THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 22, pp. 15058–15063, June 2, 2006

Thioredoxin Is Required for Deoxyribonucleotide Pool Maintenance during S Phase*

Received for publication, March 1, 2006, and in revised form, March 28, 2006 Published, JBC Papers in Press, March 29, 2006, DOI 10.1074/jbc.M601968200

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Thioredoxin was initially identified by its ability to serve as an electron donor for ribonucleotide reductase *in vitro*. Whether it serves a similar function *in vivo* is unclear. In *Saccharomyces cerevisiae*, it was previously shown that Δ*trx1* Δ*trx2* mutants lacking the two genes for cytosolic thioredoxin have a slower growth rate because of a longer S phase, but the basis for S phase elongation was not identified. The hypothesis that S phase protraction was due to inefficient dNTP synthesis was investigated by measuring dNTP levels in asynchronous and synchronized wild-type and Δ*trx1* Δ*trx2* yeast. In contrast to wild-type cells, Δ*trx1* Δ*trx2* cells were unable to accumulate or maintain high levels of dNTPs when α-factor- or *cdc15*-arrested cells were allowed to reenter the cell cycle. At 80 min after release, when the fraction of cells in S phase was maximal, the dNTP pools in Δ*trx1* Δ*trx2* cells were 60% that of wild-type cells. The data suggest that, in the absence of thioredoxin, cells cannot support the high rate of dNTP synthesis required for efficient DNA synthesis during S phase. The results constitute *in vivo* evidence for thioredoxin being a physiologically relevant electron donor for ribonucleotide reductase during DNA precursor synthesis.

Deoxyribonucleotide pools are carefully controlled in all cells to ensure efficient and yet accurate genome replication. The pools are not equimolar, but rather they have a characteristic asymmetry, with dGTP usually the smallest (1). For example, in *Saccharomyces cerevisiae*, the dGTP pool is 3-fold smaller than the dTTP pool (2, 3). Cells do not stockpile dNTPs, but rather they maintain them at levels just sufficient to support replication. For example, yeast contain fewer than 1.8×10^6 dNTP molecules/cell (3), which is less than 20% of the amount minimally needed to copy the 1.2×10^7-bp yeast genome. Limiting dNTP pool size is important for replication fidelity. Artificial pool expansion results in increased mutagenesis, perhaps because of stimulation of chain extension from DNA mismatches at high dNTP concentrations (4). The dNTP pools are dynamic, increasing severalfold as eukaryotic cells enter S phase (3). For dNTP levels to remain elevated during S phase, cells must markedly boost their rate of dNTP synthesis to compensate for the rapid consumption of dNTPs at replication forks. Replicating cells are exquisitely sensitive to dNTP depletion. When dNTP synthesis is blocked by the addition of hydroxyurea, replication stops well before any dNTP pool is exhausted (5). Presumably, cells have evolved the mechanisms to limit dNTP accumulation to support replication fidelity and yet maintain dNTP levels above the minima required for efficient DNA replication and repair.

A central enzyme controlling the rate of dNTP synthesis is ribonucleotide reductase (RNR),[3] a heterotetramer of two large and two small subunits. The enzyme is subject to extraordinary regulation. Its activity and substrate specificity are regulated by binding of different nucleoside triphosphates to two allosteric sites on the large subunit, thus preventing dNTP over-accumulation and achieving an optimal ratio of the four deoxyribonucleotides. In many eukaryotic cells, the large subunit genes are induced severalfold at late G_1/early S phase of the cell cycle (6, 7), presumably to boost RNR protein levels and support a higher rate of dNTP synthesis during S phase. In addition to allosteric and transcriptional control, RNR is bound and inhibited by Sml1, a protein phosphorylated and inactivated by the CHK1/RAD53/MEC1 pathway in response to DNA damage or S phase entry (8). Most recently, RNR activity has been shown to be regulated by subunit compartmentation (9, 10). Only during S phase or in response to DNA damage does the small subunit move from the nucleus to the cytoplasm, where it can join the large subunit and reconstitute holoenzyme (9). Together, allosteric regulation, transcriptional control, negative-acting regulatory proteins, and subunit localization converge to regulate RNR activity and ensure that cells maintain dNTPs at levels needed for efficient and yet accurate DNA synthesis.

