FEBS Letters 586 (2012) 356–361

journal homepage: www.FEBSLetters.org

Yeast ribosomal protein S3 possesses a β-lyase activity on damaged DNA

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ARTICLE INFO

Article history:
Received 31 October 2011
Revised 21 December 2011
Accepted 21 December 2011
Available online 10 January 2012

Edited by Michael Ibba

Keywords:
Yeast Rps3p
Lyase
AP sites
Pyrimidine dimer
8-Oxo-G
DNA repair

ABSTRACT

Yeast ribosomal protein S3 has multifunctional activities that are involved in both protein translation and DNA repair. Here, we report that yeast Rps3p cleaves variously damaged DNA that contains not only AP sites and pyrimidine dimers but also 7,8-hydro-8-oxoguanine. This study also revealed that Rps3p has a β-lyase activity with a broad range of substrate specificity which cleaves phosphodiester bonds of UV or oxidatively damaged DNA substrates. Mutation analysis of the yeast Rps3 protein including introduction of domain deletions and residue replacements identified the residues Asp154 and Lys200 are important for the catalytic activity. In addition, the repair enzyme activity of yeast Rps3p was confirmed by complementation in *xth, nfo Escherichia coli* cells in which the DNA repair process is defective.
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1. Introduction

Ribosomal protein S3 (rpS3) is a component of the 40S small ribosomal subunit and therefore is associated with protein synthesis [1]. In addition, it has been known that rpS3 is a multifunctional protein involved in DNA repair [2], metastasis [3], and apoptosis [4,5]. Ribosomal protein S3 is a repair protein with an endonuclease activity which acts on damaged DNA. The endonuclease activity of this protein has been reported in humans, mice, and *Drosophila* [6–9]. Mammalian ribosomal protein S3 (rpS3) was identified as UV endonuclease III, and may be associated with XP (xeroderma pigmentosum) disease [6]. It has been reported that human rpS3 protein has an endonuclease activity and cleaves DNA containing apurinic/apyrimidic (AP) sites, pyrimidine dimers, and thymine glycols [10]. However, this protein seemed to have an AP lyase activity that cleaves AP sites via a β-elimination reaction lacking δ-elimination [11].

The expression of yeast Rps3 protein is tightly regulated at the transcriptional level. A promoter study of RPS3 revealed that the expression of this protein is positively regulated by Rap1p [12] and negatively regulated by Gcn4p in stress conditions [13], which are known to be typical stress response regulators in yeasts. This protein has been shown to possess an AP endonuclease activity in which the enzyme cleaves phosphodiester bonds 3′- and adjacent to an AP site in the DNA molecule [14]. However, its activity was not evaluated on the damaged base substrates such as pyrimidine dimer and 8-oxo-G.

AP endonuclease activity can be divided into two categories according to the mechanism of action. Type I AP endonucleases cleave, more accurately called AP lyases [15], 3′ to AP sites via a β-elimination reaction to leave 3′-α,β-unsaturated aldehyde (trans-4-hydroxy-2-pentenal 5-phoshate) and deoxyribonucleoside 5′-phosphate terminal residues. *Escherichia coli* endonuclease III [16], Fpg, and T4 endonuclease V [17] are classified as type I AP endonucleases. Type II AP endonucleases such as *E. coli* exonuclease III, endonuclease IV, and the AP endonucleases of yeasts, *Drosophila*, and humans cleave the 5′ to AP sites via hydrolysis to yield 5′-deoxyribose 5-phosphate (dRP) and nucleotide-3′-hydroxyl terminal residues.

Here, we evaluated the ability of yeast Rps3 protein to cleave various damaged DNA substrates and identified the residues essential for Rps3p endonuclease activity. We describe a novel cleavage mechanism of yeast Rps3 protein involving a β-elimination reaction.

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doi:10.1016/j.febslet.2011.12.030

2. Materials and methods

2.1. Construction of the yeast RPS3 expression vector, induction, and purification

Cloning the yeast *RPS3* gene into a pGEX 2T GST-tagged expression vector, expression, and purification of yeast Rps3p were performed as previously described [14]. Yeast Rps3p expression levels were determined by Western blot analysis using a GST-polyclonal antibody. For the yeast Rps3 protein mutation analysis, N- and C-terminal deletions, and point mutations were introduced into the *RPS3* gene by PCR-based mutagenesis with specific primers (Supplementary Table 1). The mutant proteins were also expressed and purified as described above.

2.2. Endonuclease assays

2.2.1. Plasmid nicking assay

Partially depurinated DNA containing 1.5 AP sites per DNA molecule was prepared as previously reported [6] and used as the substrate for the plasmid nicking assay. The reaction mixture (20 μL) containing 0.1 μg of the plasmid, and 1 μg of purified yeast Rps3p in reaction buffer (40 mM Tris–Cl pH 8.0, 35 mM KCl, 0.001% Triton X-100, 3 mM EDTA) was incubated at 37 °C for 1 h, and then mixed with 3 μL of gel loading dye containing 1% SDS. Samples were resolved on a 1% agarose gel containing 0.1 μg/mL of ethidium bromide and visualized under UV light.

2.2.2. Nick circle assay

An endonuclease assay was performed as reported by Kim et al. [18]. Briefly, 1 nmol of [^{3}H]-labeled PM2 DNA was incubated with purified recombinant Rps3p. The number of nicks was calculated according to the Poisson distribution described by Seong et al. [19]. One unit of enzyme activity was defined as the activity to produce 1 fmol of nicked DNA per min at 37 °C. To obtain the K_m value of yeast Rps3p for the AP DNA cleavage, the assay was performed with a fixed concentration (2 units) of the purified yeast Rps3p increasing concentrations of AP DNA substrates up to 20 nmol. To measure glycosylase activity using base release and the production of AP sites, samples were treated with 0.3 M K_2HPO_4 (pH 13.2) and heated to 95 °C for 5 min.

