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Ribonucleotide Reductase Activity of *Schizosaccharomyces pombe* Is Inhibited by *Escherichia coli* RecA Antibody

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We have previoosly demonstrated that the RecA-like protein of *Schizosaccharomyces pombe* (*S. pombe*) is immunologically related to *Escherichia coli* (*E. coli*) RecA protein and that the cellular level of the protein is significantly increased by inhibitors of nucleotide pool-forming enzymes such as hydroxyurea (HU) and methotrexate (MTX) (Lee and Park, 1994; Lee *et al.*, 1994). In this study, we report that the ribonucleotide reductase activity of *S. pombe* is inhibited by *E. coli* RecA antibody, as determined by thin layer chromatography using [5-^{3}H]CDP as a substrate. The relative activity of ribonucleotide reductase was dramatically inhibited by 100 mM of HU (26.4% reduction) in *in vitro* assay, compared to that of non-treated control. The ribonucleotide reductase activity was also inhibited by immunoprecipitation with *E. coli* RecA antibody (43.3% reduction). These results indicate that the structure of *S. pombe* ribonucleotide reductase is in part similar to that of *E. coli* RecA protein.

KEY WORDS: *Schizosaccharomyces pombe*, Ribonucleotide Reductase, Methotrexate, Hydroxyurea, Thin Layer Chromatography, Polyethyleneimine Plate

Ribonucleotide reductase (E.C.1.17.4.1) catalyzes the first unique step in DNA synthesis by converting the four ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates (Kucera and Paulus, 1982; Darling *et al.*, 1987). The enzyme activities are allosterically regulated in a cell cycle-dependent manner and are transcriptionally induced in response to the stress of DNA damage (Lowdon and Vitols, 1973; Thelander and Reichard, 1979; Elledge and Davis, 1989).

In *Saccharomyces cerevisiae* (*S. cerevisiae*), *RNR2* gene encoding the small subunit of ribonucleotide reductase has been cloned by immunological screening with *Escherichia coli* (*E. coli*) RecA antibody as a probe (Elledge and Davis, 1987; Hurd *et al.*, 1987), indicating that the *E. coli* RecA protein and the *S. cerevisiae* ribonucleotide reductase is structurally similar to each other. The RNR2 protein was inducible upon treatments with DNA-damaging agents including UV-light, 4-nitroquinoline-1-oxide, and methyl methanesulfonate and agents that block DNA replication such as hydroxyurea and methotrexate (Elledge and Davis, 1989).

We have previously purified the RecA-like

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protein from *Schizosaccharomyces pombe* (*S. pombe*) and characterized the inducible nature of the protein against DNA damaging agents and inhibitors of nucleotide pool-forming enzymes (Lee and Park, 1994; Lee *et al.*, 1994). As described previously, the RecA-like protein of *S. pombe* was also inducible in cellular response to DNA damage or under the condition of deficient nucleotide pool (Angulo *et al.*, 1985; Elledge and Davis, 1987 and 1989; Lee and Park, 1994).

In this investigation, we tried to examine the structural and functional correlation of *E. coli* RecA protein and *S. pombe* ribonucleotide reductase by assaying ribonucleotide reductase activity using immunoprecipitation with *E. coli* RecA antibody. We report here that the *S. pombe* ribonucleotide reductase is at least structurally similar to that of *E. coli* RecA protein, as in *S. cerevisiae*.

Materials and Methods

Culture of yeast cells

S. pombe ura4-D18 strain was kindly provided by Dr. M. Yanagida (Department of Biophysics, Kyoto University, Japan) and cultured in YPD medium (1% yeast extract, 2% Bacto-peptone, 2% glucose, pH 5.5) at 30°C.

Enzymes and reagents

E. coli RecA protein was purchased from Sigma and alkaline phosphatase was from Hyclone. Polyethyleneimine (PEI)-cellulose plate was purchased from Sigma and [5-^{3}H]-CDP (spec. act., 10~30 Ci/mmol) was from Amersham Life Science.