Despite its central role in dNTP synthesis, the identity of the physiological electron donor used by RNR for ribonucleotide reduction is still conjectural. Both thioredoxin and glutaredoxin can support the RNR reaction *in vitro*, but it is not clear whether both or either protein serves as the major reductant *in vivo*. In yeast, the two systems complement an essential process, as mutants lacking either thioredoxin or glutathione reductase are viable, but mutants lacking both thioredoxin and glutathione reductase are not (11, 12). It is not known whether the essential process affected by thioredoxin and glutathione reductase deletion is ribonucleotide reduction, but as RNR is essential, it is clear that alternative systems can supply electrons to RNR in cells lacking either thioredoxin or glutathione reductase. Further complicating the problem of identifying the physiological electron donor, cells lacking an intact thioredoxin system often exhibit compensatory changes in gene expression and metabolite levels. For example, Δ*trr1* yeast lacking thioredoxin reductase have 3-fold higher glutathione levels (13) and significantly higher levels of transcripts encoding glutathione reductase (Glr1), glutamate cysteine ligase (Gsh1), and glutathione synthase (Gsh2) (14). Also, Northern blot results show that Δ*trx1* Δ*trx2* cells lacking thioredoxin have significantly higher levels of the mRNAs encoding the large and small RNR subunits (Rnr1, Rnr3, and Rnr2) (2), suggesting that in

* This work was supported by National Science Foundation (NSF) Grant MCB9728782 and National Institutes of Health Grant CA82633 (to G. F. M.) and by NSF Grant MCB 0137060 and Army Research Office Grant LS45039 (to C. K. M.). This work was also supported, in part, by the Cell and Tissue Analysis and Cell Culture Facility and Service Cores and the Biomolecular and Environmental Chemistry and Signaling and Oxidative Stress Research Cores of the Oregon State University Environmental Health Sciences Center, Grant P30 ES00210 from NIEHS, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[3] The abbreviations used are: RNR, ribonucleotide reductase; PBS, phosphate-buffered saline.

TABLE 1
Yeast strains

Strain	Genotype	Source
W303-1a	*MAT*-a *ade2 ura3 leu2 trp1 his3 can1*	Ref. 28
SY2626	*MAT*-a *ade2 ura3 leu2 trp1 his3 can1 BAR1*	G. Sprague (University of Oregon)
EMY56-5D	*MAT*-α *ade2 ura3 leu2 trp1 his3 lys2::HIS3 Δtrx1::lYS2 Δtrx2::LEU2*	E. Muller (University of Washington)
MY326	*MAT*-α *ade2 ura3 leu2 trp1 his3 lys2 Δtrx1::LYS2 Δtrx2::LEU2*	This study
MY401	*MAT*-a *ade2 ura3 leu2 trp1 his3 lys2 bar1*	This study
MY403	*MAT*-a *ade2 ura3 leu2 trp1 his3 lys2 Δtrx1::LYS2 bar1*	This study
MY404	*MAT*-a *ade2 ura3 leu2 trp1 his3 lys2 Δtrx2::LEU2 bar1*	This study
MY405	*MAT*-a *ade2 ura3 leu2 trp1 his3 lys2 Δtrx1::LYS2 Δtrx2::LEU2 bar1*	This study
SSC18	*MAT*-a *ade2 ura3 leu2 his3 trp1 cdc15-1*	C. Price (University of Sheffield)
MY442	*MAT*-α *ade2 ura3 leu2 his3 trp1 cdc15-1*	This study
MY443	*MAT*-α *ade2 ura3 leu2 his3 trp1 cdc15-1 Δglr1::KAN1*	This study
MY445	*MAT*-α *ade2 ura3 leu2 his3 trp1 Δtrx1::LYS2 Δtrx2::LEU2 cdc15-1*	This study

the absence of an intact thioredoxin system, normal RNR levels may not be sufficient to meet the demands of the replication forks for DNA precursors and that induction of the RNR genes may be an adaptive response. Compensatory effects of glutathione system ablation on thioredoxin system proteins or RNR levels have not been reported. Nor have compensatory effects of thioredoxin or glutathione system ablation been investigated in higher eukaryotes.