2.2.3. Endonuclease assay with DNA containing 8-oxo-G or AP sites

An oligonucleotide fragment (12-mer) containing an 8-oxo-G and an AP site at position 4 was used for the preparation of substrates (Oligo-A, 5′-CGCG*AATTCGCG-3′, G*= 8-oxo-G and Oligo-B, 5′-CGCXAATTCGCG-3′, X = spacer). Oligomers were labeled with γ-^{32}P ATP by T4 polynucleotide kinase (Promega, CA). To anneal the labeled oligomer to double-stranded DNA, each oligomer was mixed in the annealing buffer (20 mM Tris–HCl pH 7.5, 100 mM NaCl). The mixture was heated at 95 °C for 5 min and cooled overnight at room temperature. The assay mixtures (20 μL) contained 10 pmol of 5′ end-labeled 12-mer oligomer, 1 μg of GST-yeast Rps3p, 30 mM HEPES pH 7.4, 50 mM KCl, 1 μg/mL bovine serum albumin, 0.05% Triton X-100, 1 mM DTT, and 0.5 mM EDTA. The reaction samples were separated on a 20% polyacrylamide gel containing 7 M urea. The gels were dried and visualized by autoradiography (Fuji, BAS 2500 PhosphorImager).

2.3. DNA repair synthesis

DNA incision by yeast Rps3p and T4 UV endonuclease was performed by nick circle assay. The endonuclease activity was stopped by heating for 5 min at 70 °C. DNA synthesis was carried out in a mixture (200 μL) containing 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dTTP, 50 μCi of [α-^{32}P] dCTP, 20 μL of 10× reaction buffer (Kosco, South Korea), the 0.5 nmol of the incised PM2 DNA, and *E. coli* DNA polymerase I (5 units, Kosco). Unincorporated dNTPs were removed by a Sephadex G-25 column and radioactivity was measured by scintillation counter.

2.4. $NaBH_4$ trapping assay

A 34-mer oligomer containing uracil (Oligo-C, 5′-ATGCCTGCACGA(T/U)GCA ATTCGTGATCATGGTCAT-3′) and its complementary oligomer (Oligo-D, 5′-ATGACCATGATCACGAATTGCATCGTGCAGGCAT-3′) were used for $NaBH_4$ trapping assay. The commercial uracil DNA glycosylase (Promega) generated the AP site in Oligo-C which was labeled and annealed with Oligo-D as described above. Purified yeast Rps3p and T4 UV endonuclease V were incubated with 20 pmol of DNA along with 100 mM of $NaBH_4$ in reaction mixture (40 mM Tris–Cl pH 8.0, 35 mM KCl, 0.01% Triton X-100, 3 mM EDTA) for 1 h at 37 °C. The samples were then separated on a 15% non-denaturing polyacrylamide gel. After electrophoresis, the gel was dried and exposed to X-ray film (Fuji, Japan).

2.5. Pyrimidine dimer-specific endonuclease activity

Two units of yeast Rps3p or T4 UV endonuclease mutant (Ser) was incubated with UV-irradiated DNA (25 J/m^2) as performed in the nick circle assay**.** After incubation, the reaction mixture was heated at 70 °C for 15 min to inactivate the enzymes. Two units of T4 UV endonuclease mutant and/or yeast Rps3p were added to this reaction mixture and incubated for 30 min at 37 °C. Next, the reaction mixture was denatured, renatured, and filtered through a nitrocellulose membrane. The radioactivity on the membrane was measured by a scintillation counter and the concentration of nicked DNA was calculated as previously described [14].

2.6. Effect of yeast Rps3 on the survival of E. coli cells after DNA damage

BW528 *E. coli* strains transformed with GST alone or GST-yeast Rps3 derivatives were cultured overnight at 37 °C. The cultures were then diluted with fresh media and grown to mid-log phase. IPTG (0.5 mM) was added and the cultures were incubated for an additional 20 min at 30 °C. Aliquots of these cultures were incubated with 5 mM of *t*-BuO_2H or 30 mM of MMS at 30 °C for 20 min, respectively. Growth of the cells was measured by serially-diluted spots on LB plates containing ampicillin (50 μg/mL). Each survival analysis was repeated at least three times; representative results are shown.

3. Results and discussion

3.1. Yeast ribosomal protein S3 has an endonuclease activity on relaxed DNA substrates containing AP sites and 8-oxo-G

To further characterize the AP endonuclease activity, we tested whether the enzyme cleaves relaxed DNA containing AP sites. First, yeast Rps3p cleaved the depurinated supercoiled plasmid (Fig. 1a). Depurinated pBluescript plasmid was nicked to change its conformation from supercoiled to an open circular relaxed form. The nicks were increased with increasing concentration of purified yeast Rps3 (lanes 2–6). However, various concentrations of GST proteins did not affect cleavage of normal, depurinated, or UV-irradiated pBluescript plasmids (data not shown). Second, nicked and relaxed forms of depurinated PM2 DNA were evaluated in the nick circle assay. Rps3 proteins showed an endonuclease activity in a dose-dependent manner. But, it was not observed in normal unrelaxed PM2 DNA showing that the activity works on relaxed

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