Purification of *Saccharomyces cerevisiae* recombinant Crp1

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Abstract

A complex Mus81-Mm4 is a DNA structure–specific endonuclease in *Saccharomyces cerevisiae*. Mus81-Mms4 functions in processing of recombination intermediates that could arise during the repair of stalled and blocked replication forks and double stranded breaks. Mus81-Mms4 works with many proteins involved in DNA repair, replication fork stability, and joint molecule formation/resolution during homologous recombination repair. A biochemical screening of protein(s) that enhances the Mus81-Mms4 endonuclease activity on its preferable substrates *in vitro* revealed that Crp1, a cruciform DNA-recognizing protein, which can specifically bind to DNA four-way junction structures like Holliday junctions could be the potential factor. To further demonstrate that Crp1 interacts functionally with Mus81-Mms4 *in vitro*, we carried out the purification of recombinant Crp1 using *Escherichia coli* system. Our results showed that the purified Crp1 was highly homogenous and active that is ready for biochemical use.

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Crp1, DNA binding, Mus81, purification.

1 Introduction

Mus81 is a conserved DNA structure–specific endonuclease which belongs to the XPF/Rad1 family of proteins involved in DNA nucleotide excision repair [1]. XPF/Rad1 family members typically contain a pair of helix-hairpin-helix (HhH) motifs and a conserved catalytic domain in their C-terminal region with an ERKX3D active site motif [1]. In Mus81, the HhH motifs are positioned at both ends, whereas in XPF they are positioned at the C-terminal end. The HhH motifs may play a role in DNA binding and dimer formation, and are also required for nuclease activity [2, 3].

Mus81 was shown to be active as a structure-specific endonuclease only when it forms a heterodimer with a partner protein, namely Eme1 in humans and *Schizosaccharomyces pombe*, andmMs4 in budding yeast. Mus81-Mms4 complex can catalyze 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 [4-8]. The in vivo findings above and the fact that Mus81-Mms4 alone hardly cleaves the intact HJs suggests that Mus81-Mms4 may require some other factors to resolve HJs in vivo. Rad54 has been shown to be the stimulation factor of both Mus81-Eme1 and Mus81-Mms4 endonuclease activity [9, 10]. Recently, our previous study had shown genetic and biochemical interaction of Rad27 and the Mus81-Mms4 complex [11], implicating the joint function of both enzymes in DNA replication or/and recombination. Taking together all, Mus81-Mms4 is most likely to work with other proteins involved in a variety of DNA metabolisms including DNA repair, replication fork stability, and joint molecule formation/resolution during meiotic recombination in order to safeguard the genome integrity.

A biochemical screenings to seek for protein(s) which elevates the Mus81-Mms4 endonuclease activity on its preferable substrates was carried out. The screening led us to the potential candidate, namely Crp1, a cruciform DNA-recognizing protein, which has the high possibility to enhance the endonuclease activity of Mus81-Mms4 in vitro. Crp1 (YHR146W) is a poorly characterized nuclear protein [12] reported to bind cruciform DNA structure [13]. Crp1 was found to belong to the telomerase deletion signature genes, a small set of genes which are specifically induced when telomeres can no longer be maintained by telomerase, but rarely induced under the diverse environmental stress conditions and DNA-damaging agents. This implies that Crp1 might be involved in telomere recombination/repair processes [14]. Crp1 also was shown to interact with the N-terminal FHA1 domain of Rad53 independently ofmMS treatment [15]. Besides, Crp1 was found among genes

induced by the hydrostatic pressure shock which decreases the viability of yeast cells [16].

Crp1 deletion strain is fully viable and does not show any visible phenotype at normal or non-permissive temperatures. Furthermore, the deletion strain does not react differently from wild type to UV irradiation ormMS treatment [13]. While Crp1 shows no similarity to other X-DNA-binding proteins, database searches for the homology to the lysine rich X-DNA-binding domain reveal that Crp1 shows the homology to the yeast protein Mdg1, with 41% identity in the N-terminal part from residues 1 to 160 of Crp1. Mdg1 is the plasma membrane protein involved in G-protein mediated pheromone signaling pathway [17]. Mdg1 was also found to bind specifically the branched DNA substrates. Furthermore, the Mdg1 deletion strain behaves the same as Crp1 null mutant with regards to temperature test or DNA damage agent treatment [13]. Here, we successfully purified the recombinant Crp1 using *E. coli* system and auto-induction method, and demonstrated that the Crp1 purified was active and specifically binds to cruciform DNA structure as previously reported.