In yeast, deletion of the two genes encoding cytosolic thioredoxin (*TRX1* and *TRX2*) results in slower growth, which is due exclusively to an elongated S phase (15). The possibility that impaired ribonucleotide reduction was responsible for S phase protraction was investigated by measuring the dNTP pools in asynchronously growing wild-type yeast and Δ*trx1* Δ*trx2* double mutants lacking thioredoxin. The asynchronous cell measurements indicated that, rather than being depressed in the double mutant, dNTP levels were actually elevated, and it was concluded that thioredoxin is required for an S phase function other than ribonucleotide reduction (2). However, we and others have shown that dNTP levels are not static during the yeast cell cycle (3, 8). The dNTP pools increase about 5-fold when cells are released from an α-factor-induced G_1 arrest and enter S phase (3). The increase in dNTP levels during S phase is particularly remarkable when one considers that dNTPs are rapidly being consumed by replication forks in S phase cells. Thus, only during S phase, when there is a high flux of substrate through RNR, may a deficiency in dNTP synthesis imposed by thioredoxin deletion be observed. Unable to make dNTPs at a sufficiently fast rate, Δ*trx1* Δ*trx2* cells may require more time to complete replication, leading to the observed elongation of S phase. To investigate this explanation for S phase protraction, we measured dNTP levels in wild-type and Δ*trx1* Δ*trx2* yeast that were synchronized using α-factor or a temperature-sensitive *cdc15* mutation. Using both synchrony methods, we found that yeast lacking thioredoxin had significantly lower dNTP levels during S phase. The results are consistent with the conclusion that S phase protraction in cells lacking thioredoxin is due to a deficiency in dNTP synthesis and supports the idea that thioredoxin is the physiologically relevant RNR reductant in eukaryotes.

MATERIALS AND METHODS

Strains, Media, and Cell Synchrony—Standard yeast genetic methods were used for the analysis of strains and crosses (16). Cells were grown in YEPD (2% bacto-yeast extract, 2% bacto-peptone, 2% glucose). Unless specified otherwise, cells were grown at 25 °C. The strains used are listed in Table 1. SY2626 is a W303 derivative that carries a *bar1* mutation to facilitate α-factor synchronization. MY326 is a derivative of EMY56-5D. MY401, MY403, MY404, and MY405 were derived by mating MY326 to SY2626 and isolating appropriate segregants. MY445 was derived by mating SSC18 (17) to EMY56-5D and back-crossing *cdc15* Δ*trx1*::*TRP1* Δ*trx2*::*LEU2* segregants to SSC18. MY442 and MY443 were derived by mating BY4742 Δ*glr1*::*KAN* (18, 19) (Open Biosystems) to SSC18 and back-crossing *cdc15* Δ*glr1*::*KAN* segregants to SSC18.

For α-factor synchronization, exponentially growing *bar1* cells (~10^7 cells/ml) were incubated with 100 ng/ml α-factor for 3 h at 25 °C. Arrested cells, collected either by filtration or centrifugation (5 min at 1000 rpm), were washed once with fresh YEPD, once with 100% conditioned YEPD, and resuspended in 25% conditioned YEPD. Conditioned YEPD was prepared by growing W303-1a cells to saturation in YEPD and removing the cells by filtration. For *cdc15* synchronization, exponentially growing *cdc15* cells were incubated at the nonpermissive temperature (37 °C) for 3.75 h. Arrested cells were released to the permissive temperature by adding an equal volume of 15 °C medium and incubating cells at 25 °C.

Flow Cytometry—Culture aliquots containing ~5 × 10^6 cells were mixed with 2 volumes of 95% ethanol and fixed for at least 15 min at 25 °C or overnight at 4 °C. After microcentrifugation (6 s at 10,000 rpm), cell pellets were washed with 100 μl of 50 mM sodium citrate buffer (pH 7), resuspended in 100 μl of sodium citrate buffer and sonicated on ice for 6 s at 10 watts using a Fisher Scientific 60 Sonic Dismembrator. After a 1-h incubation at 37 °C with RNase A (0.25 mg/ml) and a 1-h incubation at 55 °C with proteinase K (1 μg/ml), 100 μl of sodium citrate buffer containing 16 μg/ml propidium iodide was added, and samples were incubated overnight in the dark at 4 °C. A Beckman Coulter Epics XL flow cytometer (Hialeah, FL) and WinList software (Verity Software House, Topsham, ME) were used to analyze the samples. For each sample, 25,000 events were assayed. To determine the percentage of cells in each cell cycle phase, histograms were analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA), and the G_2 and G_1 peaks were corrected by subtracting the number of binucleated cells (determined by microscopy, as described below) from the G_2 peak and adding twice this number to the G_1 peak.

