Ribonucleotide reductase (RNR) is an essential enzyme that provides the cell with a balanced supply of deoxyribonucleoside triphosphates for DNA replication and repair. Mutations that affect the regulation of RNR in yeast and mammalian cells can lead to genetic abnormalities and cell death. We have expressed and purified the components of the RNR system in fission yeast, the large subunit Cdc22p, the small subunit Suc22p, and the replication inhibitor Spd1p. It was proposed (Liu, C., Powell, K. A., Mundt, K., Wu, L., Carr, A. M., and Caspary, T. (2003) *Genes Dev.* 17, 1130–1140) that Spd1 is an RNR inhibitor, acting by anchoring the Suc22p inside the nucleus during G1 phase. Using *in vitro* assays with highly purified proteins we have demonstrated that Spd1 indeed is a very efficient inhibitor of fission yeast RNR, but acting on Cdc22p. Furthermore, biosensor technique showed that Spd1p binds to the Cdc22p with a $K_D$ of 2.4 μM, whereas the affinity to Suc22p is negligible. Therefore, Spd1p inhibits fission yeast RNR activity by interacting with the Cdc22p. Similar to the situation in budding yeast, logarithmically growing fission yeast increases the dNTP pools 2-fold after 3 h of incubation in the UV mimetic 4-nitroquinoline-N-oxide. This increase is smaller than the increase observed in budding yeast but of the same order as the dNTP pool increase when synchronous *Schizosaccharomyces pombe* cdc10 cells are going from G1 to S-phase.

Looking for novel fission yeast genes that cause cell cycle arrest when overexpressed, Woollard *et al.* (1) cloned *spd1* (for S-phase delayed). The encoded 14-kDa protein is cell cycle-regulated, and the levels decline during S-phase. Overexpression of *spd1* inhibits G1/S progression downstream of Start, but it is not an essential gene. In a later publication, Borgne and Nurse (2) demonstrated that overexpression of Spd1p blocks the onset of both S-phase and mitosis, and they suggested that the protein might act by interfering directly with DNA replication giving the same phenotype as that seen using the ribonucleotide reductase (RNR) inhibitor hydroxyurea.

Null mutants of the fission yeast *csn1-d* and *csn2-d* signalosome subunits are slow growing and have a prolonged S-phase (3). In a screen for multicopy suppressors of *rad3-ts csn1-d* lethality to identify the cause of the slow S-phase in signalosome mutants, Liu *et al.* (4) identified multiple independent clones of *suc22* that rescued the growth defect. *Suc22* encodes the small subunit of *Schizosaccharomyces pombe* RNR that together with the large subunit Cdc22p forms the active enzyme complex (5). Because the phenotype of *spd1* overexpression resembled the phenotype of *csn1-d* or *csn2-d* mutants, Liu *et al.* combined the deletion of *spd1* with either of the signalosome subunit mutants, and in both cases, loss of *spd1* restored the normal cell cycle profile. They concluded from these experiments that Spd1p inhibits RNR activity and that the signalosome complex is required to release the inhibition during DNA replication or repair.

Using indirect immunofluorescence microscopy, Liu *et al.* observed nuclear co-localization of Suc22p and Spd1p. Although no direct evidence was presented for interaction between the proteins, such an interaction was assumed in a model for the regulation of fission yeast RNR activity during S-phase and DNA repair. According to this model, Spd1p anchors the Suc22p inside the nucleus, thereby inhibiting RNR activity. To increase the supply of deoxyribonucleotides during DNA replication or repair, Spd1p is destroyed by the signalosome; this leads to a delocalization of the Suc22p from the nucleus to the cytoplasm where it combines with the Cdc22p to form active RNR. This model is cited in a number of publications and reviews (6–9).

In this report, we describe the expression and purification of the Cdc22p, Suc22p, and Spd1p and demonstrate by *in vitro* assays with highly purified recombinant proteins that Spd1p is a strong inhibitor of fission yeast RNR acting on Cdc22p. Furthermore, biosensor technique using chips with immobilized Spd1p shows that the inhibitor specifically binds to Cdc22p with a dissociation constant of 2.4 μM, whereas the affinity to Suc22p is negligible. Our data support a model where Spd1 regulates RNR activity by binding to the Cdc22p and not to the Suc22p. This mode of regulation is similar to the regulation of RNR activity in budding yeast, where binding of the low molecular weight inhibitor Sm11 to the Cdc22p homologue, the Rnr1p, controls RNR activity.

