Yeast Sml1, A Protein Inhibitor of Ribonucleotide Reductase*

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Ribonucleotide reductase (RNR) catalyzes the reduction of ribonucleotides to deoxyribonucleotides; this step is rate-limiting in DNA precursor synthesis. A number of regulatory mechanisms ensure optimal deoxyribonucleotide pools, which are essential for cell viability. The best studied mechanisms are transcriptional regulation of the RNR genes during the cell cycle and in the response to DNA damage, and the allosteric regulation of ribonucleotide reductase by nucleoside triphosphates. Recently, another mode of RNR regulation has been hypothesized in yeast. A novel protein, Sml1, was shown to bind to the Rn1 protein of the yeast ribonucleotide reductase; this interaction was proposed to inhibit ribonucleotide reductase activity when DNA synthesis is not required (Zhao, X., Muller, E.G.D., and Rothstein, R. (1998) Mol. Cell 2, 329–340). Here, we use highly purified recombinant proteins to directly demonstrate that the Sml1 protein is a strong inhibitor of yeast RNR. The Sml1p specifically binds to the yeast Rnr1p in a 1:1 ratio with a dissociation constant of 0.4 μM. Interestingly, Sml1p also specifically binds to the mouse ribonucleotide reductase R1 protein. However, the inhibition observed in an in vitro mouse ribonucleotide reductase assay is less pronounced than the inhibition in yeast and probably occurs via a different mechanism.

Ribonucleotide reductase (RNR) plays a crucial role in DNA synthesis, by catalyzing the direct reduction of all four ribonucleotides to deoxyribonucleotides. Both the yeast and mammalian ribonucleotide reductases belong to the ribonucleotide reductase class I; in this class, the active enzymes consist of a large subunit and a small one (1). In mammalian cells, these two non-identical homodimeric subunits are encoded by the R1 and R2 genes, respectively. The R1 protein contains reductive active dithiols, the active site binding nucleoside diphosphate substrates and binding sites for nucleoside triphosphates that act as allosteric effectors. Substrate specificity is controlled by binding of ATP, dATP, dTTP, or dGTP to a specificity site, whereas overall activity is controlled by binding of ATP (active) or dATP (inactive) to an activity site (2). Failure to control the size of dNTP pools and/or their relative amounts leads to cell death or genetic abnormalities (3).

The R2 protein contributes a tyrosyl-free radical, which is essential for RNR activity; this radical is generated by a binuclear iron center (2). The flexible C-terminal tail of the R2 polypeptide chain is essential for the R1 and R2 interaction, and upon binding to the R1 protein, the flexible R2 protein tail becomes rigid (4). Peptides and peptidomimetics corresponding to the R2 protein C-terminal inhibit ribonucleotide reductase in a species specific way (5).

In Saccharomyces cerevisiae, there are two genes encoding R1-like proteins, RNR1 and RNR3 (6); there are also two genes encoding R2-like proteins, RNR2 (7, 8) and RNR4 (9, 10). The yeast ribonucleotide reductase genes are one of the targets of the Mec1-Rad53-dependent DNA damage/cell cycle checkpoint pathway (11). Overexpression of the yeast RNR genes suppresses the lethality of strains lacking Mec1 or Rad53, supposedly by increasing the dNTP pools (12). Unlike the mammalian enzyme, the yeast RNR is not inhibited by physiological concentrations of dATP; this observation explains the positive correlation between enzyme and dNTP levels in yeast. Recently, Zhao et al. (13) identified a novel protein that negatively affects dNTP pools in yeast, and they called it Sml1p. Deletion of SML1 rescued the lethality of a mec1 or rad53 strain. Sml1p was shown to interact with the yeast Rnr1 protein using the two-hybrid system and co-immunoprecipitations. For this reason, a novel mode of RNR regulation was suggested, where Sml1p binding to the Rnr1 protein would inhibit the enzyme.

Using highly purified recombinant proteins, we now directly demonstrate in an in vitro yeast ribonucleotide reductase assay that Sml1p is indeed a very potent inhibitor of yeast RNR. It specifically binds to the yeast Rnr1p as shown by biosensor technique using sensor chips with immobilized Sml1p. Interestingly, the Sml1p also specifically binds to the mouse R1 protein with high affinity. However, the inhibition seen in an in vitro mouse ribonucleotide reductase assay is less pronounced than in the yeast system, which indicates an inhibition mechanism different from the one in yeast. These findings may be used to develop a new generation of antiproliferative drugs targeted to RNR.