2 Materials and Methods

- Nucleotides, enzymes, and plasmids

pET vector used for protein expression in *E. coli* were from Novagen (Darmstadt, Germany). [γ-^{32}P] ATP (>3000 Ci/mmol) was purchased from IZOTOP (Budapest, Hungary). The oligonucleotides used to construct DNA substrate were synthesized commercially from Genotech (Daejeon, South Korea). T4 polynucleotide kinase was purchased from Enzynomics (Daejeon, Korea).

- Purification of recombinant Crp1

We constructed pET28b inserted *CRP1* tagged 6XHis tag at C-terminal to express Crp1 in *E. coli* BL21-CodonPlus (DE3)-RIL strain. Cells were incubated at 37°C in auto-induction culture media for 16 hr. Cells were harvested by centrifugation, washed with Tris-HCl-buffered saline, and stored at -80°C. The cell pellet was resuspended in lysis buffer T_{100} (50mM Tris-HCl/pH 8.0, 100mM NaCl, 10% glycerol, 0.01% NP-40, and protease inhibitors). Following sonication, the crude lysate was clarified by centrifugation at 45,000rpm for 30min. The supernatant was applied onto pre-equilibrated SP-Sepharose column. After washing step by 10-column volumes of buffer T_{100}, the bound protein was eluted by buffer containing NaCl gradient from 100mM to 1000mM. The fractions containing Crp1 (confirmed by western blot using anti-His antibody) were pooled and loaded on Ni-IDA column. After two steps of washing with 10-column volumes each of buffers T_{1000} plus 100mM IDZ and T_{1000} plus 250mM IDZ sequentially, the bound proteins were eluted by buffer T_{1000} containing IDZ gradient from 250mM to 500mM. Peak fractions were then pooled, dialyzed to T_{100} containing 50% glycerol, and stored at -80°C.

- DNA structure-specific binding activity of Crp1

The electrophoretic mobility shift assay (EMSA) was carried out as follows: the reaction mixtures (10 µl) containing 50mM Tris–HCl/pH 8.0, 100mM NaCl (final concentration), 10mM $MgCl_2$, 1mM DTT, 0.1 mg/ml BSA, 5mM EDTA pH 8.0, 10 fmol of intact HJ substrate, and indicated amounts of Crp1 and competitors were incubated on ice for 5min, followed by the further incubation at room temperature for 15min. After incubation, 10% glycerol (v/v, final concentration) and 0.05% BPB (w/v, final concentration) were added to the reaction mixtures. The formed nucleoprotein complexes were separated through a 3% native polyacrylamide gel in 0.5X TBE (45mM Tris-HCl, 45mM boric acid, and 1mM EDTA) at 100 V for 1.5 hr. The gels were dried on a DEAE-cellulose paper and autoradiographed. The amount of complexes were quantified and analyzed with the use of a phosphor-imager (BAS-1500, FUJIFILM). The intact HJ is made of 1, 2, 4, and 6 oligonucleotides, where **1** (5′-CGA ACA ATT CAG CGG CTT TAA CCG GAC GCT CGA CGC CAT TAA TAA TGT TTT C-3′), **2** (5′-CGC ATC CTA TCA GTT CGT ATG CAG TGT CCG GTT AAA GCC GCT GAA TTG TTC G-3′), **4** (5′-GAA AAC ATT ATT AAT GGC GTC GAG CTA GGC ACA AGG CGA ACT GCT AAC GG-3′), and **6** (5′-CCG TTA GCA GTT CGC CTT GTG CCT A ACT GCA TAC GAA CTG ATA GGA TGC G -3′).