Preparation of the *E. coli* RecA antisera and the *S. pombe* cell-free extract

The RecA antisera and the cell-free extract of *S. pombe* were prepared as described previously (Lee and Park, 1994; Lee *et al.*, 1994). From the rabbit RecA antisera, IgGs were purified by Affi-Gel10 chromatography (Lee *et al.*, 1994) and 1 mg of the IgGs per reaction was used for the immunoprecipitation of ribonucleotide reductase.

Ribonucleotide reductase assay

The ribonucleotide reductase activity was assayed according to the method of Schrecker *et al.* (1968) with a minor modification. In a final reaction volume of 100 μl, 200 mM of Hepes, pH 8.2, 10 mM of DTT, 100 μM of CDP, 1 μCi of ^{3}H-CDP (spec. act., 10 - 30 Ci/mmol), and 1 mg of *S. pombe* protein were included. After incubation at 30°C for 30 mins, the reaction was stopped by heating to 100°C for 2 mins. Hydrolysis of nucleotides was then performed by addition of 4U alkaline phosphatase (Sigma). After incubation at 37°C for 2 hrs, samples were heated at 100°C for 2 mins and the precipitate formed was removed by centrifugation at 2,000 $\times$g for 5 mins. The resulting supernatant was used for thin layer chromatography.

Thin layer chromatography

Thin layer chromatography (TLC) was performed on PEI-cellulose plate prepared as follows: the plate was washed with 10% NaCl and then the chloride form of the anion exchanger was converted to the borate form by soaking the dried sheet for 5 min in 0.4 M triethylammonium tetraborate. The sheet was immersed in excess distilled water for 1 min, in absolute methanol for 1 min, dried overnight, and stored below 0°C until used.

Samples were applied on the prepared PEI-cellulose plate and ascending development was then carried out in a closed glass tank with 0.1 M boric acid (Steeper and Steuart, 1970). After drying, the cellulose plate was cut out in an interval of 0.5 cm and the radioactivities were counted with a liquid scintillation spectrophotometer. The relative ribonucleotide reductase activity was calculated by the conversion rate of ^{3}H-cytidine to ^{3}H-deoxycytidine in percent.

Results and Discussion

As described previously, the small subunit of ribonucleotide reductase of *Saccharmyces cerevisiae* (*S. cerevisiae*) has homology with *Escherichia coli* (*E. coli*) RecA protein at the amino acid sequence level (Elledge and Davis,

1989). Indeed, the *RNR2* gene encoding the protein has been cloned by immunological screening with *E. coli* RecA antibody (Elledge and Davis, 1987; Hurd *et al.*, 1987). In this study we tried to examine whether the ribonucleotide reductase of *S. pombe* is also structurally related to that of *E. coli* RecA protein, as in *S. cerevisiae*.

To compare the structural correlation of *S. pombe* ribonucleotide reductase with *E. coli* RecA protein, ribonucleotide reductase activity assay was performed by thin layer chromatography on