MATERIALS AND METHODS

Recombinant Proteins and Peptides—The recombinant yeast proteins Rnr1, Rnr2, and Rnr4 were expressed in Escherichia coli BL21(DE3) bacteria using the pET3a expression vector; mouse recombinant proteins R1 and R2 were expressed in E. coli BL21(DE3)pLysS bacteria using the same vector (14). Purification of the recombinant mouse and yeast R1 proteins, and of the recombinant mouse R2 protein, was made as described earlier (15, 16). The yeast Rnr2 and Rnr4 proteins were coexpressed and purified as a heterodimer. The SML1 coding sequence (13) was amplified by polymerase chain reaction from yeast genomic DNA using the following oligonucleotides: 5′-CAA TAA TTC CCC CAT ATG CAA AAT TCC-3′ and 5′-AAA GGA TCC TTC TTA GAA GTG CAT TTC CTC GAC-3′. After the polymerase chain reaction product was cleaved with NdeI and BamHI restriction endonucleases, it was

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The abbreviations used are: RNR, ribonucleotide reductase; RU, resonance unit; PAGE, polyacrylamide gel electrophoresis.

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Inhibition of Yeast Ribonucleotide Reductase by Nonapeptides Corresponding to the C-terminal Ends of Sml1p, Rnr2p, or Rnr4p. The C-terminal sequence of the Sml1p shows some homology to the C-terminal peptides of the Rnr2 or Rnr4 proteins. Because such peptides are known to inhibit RNR activity by binding to the R1 protein and preventing R1/R2 interaction, we wanted to study the influence of a C-terminal nonapeptide of Sml1p on yeast RNR activity. As shown in Fig. 3, nonapeptides from the C terminus of Rnr2p or Rnr4p inhibited the in vitro yeast RNR assay to about the same extent with an IC50 of 44 and 30 μM, respectively. In contrast, the nonapeptide corresponding to the C terminus of Sml1p showed an inhibition with an IC50 of only about 300 μM.

Interaction between Yeast Rnr1 Protein and Sml1 Protein Assayed by Sucrose Gradient Centrifugation—For enzymatic activity, ribonucleotide reductases of the class Ia-type must form a heterodimeric complex composed of homodimeric R1 and R2 proteins. It was previously demonstrated for the mouse RNR that binding of effectors to the substrate specificity site promotes formation of the R1 dimer, which is believed to be a prerequisite for binding to the R2 dimer (18, 23). To test if Sml1p binding to the Rnr1p might interfere with Rnr1p dimer formation and thereby inhibit RNR activity, we preincubated Rnr1p and Sml1p in the presence of the allosteric effector dTTP; we then analyzed the mixture on a sucrose gradient (Fig. 4). The addition of dTTP induced formation of dimers and tetramers of the Rnr1p, and no monomer peak could be seen in the gradient. This pattern is quite different from the one shown in the absence of dTTP, where the Rnr1p monomers dominate (Fig. 4). Addition of Sml1p did not influence the distribution of Rnr1p in the gradient, and no shift from dimers to monomers...
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Could be observed. At the same time, analysis of the fractions by SDS-PAGE clearly demonstrated two peaks of Sm1p, one minor peak co-sedimenting with the dimer peak of Rnr1p, and one major peak representing free Sm1p sedimenting at the top of the gradient. The fact that a portion of Sm1p protein was present in the fractions containing the Rnr1p dimers indicates that the Sm1p can bind to the Rnr1p dimer without dissociating it into monomers. No shift toward Rnr1p monomers was observed under the following conditions: the Rnr1p/Sm1p/dTTP incubation mixture was sedimented through a gradient containing Sm1p at a concentration of 0.15 mg/ml throughout to minimize Rnr1p/Sm1p dissociation (data not shown). Therefore, binding of Sm1p did not influence the monomer/dimer equilibrium of the Rnr1p.

Kinetic Studies of the Interaction between the Rnr1 and Sm1p Proteins Using a Biosensor Technique—To obtain a quantitative description of the Rnr1p and Sm1p interaction, we immobilized the Sm1p protein to the dextran layer of a sensor chip and then injected a series of solutions containing increasing concentrations of Rnr1p to the same sensor chip. The immobilization of Sm1p at a concentration of 0.44 mg/ml gave an increase of 91 RU, which correspond to 91 pg/mm². With a surface of 0.8 mm², 91 RU corresponds to a total of 72.8 pg of bound Sm1p, which can be compared with the total injected amount of 15.4 μg. This low degree of attachment (only 0.0005%) makes it unlikely that the protein is attached to multiple binding sites.