Logarithmically growing budding yeast dramatically improves survival following DNA damage by increasing the deoxyribonucleotide pools to values much higher than in S-phase cells (10). We also observe an increase in deoxyribonucleotide pools in fission yeast after DNA damage. However, the increase is only 2-fold and of the same order as the increase in dNTP pools in synchronized, undamaged S-phase cells compared with G1 cells.

**EXPERIMENTAL PROCEDURES**

**Growth of Fission Yeast**—Wt and the ts mutant strain *cdc10-V50* were grown in YE medium. Synchronization of the *cdc10* strain was accomplished by first growing the cells at 25 °C, shifting to 36 °C for 4 h, and then shifting back again to 25 °C (1). Flow cytometry was made according to Ref. 11 using a Beckman Cytomics FC 500 machine.

**Protein Expression and Purification**—Cdc22p, Suc22p, and Spd1p were expressed in *Escherichia coli* BL21(DE3)pLysS bacteria using the
pET3a expression vector (Novagen). Plasmids containing the cdc22 and suc22 genes, pCDC22–1, and pSUC22–1, were gifts from Christopher J. McInerney, University of Glasgow, UK. A cDNA clone of spd1 (ARC 640:Spd1cDNA) in pREP41X was obtained from Paul Nurse. To remove the intron in cdc22, the Aatt site in pET 3a was first destroyed by cutting, removing the protruding 3′-tail with T4 DNA polymerase, and religation. A synthetic linker encoding the first 9 amino acid residues in Cdc22p including an Aatt site was cloned into the pET3a opened with NdeI and BamH1. A fragment lacking the intron but containing the major part of the Cdc22p coding sequence was prepared by digesting the pCDC22–1 with AattII and NsiI (cutting downstream of the stop codon) and ligating into the pET vector opened with the same restriction enzymes.

The Cdc22p was expressed by growing the bacterial culture in TB medium plus 100 µg/ml of carbenicillin and 34 µg/ml of chloramphenicol at 30 °C to an A600 = 0.7 when isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 0.2 mM, and the culture was allowed to grow at 15 °C overnight. Cdc22p was purified by ammonium sulfate fractionation and affinity chromatography on dATP-Sepharose as described (12).

Suc22p-expressing bacteria were grown in Luria Bertani medium containing the same antibiotics as above at 30 °C to an A600 = 0.8 when isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 0.4 mM and the culture incubated for another 3 h at 30 °C. Suc22p was purified by streptomycin sulfate precipitation, ammonium sulfate precipitation to 50% saturation (0.25 g/ml), and chromatography on DEAE-Sepharose fast flow essentially as described earlier (13). Instead of stepwise elution, the protein was eluted by a linear gradient of 0–500 mM NaCl in 10 mM potassium phosphate buffer, pH 7.0, where Suc22p eluted around 250 mM NaCl. The final Suc22p preparations contained 0.2–0.4 mol tyrosyl radical/mol of polypeptide chain.