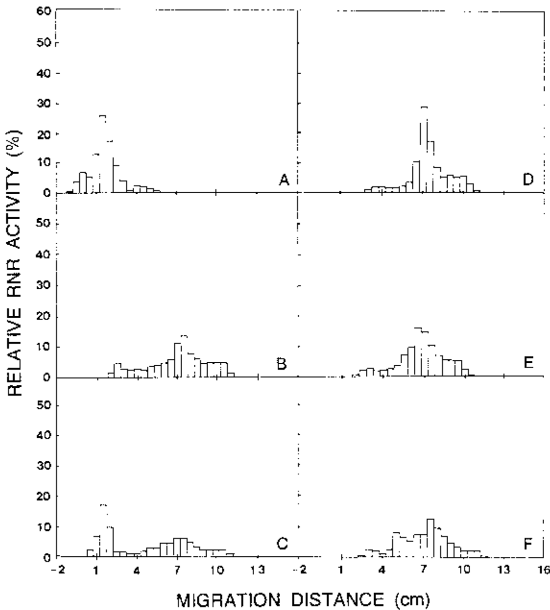

Fig. 1. Ribonucleotide reductase activity assay by thin layer chromatography. *S. pombe* cells were non-treated or treated with 15 μg/ml of methotrexate (MTX) for 6 hrs and cell-free extracts were prepared as described previously (Lee *et al.*, 1994). For the enzymatic assay, one milligram protein per reaction was used and [5-^{3}H]-CDP was added to the reaction mixture as substrate. Enzyme reaction was performed at 30°C for 30 mins, after which the phosphate group from dCDP produced was removed with alkaline phosphatase as described in Materials and Methods. PEI-cellulose plate was used for the separation of cytidine and deoxycytidine by thin layer chromatography. A, [5-^{3}H]-CDP treated only with alkaline phosphatase; B, Non-treated cell-free extract; C, Non-treated cell-free extract containing 100 mM of hydroxyurea (HU); D, MTX-treated cell-free extract; E, MTX-treated cell-free extract containing 100 mM of HU; F, MTX-treated cell-free extract immunoprecipitated with *E. coli* RecA antibody.

PEI-cellulose plate (Schrecker *et al.*, 1968). Because negative charged molecules do not migrate on PEI-cellulose plate during thin layer chromatography (Darling *et al.*, 1987; data not shown), the phosphate group from [5-^{3}H]CDP was removed by treatment with alkaline phosphatase (Eriksson *et al.*, 1981; Ullman *et al.*, 1981) and then thin layer chromatography (TLC) was carried out as described in Materials and Methods. Fig. 1 shows a typical TLC chromatogram on which the actual migration distance of [5-^{3}H]-cytidine was approximately 1.0 to 2 cm (Fig. 1A) and that of deoxycytidine was about 6.5 to 7.5 cm (Fig. 1B).

When ribonucleotide reductase activity was assayed in non-treated *S. pombe* cell-free extract, about 38% of CDP added in the reaction mixture was converted to dCDP (Fig. 1B). However, as shown in Fig. 1C, only 18% CDP was converted to dCDP by non-treated cell-free extract containing 100 mM of hydroxyurea (HU) which prevents the synthesis of deoxyribonucleotides by specifically inhibiting ribonucleoside diphosphate reductase, implying that HU remarkably inhibits the *S. pombe* ribonucleotide reductase as shown in all organisms tested until now (Table 1).

However, methotrexate (MTX) which acts as a structural analogue of folic acid and which binds to and inhibits the enzyme dihydrofolate reductase (DHFR) increased the cellular activity of ribonucleotide reductase to 1.6-fold, compared to that of non-treated control (Fig. 1D; Table 1). Since the inhibition of DHFR by MTX leads to preventing the reduction of dihydrofolate (or folic acid) to tetrahydrofolate (THF) and thereby inhibiting THF-dependent reactions (e.g. synthesis of deoxythymidine), the result shown in Fig. 1D suggests that the depletion of cellular deoxynucleotide triphosphates, especially TTP starvation by methotrexate, is a signal for induction of *S. pombe* ribonucleotide reductase activity (Lammers and Follmann, 1984).

In addition, HU also exerted an inhibitory effect on ribonucleotide reductase activity *in vitro* (Fig. 1E). When CDP was incubated with MTX-treated cell-free extract in the presence of 100 mM HU, 46% of CDP added was converted to dCDP (Fig. 1E). The ribonucleotide reductase activity was also

Table 1. Inhibition of ribonucleotide reductase activity of *S. pombe* by various inhibitors and *E. coli* RecA antibody.