3 Results

- Purification of recombinant Crp1

The biochemical screening revealed five proteins which might be the factor(s) enhancing the endonuclease activity of Mus81-Mms4, namely Hcs1, Crp1**,** Cdc9, Rad52, and Top2 because their substrates are DNA. Markedly, we specifically focus on Crp1, a cruciform DNA-recognizing protein, because this protein possesses the ability of specific binding to cruciform DNA structure, such as HJs [13]. Since both Crp1 and Mus81 work together on the same DNA substrate *in vivo*, Crp1 is a most likely candidate that interacts functionally with Mus81.

The reason why we decided to further study on Crp1 is due to its ability to bind specifically on cruciform DNA structure. In order to confirm the structure specific binding of Crp1 and the stimulation of Crp1 to Mus81-Mms4 endonuclease, recombinant Crp1 protein was purified to near homogeneity as described in Materials and Methods (Fig. 1A). With the aim of confirmation of structure specific binding of recombinant Crp1 that we purified, we carried out the EMSA with all the fractions collected from Ni-IDA column. The results prove that the binding activity follows the Crp1 protein amount present in each fraction (Fig. 1B).

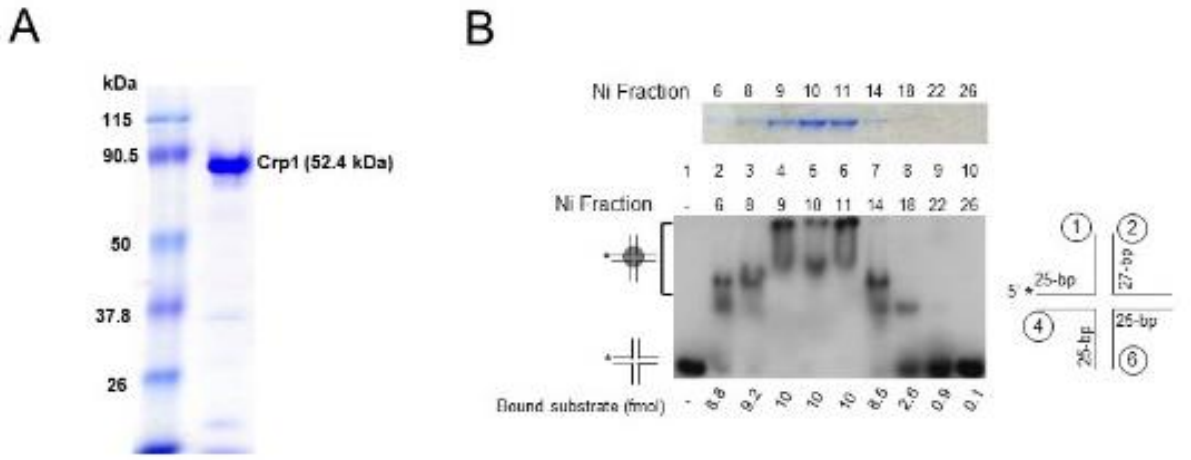

Figure 1 Purification of Crp1

A. SDS-10% PAGE stained by Coomasie brilliant blue shows the peak fraction from Ni-IDA column of Crp1 (5µg, lane 2); B. Native polyacrylamide gel shows the binding of Crp1 present in Ni-IDA fractions onto intact HJ substrate. 0.25µl of each fraction was used.