The Spd1p-expressing bacteria were grown the same way as the Suc22p-expressing bacteria but at 37 °C, and they were induced with isopropyl 1-thio-β-D-galactopyranoside at a final concentration of 0.5 mM. After lysing the bacteria by freezing and thawing, the crude extract was centrifuged for 60 min at 45,000 rpm at 4 °C in a Beckman Ti-70 rotor, and the supernatant was discarded. The pellet was washed three times with 50 mM Tris-Cl, pH 7.4, treated with DNase 1 (5 µg/ml), and dissolved in 0.1 M Tris-Cl, pH 8.5, 5 mM NaCl in 10 mM potassium phosphate buffer, pH 7.0, where Spd1p formed almost exclusively inclusion bodies when expressed at 37 °C. Initially, we therefore grew the bacteria at 20 °C for 3 h after isopropyl 1-thio-β-D-galactopyranoside was added and then incubated at 37 °C. The soluble and the solubilized forms of Spd1p both were used for the interaction studies. However, the yield was much better and the purity of the final preparation higher when we expressed Spd1p as inclusion bodies (Fig. 1). The soluble and the solubilized forms of Spd1p formed almost exclusively inclusion bodies when expressed at 37 °C. Initially, we therefore grew the bacteria at 20 °C for 3 h after isopropyl 1-thio-β-D-galactopyranoside was added and then incubated at 37 °C. The soluble and the solubilized forms of Spd1p formed almost exclusively inclusion bodies when expressed at 37 °C. Initially, we therefore grew the bacteria at 20 °C for 3 h after isopropyl 1-thio-β-D-galactopyranoside was added and then incubated at 37 °C. The soluble and the solubilized forms of Spd1p formed almost exclusively inclusion bodies when expressed at 37 °C.
budding yeast Rnr1p and Rnr2p/Rnr4p, 250 and 2250, respectively, assayed at 30 °C (21, 22). Like other class 1 RNRs, the S. pombe enzyme is feedback inhibited by dATP with a 50% inhibition of the 5-mm ATP-stimulated reaction at 40 μM dATP.

**Inhibition by Spd1p**—Recombinant Cdc22p plus Suc22p was assayed for activity using the CDP reduction assay, with ATP as a positive effector in the presence of increasing amounts of Spd1 protein (Fig. 2). Under these conditions 50% inhibition was already observed at 1 μM concentration of Spd1p. The inhibition was highly specific for S. pombe RNR because very little inhibition was observed when Spd1p was added to an assay system containing Rnr1p plus Rnr2p/Rnr4p of S. cerevisiae or an assay system containing mouse R1 plus R2 proteins (data not shown).

To differentiate between inhibition of Suc22p and Cdc22p activity by Spd1p, we took advantage of the observation that budding yeast Rnr1p forms an active RNR complex with fission yeast Suc22p, although the amino acid sequence identity is only 66% between Rnr1p and Cdc22p. Assaying recombinant budding yeast Rnr1p plus Suc22p for activity using the CDP reduction assay in the presence of increasing amounts of Spd1 protein showed very little inhibition compared with the results with Cdc22p and Suc22p (Fig. 2). Because Spd1 is a very weak inhibitor of the budding yeast RNR, these results strongly indicate that Spd1 inhibits the fission yeast RNR by specifically interacting with Cdc22p.

To find out more about the inhibition mechanism, we prepared a series of assay tubes containing a fixed amount of Cdc22p (85 pmol) and increasing amounts of Suc22p with and without a constant amount of Spd1p (90 pmol) (Fig. 3A). In the absence of added Spd1p, the activity increased with increasing amounts of Suc22p up to 330 pmol, when a plateau was reached. Addition of Spd1 resulted in an almost constant increased activity for 25 min. In addition, the tubes contained increasing amounts of Spd1p. 100% activity corresponds to the formation of 1.9 nmol dCDP. The assay was also made with a heterologous system consisting of budding yeast Rnr1p (100 pmol) and fission yeast Suc22p (550 pmol) (Fig. 3B). In this combination, 100% activity corresponds to the formation of 4.9 nmol dCDP.

**Kinetic Studies of the Interaction between the Cdc22p, Suc22p, and Spd1p Using a Biosensor Technique**—To determine whether Spd1p interacts with Cdc22p, Suc22p, or both, we immobilized the Spd1 protein to a dextran layer on a sensor chip and then injected a series of solutions containing increasing concentrations of Cdc22p or Suc22p to the same sensor chip. The immobilization of Spd1p at a concentration of 0.07 mg/ml gave an increase of 200 RU, which corresponds to 200 pg/mm². With a surface of 0.8 mm², 200 RU corresponds to a total of 160 pg of bound Spd1p, which can be compared with the total injected amount of 210 ng. This low degree of attachment (only 0.08%) makes it unlikely that the Spd1p is attached to multiple binding sites.