For microscopy and budding index determination, ~5 × 10^6 cells were fixed in ethanol as described above, microcentrifuged, resuspended in PBS (10 mM sodium phosphate, pH 7.2, 150 mM NaCl), microcentrifuged, and resuspended in 50 μl of PBS containing 1 μg/ml Hoechst 33258 and a 1:100 dilution of Alexa Fluor 488-phalloidin (Molecular Probes, Eugene, OR). After 10 min, cells were diluted with 400 μl of PBS, microcentrifuged and washed twice with 400 μl of PBS, and resuspended in 50 μl of PBS containing 50% glycerol. Cells were examined using a Zeiss Axiovert S100TV fluorescence microscope with ×100 objective and zoom set at ×0.63, and CoolSNAP HQ charge-coupled device camera. For each sample, multiple fields were photographed using Nomarski optics and fluorescence (480ex/535em filter for phalloidin staining of f-actin and 360ex/460em filter for Hoechst staining of DNA). Photographs were analyzed using MetaMorph software (Universal Imaging, Philadelphia) and scored for budded and

unbudded cells and nuclear morphology. Fields totaling at least 50 cells were scored in triplicate for each sample.

Dexoxyribonucleoside Triphosphate Pool Measurements—Approximately 3×10^8 cells were harvested by centrifugation (5 min at 1000 rpm), and nucleotide extracts were prepared either by the trichloroacetic acid extraction and Freon-amine neutralization method (2) or by an adaptation of the methanol extraction and boiling method (20). The latter involved resuspending harvested cells in 60% cold methanol, vortexing suspensions 10 times for 30 s during a 2-h incubation on ice, boiling samples for 3 min, and clarifying extracts by centrifugation (20 min at 12,000 × *g*). Extracts were dried using a Savant SpeedVac Concentrator (Thermo Electron Corp., East Greenbush, NY), and the residue was resuspended in 200 μl of cold deionized H_2O and assayed for each of the four dNTPs by the DNA polymerase-based enzymatic method (21). Cell volume was determined from A_{600}, where 1 absorbance unit of cell suspension was empirically determined to correspond to 1 μl of cell volume.

RESULTS

Thioredoxin Deletion Results in an Elongated S Phase in Several Genetic Backgrounds—It was previously shown that deletion of the *TRX1* and *TRX2* genes encoding cytosolic thioredoxin results in S phase protraction in yeast (15). In contrast, deletion of the *GLR1* gene encoding glutathione reductase has no effect on S phase length, even though it results in a severe (27-fold) increase in the GSSG:GSH ratio (11). To confirm these earlier findings and extend them to strains carrying mutations that facilitate cell synchronization, we used flow cytometry to determine the DNA profile of asynchronously growing *bar1* yeast, which lack the protease that degrades α-factor, and *cdc15* yeast, which arrest at M/G_1 at the nonpermissive temperature. Fig. 1, *A–C*, shows that *bar1* yeast carrying a Δ*trx1* deletion mutation (strain MY403) or a Δ*trx2* deletion mutation (strain MY404) had DNA profiles similar to isogenic wild-type yeast (strain MY401). In all three strains, as well as in wild-type strain SY2626 (data not shown), most of the cells in the asynchronously growing population had either a 1N or 2N DNA content, where N refers to one genome equivalent of DNA. In contrast, Fig. 1*D* shows that *bar1* yeast carrying a Δ*trx1* Δ*trx2* double deletion mutation (strain MY405) had a higher fraction of cells with an intermediate DNA content, indicative of an elongated S phase. Fig. 1, *E–F*, shows that S phase protraction was also observed in *cdc15* yeast lacking thioredoxin (compare Δ*trx1* Δ*trx2* strain MY445 with isogenic wild-type strain SSC18). In contrast, Fig. 1, *G–H*, shows that S phase protraction was not observed in *cdc15* yeast lacking glutathione reductase (compare Δ*glr1* strain MY443 with isogenic wild-type strain MY442). These results confirm earlier findings that disruption of the thioredoxin system, but not the glutathione system, results in S phase protraction in budding yeast.

Note that the fraction of cells in the G_1, S, and G_2/M phases cannot be directly determined from the DNA profiles shown in Fig. 1. Microscopy showed that a significant number of the cells in the populations were binucleated doublets because of a lag between mitotic division and cell separation. For example, 15% of the SSC18 population and 9% of the MY445 population were binucleated doublets. These doublets would register as having a 2N DNA content during flow cytometry, when actually they represented two G_1 cells. Taking the G_1 cell doublets into account, the fractions of cells in G_1, S, and G_2/M were 0.31, 0.26, and 0.43 for wild-type strain SSC18, and 0.15, 0.51 and 0.34 for Δ*trx1* Δ*trx2* strain MY445. The proportion of cells in a given phase of the cell cycle is related to the length of the time spent in that phase (22). Given the 140-min generation time of SSC18 and the 190-min generation time of MY445, the calculated lengths of the G_1, S, and G_2/M phases were 43, 36, and 60 min for SSC18, and 29, 97, and 65 min for MY445. Thus, the 50-min-longer generation time of the Δ*trx1* Δ*trx2* strain was due to a 61-min-longer S phase, which was partially compensated for by a 14-min-shorter G_1 phase. A similar 2.7-fold protraction of S phase was observed in *bar1* Δ*trx1* Δ*trx2* strain MY405 (data not shown) and in Δ*trx1* Δ*trx2* strains EMY21–8D (15) and EMY63 (11).