- DNA structure-specific binding activity of Crp1

EMSA was also performed to compare the competition ability of HJ competitor and the double-stranded DNA competitor to the binding activity of Crp1. HJ is considered as the specific competitor, while double-stranded DNA is the non-specific one. Therefore, HJ competitor should affect dramatically the binding of Crp1 on the HJ substrate while double-stranded DNA competitor insignificantly reduces the binding activity of Crp1. To make the data convincing, we prepared two kinds of double-stranded non-specific competitors. One was generated by restricting the plasmid pRS325 with SmaI producing blunt end, and the other is the collection of equal amount of two DNA duplexes which are produced by annealing oligo 7 and 8, 9 and 10. The sequence of those oligos are as follows: 7 (5'-CGC ATC CTA TCA GTT CGT ATG CAG TGC TCG ACG CCA TTA ATA ATG TTT TC-3'), 8 (5'- GAA AAC ATT ATT AAT GGC GTC GAG CAC TGC ATA CGA ACT GAT AGG ATG CG-3'), 9 (5'- CGA ACA ATT CAG CGG CTT TAA CCG GAC TAG GCA CAA GGC GAA CTG CTA ACG G-3'), and 10 (5'- CCG TTA GCA GTT CGC CTT GTG CCT AGT CCG GTT AAA GCC GCT GAA TTG TTC G-3'). The HJ competitor is 102 base pairs (bp) while the first double-stranded competitor is about 6800bp and the second consists of equal amount of two duplexes with the same sequence to the HJ, one is 50bp and the other is 52bp. Each comparison assay between the HJ and one kind of non-specific competitor was repeated 3 times independently. The EMSA results turned out as we expected, proving that the recombinant Crp1 that we purified is fully active and ready for the next biochemical assay (Fig. 2A, B, and C).

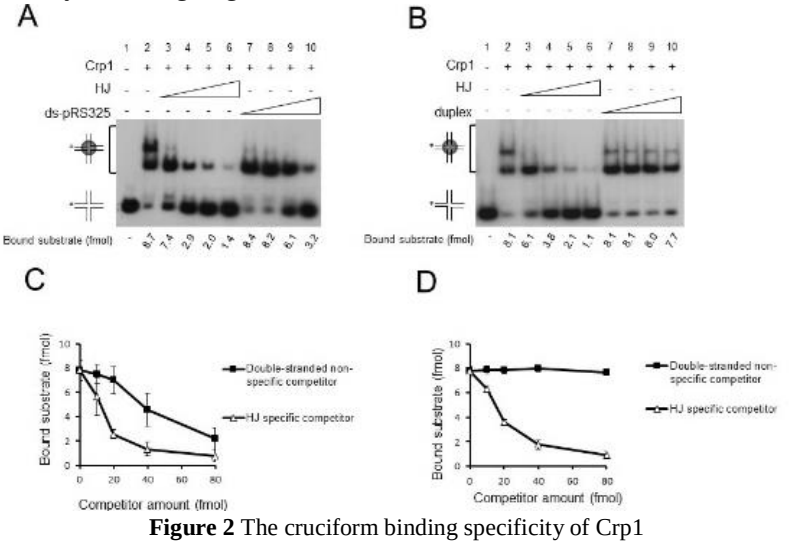

Figure 2 The cruciform binding specificity of Crp1

A. Comparison of the competition ability between HJ-specific competitor and double-stranded DNA non-specific competitor pRS325 to the binding activity of Crp1 onto HJ substrate. HJ competitor was generated by annealing 4 oligos 1, 2, 4, and 6 with

1:1:1:1 molar ratio. Double-stranded DNA was produced by restricting the pRS325 plasmid with SmaI producing blunt end; B. Comparison of the competition ability between HJ-specific competitor and duplex-DNA non-specific competitor to the binding activity of Crp1 onto HJ substrate. The duplex-DNA was produced by annealing oligo 7 and 8, 9 and 10 with 1:1 molar ratio; C. The amount of bound substrates (fmol) in A were plotted against the amount of competitor (ng) used. The graph obtained by 3 independent experiments; D. The amount of bound substrates (fmol) in B were plotted against the amount of competitor (ng) used. The graph obtained by 3 independent experiments.

4 Discussion

Firstly, Crp1 was identified as N-terminal fragments, which possess exclusively the DNA cruciform structure (X-DNA) binding ability, originating from the same putative protein from yeast extract, encoded by open reading frame YHR146W [13]. The binding specificity of these protein fragments was defined as the DNA-branched structures binding [13]. Expression of Crp1 in *E. coli* showed that Crp1 is subjected to efficient proteolysis post-translation at one specific site and by generating truncations of Crp1, this site was clarified at approximately position 160, and the cleavage of Crp1 is not a prerequisite for the X-DNA binding activity [13]. The X-DNA binding domain of Crp1 is mapped in the N-terminal part of the protein, and more specifically, is dependent on residues 120-141, a region that can act independently as an X-DNA binding peptide [13]. This short lysine-rich stretch might account for a new kind of X-DNA binding protein. Interestingly, Crp1 enhances the cleavage of X-DNA by T4 Endo VII, the endonuclease VII from bacteriophage, and the site specificity of T4 Endo VII-cleavage was not affected by Crp1. This is the first clue implying that Crp1 has the potential to act on HJ as part of processing machinery [13].