Injection of increasing concentrations of Cdc22p resulted in increasing equilibrium values (Fig. 4A). A Scatchard plot of the response at equilibrium of increasing concentrations of Cdc22p interacting with a constant amount of immobilized Spd1p gave an equilibrium dissociation constant Kd of 2.4 μM and a maximal binding of 0.43 mol Cdc22p monomer/mol of immobilized Spd1p (data not shown). Injection of Suc22p resulted in very low binding; low binding was also observed after the injection of S. cerevisiae Rnr1p or mouse R1 protein (Fig. 4A). Injection of an equimolar solution of Cdc22p and Suc22p resulted in a small but reproducible decrease in equilibrium value compared with the injection of Cdc22p alone. Addition of 0.1 mM dTTP to the injection mixture, known to induce dimerization of the large subunit of mouse and budding yeast RNR (16), had no major effect on the sensogram. As a comparison we immobilized S. cerevisiae Sml1p to a sensor chip (Fig. 4B). Injection of S. cerevisiae Rnr1p resulted in an efficient binding, whereas injection of Cdc22p or Suc22p gave no significant binding.

**Changes in Deoxyribonucleoside Triphosphate Pools in Fission Yeast after DNA Damage and during the Cell Cycle**—Budding yeast regulates RNR activity during DNA replication and repair by binding of the low molecular weight protein inhibitor Sml1p to Rnr1p (16, 23). When budding yeast enters S-phase or after DNA damage, Sml1p is degraded, releasing RNR from inhibition and resulting in elevated dNTP pools (10, 23, 24). It was reported that Ddb1/Csn1/Csn2/Cullin 4-mediated degradation of Spd1p is essential for DNA replication and repair in fission
yeast and that deletion of Ddb1 led to decreased dNTP pools in undamaged cells (4, 8).

Because Spd1p similar to Sm1p specifically inhibits RNR, we were interested to study whether and to what extent DNA damage affected the dNTP pools in logarithmically growing fission yeast. Like budding yeast (10), an increase in dNTP pools was already observed 1 h after the addition of 1 mg/liter of 4-nitroquinoline-N-oxide (4-NQO), a mutagen that acts as a UV mimetic (25), and after 3 h all four dNTP pools showed a 2-fold increase (Fig. 5B). Separate experiments using 0.2 mg/liter of 4-NQO indicated that maximal pool increase was obtained at 1 mg/liter (data not shown). Flow cytometry of the culture before and 3 h after the addition of 4-NQO showed a small increase in G1 cells and a small decrease in the number of S-phase cells after drug addition (Fig. 5A).

To be able to relate this DNA damage-induced dNTP pool increase to the dNTP levels in fission yeast during the cell cycle, we measured the pools in temperature-sensitive cdc10 mutant cells shifted to 25 °C after 4 h of synchronization at 36 °C. Cells were harvested for flow cytometry and dNTP pool determinations 0, 45, 60, 75, and 90 min after the down-shift in incubation temperature. A 2-fold increase in all dNTP pools was observed when the 0-min sample was compared with the 90-min sample (Fig. 5D). Flow cytometry showed that the cells were blocked in G1 after 4 h at the restrictive temperature, proceeded into S-phase after 45 min at 25 °C, and after 90 min the majority of the cells had a DNA content >1C indicative of being in or just after S-phase (Fig. 5C). The dNTP pool increase observed in logarithmically growing wt cells after the addition of 4-NQO (Fig. 5B) was similar to the increase observed in 4-NQO-treated logarithmically growing cdc10 cells.

**DISCUSSION**

We have demonstrated in in vitro assays using highly purified proteins that Spd1p is an efficient inhibitor of fission yeast ribonucleotide reductase. However, in strong contrast to the model proposed by Liu et al. (4), biosensor technique showed that Spd1p has no affinity for the Suc22p but instead binds the Cdc22p with a $K_D$ of 2.4 μM. The binding data are in full agreement with data from inhibition assays with the homologous Cdc22p/Suc22p system and the heterologous Rnr1p/Suc22p system where significant inhibition was only observed in the system containing Cdc22p. Therefore, it is highly unlikely that Spd1p acts by anchoring Suc22p inside the nucleus during the G1 phase of the cell cycle as was proposed. Instead, our results are in agreement with the demonstration by Borgne and Nurse (2) that Spd1p is mainly localized...
in the cytoplasm where it can directly control RNR activity by binding to the Cdc22p.

