analyzed by 10% native polyacrylamide gel electrophoresis.

4. Discussion

In *Saccharomyces cerevisiae*, Mus81-Mms4 complex is responsible for the process of DNA intermediates in HR as well as those arisen from blocked and collapsed replication forks. All known and efficient HR mechanisms require RAD52 epistasis gene group which includes Rad52. We have presented data showing the strong stimulation on a broad range of different substrates of the yeast Mus81-Mms4 endonuclease activity by yeast Rad52 in vitro. Besides, human and yeast Rad52 inhibited yeast Mus81-Mms4 and human Mus81-

Eme1, respectively. Therefore, the yeast Rad52-mediated enhancement of yeast Mus81-Mms4 nuclease is species-specific.

The Δ*mus81* mutants were not viable in the absence of the Sgs1, which acts jointly with Top3 to resolve late recombination intermediates, such as double HJs, to produce non-crossover recombinants [9], [20]. However, all of the Δ*rad52*Δ*sgs1*Δ*mus81* triple-mutant spores grew into colonies, similarly to the Δ*rad52*Δ*mus81* double-mutant spores, confirming that the abolishment of homologous recombination suppresses the lethality of Δ*sgs1*Δ*mus81* cells [20]. Besides, Mus81 was found in the screening for the increase of spontaneous Rad52-YFP foci [31]. Moreover, Δ*rad52*Δ*mus81* double mutants grew at the same rate as the single Δ*rad52* mutants did, that is, in a Δ*rad52* background, Δ*mus81* mutations do not induce any important growth defect [20]. These results provide the evidence that the two enzymes work in the same pathway. Thus, Mus81-Mms4 functioning downstream of Rad52 constitutes an alternative mechanism paralleling to the Sgs1-Top3 pathway for the resolution of toxic intermediates. Furthermore, the assembly of DNA lesion-induced Mus81 foci likely depends on Rad52. The observed stimulation of Mus81-Mms4 endonuclease activity by Rad52 has important significance, allowing Mus81-Mms4 complex rapidly resolve recombinant intermediates which are accumulated by upstream action of Rad52. Especially, this functional interaction becomes critical when Sgs1 is dysfunctional or in the presence of DNA damaging agents inducing a lot of DNA lesions that activate DNA repair pathway by HR and generate high amount of intermediates. Together with our findings, it suggests that Rad52 and Mus81-Mms4 should work conjointly in the repair of DNA damage and stalled replication fork. These findings, with previous studies of functional interaction of Mus81 and Rad54, may serve as the primarily important pieces of evidence for the higher-order complex of Mus81-Mms4 and Rad52 and Rad54.

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