Increasing concentrations of Rnr1p resulted in increasing equilibrium values, which approached a maximal value (Fig. 5A). In control experiments, where the same series of Rnr1p solutions passed a sensor chip without immobilized Sm1p, a “bulk effect” gave a response of only about 60 RU. The same background value of about 60 RU was observed when bovine serum albumin or Rnr2p/Rnr4p heterodimer was injected at a concentration of 0.1 mg/ml (data not shown). In Fig. 5B, the response at equilibrium is plotted against the concentration of injected Rnr1p. Using the GraphPad Prism program (GraphPad Software, Inc.), an equilibrium dissociation constant (K_D) of 0.41 ± 0.1 μM and a maximal binding of 691 RU were obtained. These values correspond to 691 pg/mm² or a total of 553 pg or 5.5 fmol of Rnr1p monomer bound to about 6.2 fmol of immobilized Sm1p, i.e. nearly one Rnr1p monomer bound per molecule of immobilized Sm1p. Using the BIAevaluation software (Biacore AB), an association rate constant of about 153,000 M⁻¹ s⁻¹ and a dissociation rate constant of 0.04 s⁻¹ were obtained from the lower curves in Fig. 5A. Calculating the equilibrium dissociation constant from these rate constants gave a K_D of about 0.25 μM, which is close to the directly determined K_D. Mixing the Rnr1p with Rnr2p/Rnr4p heterodimer, with or without dTTP before injection, did not affect the curves; the same results were obtained as when Rnr1p was injected alone.

Inhibition of Mouse Ribonucleotide Reductase by the Sm11 Protein—The effects of Sm11 protein on pure recombinant mouse ribonucleotide reductase were tested in a CDP assay in the presence of ATP as a positive effector (Fig. 6). In the figure, RNR activity in the presence of a fixed amount of R1 protein, and in the presence or absence of a 300-fold molar excess of Sm11p, are plotted against increasing concentrations of the R2 protein. In contrast to the situation with the yeast RNR, this inhibition is less pronounced and dependent on the R1 to R2 ratio. In a double reciprocal plot, clear competition is observed between Sm11p and the R2 protein (data not shown).

Kinetic Studies of the Interaction between the Mouse RNR R1 Protein and the Sm11 Protein Using a Biosensor Technique—After observing inhibition of the mouse RNR by Sm11p, we wanted to characterize the binding between the mouse R1 protein and Sm11p. As before, the Sm11p was immobilized on a
sensor chip, and a solution containing 0.1 mg/ml of mouse R1 protein was injected (Fig. 7). On injection, a very rapid association phase was observed; this phase was immediately followed by a prolonged dissociation phase that never reached an equilibrium plateau. Injecting the R1 solution over an empty sensor chip gave the same low background value as observed earlier. Injection of mouse R2 protein also resulted in only background values (data not shown). To exclude the possibility that the unexpected behavior of the mouse R1 protein was due to an improperly immobilized Sml1p, we injected 0.1 mg/ml of the yeast Rnr1 protein; we observed the same type of response as in Fig. 5A, with a clear equilibrium plateau. Knowing that allosteric effectors affect the conformation of R1 proteins, we next mixed the mouse R1 protein with dTTP before injection. This time, a rapid association phase was followed by a clear equilibrium plateau; this sensogram resembled the sensogram obtained with the yeast Rnr1p. Finally, we mixed the mouse R1 protein plus dTTP with R2 protein before injection, expecting to see an R1-R2 complex bound to Sml1p. However, the R2 protein addition almost abolished the specific R1 binding, and resulted in almost background values. No attempts were made to quantify the mouse R1 binding data, because the sensograms deviated widely from standard curves.

DISCUSSION

Our in vitro data directly prove the hypothesis of Zhao et al. (13) that the yeast Sml1 protein is a physiological inhibitor of ribonucleotide reductase; this is a new concept in the RNR field. So far, no mammalian homologue has been identified, and no homologous proteins could be found in available data bases. This situation may reflect different control of ribonucleotide reductase activity in yeast and mammalian cells. In yeast, transcriptional activation of the RNR genes and suppression of the Sml1 protein lead to increased RNR activity and deoxyribonucleotide pools after DNA damage (12, 13). In contrast, mammalian cells control RNR activity by an S-phase/DNA-damage specific stabilization of the R2 protein, until cells enter mitosis, in combination with negative feedback regulation by dATP. We suggest that the Sml1 protein may substitute for dATP feedback regulation, since yeast RNR is not inhibited by physiological concentrations of dATP. Therefore, the inhibition mechanism may differ for yeast and mammalian RNR enzymes. Although inhibition in the yeast system is very efficient and may involve blocking the entrance to the active site, inhibition in the mouse system is rather inefficient and may reflect blocking R2 protein binding to the R1 protein, perhaps in the same way as R2 protein C-terminal peptides.

We hope that structural studies of the Sml1 protein, alone and bound to the R1 protein will reveal the mechanism of inhibition. This finding should be useful in developing specific antiproliferative inhibitors of RNR; such new inhibitors could complement existing ones, which include radical scavengers, iron chelators, and peptidomimetics (2).

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