Moreover, mChIP-MS of Crp1-TAP successfully detected Pep4, Prc1, and Prb1, three proteinase involved in vacuolar degradation, the glycogen synthases Gsy1 and Gsy2, as well as the phosphatase Glc7 and its targeting subunit Pig2. Surprisingly, six proteins associated with Crp1-TAP (Glc7, Pep4, Gsy2, Pig2, Htd2 and Prb1) are required for proper glycogen accumulation (Francois and Parrou, 2001), which suggests that Crp1 may have a critical role in this process (Jean-Philippe *et. al.*, 2010). Interestingly, Crp1 is an ortholog to the mammalian AMP-activated protein kinase β-2 subunit, which is known to directly bind glycogen and coordinate cellular metabolism in response to energy demands (Polekhina *et. al.*, 2003). The polypeptide consisting of the residues from 171 to 185 of Crp1 protein was found in differently regulated phosphopeptides screening (Gruhler *et. al.*, 2005). Moreover, *CRP1* is one of the genes that showed significant expression divergence between laboratory strain and a wild strain in co-culture (Wang *et. al.*, 2007).

Crp1 possesses the structure specific binding to cruciform DNA which is also the substrate of Mus81-Mms4 endonuclease. Thus, Crp1 could stimulate Mus81-Mms4 by some different mechanism as follows: (i) recruitment in which Crp1 first binds the substrate and elevates the recruitment speed of Mus81-Mms4 to the cleavage site, (ii) turn-over in which the binding of Crp1 helps dissociate Mus81-Mms4 from cleavage products, thereby rapidly recycling Mus81–Mms4, and (iii) protein-protein interaction in which the stimulation effect is due to the directly specific interaction between Crp1 and Mus81-Mms4 complex. Therefore, purification of high homogeneous recombinant Crp1 is the critical step required to investigate the functional interaction between these two enzymes.

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Tinh sạch protein *Saccharomyces cerevisiae* Crp1 tái tổ hợp

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Tóm tắt Phức hợp Mus81-Mms4 là một endonuclease cắt các cấu trúc ADN đặc hiệu ở *Saccharomyces cerevisiae*. Mus81-Mms4 tham gia vào việc xử lí các cấu trúc trung gian tái tổ hợp mà được hình thành trong quá trình sửa chữa chạc sao chép dừng/lỗi và đứt gãy sợi đôi ADN. Mus81-Mms4 hoạt động với nhiều protein liên quan đến sửa chữa ADN, ổn định chạc sao chép, và hình thành/phân giải các phân tử nối trong con đường sửa chữa bằng tái tổ hợp tương đồng. Một nghiên cứu sàng lọc hóa sinh nhằm tìm kiếm protein có khả năng tăng cường hoạt tính endonuclease của Mus81-Mms4 trên cơ chất ưa thích của phức hợp in vitro đã cho kết quả rằng Crp1, protein nhận biết ADN có cấu trúc cruciform mà có khả năng bám một cách đặc hiệu lên các cấu trúc ADN bốn nhánh tương tự như mối nối Holliday, có thể là kích thích tố tiềm năng của hoạt tính endonuclease của Mus81-Mms4. Nhằm chứng minh tương tác chức năng giữa Crp1 và Mus81-Mms4 in vitro, chúng tôi tiến hành tinh sạch protein Crp1 tái tổ hợp sử dụng hệ thống *Escherichia coli*. Kết quả thu được thể hiện rằng Crp1 tinh sạch có độ đồng thể cao và có hoạt tính bám ADN cấu trúc đặc hiệu, phù hợp với yêu cầu của các thí nghiệm hóa sinh tiếp theo.

Từ khóa bám ADN, Crp1, Mus81, tinh sạch.