Liu et al. (4) observed that loss of spd1 phenocopied suc22 overexpression in min1-1I cells and interpreted their data to mean that Spd1p directly acted on Suc22p. However, as clearly seen in Fig. 3, the low affinity between the two subunits of fission yeast RNR, Cdc22p and Suc22p, similar to most type I RNRs, results in increased enzyme activity independent of which subunit is overexpressed when one subunit is present in low amounts.

Our results emphasize the similarities between the RNR systems in fission and budding yeast in that both regulate RNR activity during DNA replication and repair by a low molecular weight protein inhibitor, Spd1p and Sml1p, respectively (4, 16, 23). There is no sequence homology between the two inhibitors, and in the case of Sml1p, deletion of amino acid residues 2–50 from the total 104 amino acid residues had very little effect on the inhibition efficiency (26). In contrast, deletion of the first 29 amino acid residues from the total 124 amino acid residues in Spd1p resulted in a protein much less effective in causing cell cycle arrest when overexpressed than the intact protein (1). Sml1p is a target of the Mecl1/Rad53 kinase cascade, and its degradation after DNA damage is initiated by phosphorylation by the Dun1 kinase at serine 56, serine 58, and serine 60 (24, 27). In fission yeast, Ddb1, Cullin4, and the signalosome subunits Csn1 and Csn2 are required for the degradation of Spd1p and Sml1p, respectively (4, 16, 23). There is no sequence homology between Spd1p and Sml1p, respectively (1, 4, 8). The exact mechanism by which Spd1/Sml1 inhibits RNR activity is still unknown, but our results from in vitro assays indicate that there is no simple competition between Spd1/Sml1 and the small RNR subunit for binding to the large subunit (16).

In both budding and fission yeast, regulation of export of the small subunit from the nucleus to the cytoplasmically localized large subunit was suggested to regulate RNR activity during S-phase and after DNA damage (4, 28). In mammalian cells, both RNR subunits were demonstrated to be localized to the cytoplasm during the cell cycle (29, 30). In contrast, the p53-inducible small subunit homologue, p53R2, was translocated from the cytoplasm to the nucleus after DNA damage (31). Our present results do not exclude transport of the small RNR subunit from the nucleus to the cytoplasm as a mechanism to regulate RNR activity in yeast cells.

In budding yeast, DNA damage was shown to increase the dNTP pools 6–8-fold, which resulted in dNTP levels 3–5-fold higher than the dNTP pools in untreated synchronized S-phase cells (10). This increase is possible because of a relaxed dATP feedback inhibition of budding yeast RNR. Even further increased pools strongly improved survival following DNA damage, but they also led to higher mutation rates. The fission yeast RNR shows a 50% feedback inhibition at 40 μM dATP compared with 10 and 80 μM for the mouse and budding yeast enzyme, respectively, all assayed in the presence of 5 mM ATP (10, 21). This relatively tight dATP feedback inhibition of the fission yeast RNR may be one explanation why the dNTP pools in logarithmically growing fission yeast only increased ~2-fold after DNA damage. Also, the fluctuations during the cell cycle are much less pronounced in fission yeast than in budding yeast, an ~2-fold increase for all dNTPs in fission yeast compared with a 6-fold increase in the purine dNTPs and a 3-fold increase in the pyrimidine dNTPs during S-phase in budding yeast (10, 32).

How does the regulation of RNR activity in fission and budding yeast compare with the regulation in mammalian cells? Common for all three organisms is that a failure to provide sufficient and balanced dNTP pools can lead to misincorporation of dNTPs into DNA, resulting in genetic abnormalities and cell death. So far, no low molecular weight protein inhibitor of the mammalian RNR has been identified, but the small size and the very low amino acid sequence conservation between Spd1p and Sml1p make it difficult to identify such an inhibitor in mammalian genome sequences. There are conflicting results in the literature regarding the effects of DNA damage on dNTP pools in mammalian cells (33). However, recent data from our laboratory show that mammalian cells do not increase their dNTP pools after DNA damage and still the dNTP pools in G1 cells are only 1/20 of the pools in S-phase cells,3 indicating a regulation different from the regulation in yeast.

Acknowledgments—We thank Christopher J. McNerny and Paul Nurse for providing plasmids, Karl Ewbank for providing fission yeast strains, Erik Boye for help with flow cytometry, and Nina Voevodskaya for performing the EPR analysis.

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