Experimental group	dCDP formed (%)[e]	Relative RNR activity (%)
Non-treated control	38±2.5	100.0
HU[a]	18±3.7	26.3
MTX[b]	61±5.8	160.5
MTX + HU[c]	46±4.6	75.4
MTX + *E. coli* RecA Ab[d]	26±2.1	43.3

a, Hydroxyurea (HU) was added in the enzyme reaction mixture to a final concentration of 100 mM for 30 mins; b, Ribonucleotide reductase (RNR) activity was assayed with cell-free extract prepared from *S. pombe* cells pretreated with 15 μg/ml of methotrexate (MTX) for 6 hrs; c, Enzyme assay was carried out with the MTX-treated cell-free extract containing 100 mM of HU for 30 mins; d, The MTX-treated cell-free extract was used for the immunoprecipitation experiment with IgGs purified from rabbit antiserum raised against *E. coli* RecA protein; e, Relative conversion rate of CDP to dCDP was calculated by the mean value ± SD obtained from at least four independent experiments.

inhibited by immunoprecipitation with *E. coli* RecA antibody (Fig. 1F). By the immunoprecipitation, the relative activity of ribonucleotide reductase was reduced to 43.3% (Table 1). This result suggests that the ribonucleotide reductase of *S. pombe* is in part structurally related to that of *E. coli* RecA protein, as in *S. cerevisiae* (Hurd *et al.*, 1987; Elledge and Davis, 1987 and 1989).

Elledge and Davis (1987) have reported that the amino acid sequences of *S. cerevisiae* RNR2 protein and *E. coli* RecA protein have significant homology at the carboxy terminus of each protein. This suggests that the presence of homology to carboxy terminus suffices to form a strong epitope for RecA protein. Therefore, to provide further evidence that the carboxy terminus of *S. pombe* ribonucleotide reductase is the reactive part to *E. coli* RecA antibody as in the case of *S. cerevisiae*, an approach should be taken in order as follows: firstly, ribonucleotide reductase should be homogeneously purified from *S. pombe*.; secondly, the C-terminal region of the purified protein should be cleaved with protease such as chymotrypsin; finally, the immunological cross-reactivity of the digested protein to *E. coli* RecA antibody should be checked by Western blot analysis.

On the other hand, we have previously demonstrated that RecA-like protein from *S. pombe* was significantly induced by a wide variety of agents that either damage the DNA directly or induce stress by blocking DNA replication (Lee and Park, 1994). From these results we have proposed basic models accounting for specific induction of *S. pombe* RecA-like protein: (1) feedback regulation in response to depletion of the dNTP pools by repair processes or (2) direct induction in response to DNA damage. As presented in this study, the first model is in part supported by the fact that the ribonucleotide reductase activity is enhanced by MTX. However, on the basis of the results presented in this study and described previously we could not conclude that *S. pombe* RecA-like protein have a functional relationship with ribonucleotide reductase. Therefore, the structural similities and the functional relationships between two proteins should be further elucidated in the future.

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*Schizosaccharomyces pombe*에서 *Escherichia coli* RecA 항체에 의한 Ribonucleotide Reductase 효소활성 저해

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분열형 효모인 *Schizosaccharomyces pombe*(*S. pombe*)에서 RecA 유사 단백질은 hydroxyurea(HU)나 methotrexate(MTX)와 같은 nucleotide pool 형성에 관여하는 효소의 저해제에 의해 유도발현된다(Lee and Park, 1994; Lee *et al.*, 1994). 본 연구에서는 대장균의 RecA 단백질과 *S. pombe*의 ribonucleotide reductase가 구조적으로 유사한지를 RecA 항체를 이용한 면역침전법과 [5-^{3}H]CDP를 기질로하는 thin layerchromatography방법을 통하여 알아보고자 하였다. *S. pombe*의 ribonucleotide reductase활성은 100 mM HU에 의해 대조군에 비해 26.3% 저해되었으며, RecA 항체를 이용한 면역침전에 의해서도 43.3% 저해되었다. 이와같은 결과는 *S. pombe*의 ribonucleotide reductase가 대장균의 RecA 단백질과 구조적으로 유사함을 시사하는 것이다.