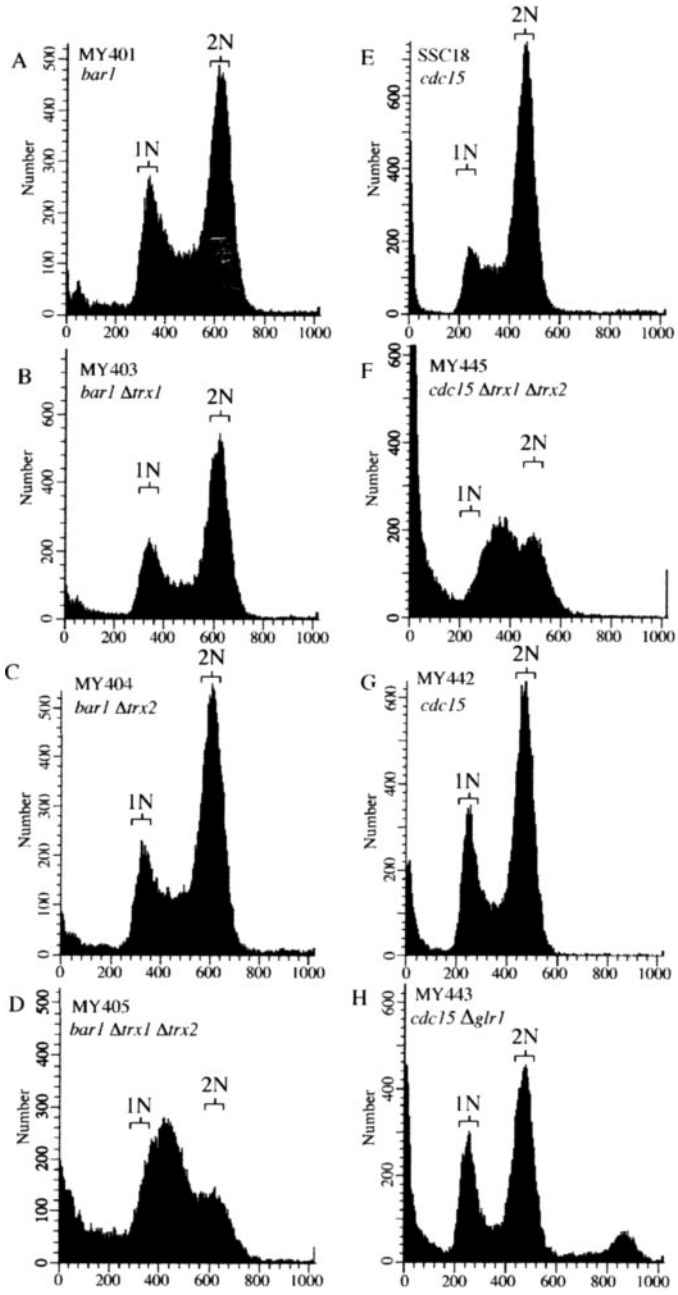

FIGURE 1. **Effect of deleting thioredoxin genes *TRX1* and *TRX2* on yeast cell cycle kinetics.** *A–H*, growing asynchronous yeast with the indicated relevant genotypes were processed for flow cytometry as described under "Materials and Methods." Cell counts are plotted as a function of propidium iodide staining intensity. Cells with G_1 and G_2 equivalents of DNA are labeled *1N* and *2N*, respectively. In both *bar1* and *cdc15* genetic backgrounds, only Δ*trx1* Δ*trx2* double deletion mutations resulted in S phase protraction.

From the data shown in Fig. 1, we conclude that S phase was protracted in *bar1* and *cdc15* strains carrying the Δ*trx1* Δ*trx2* double deletion mutation but not in strains carrying Δ*trx1*, Δ*trx2* or Δ*glr1* single deletion mutations. Therefore, in investigating whether S phase pro-

traction is associated with a deficiency in dNTP synthesis, we confined our comparisons to wild-type and Δ*trx1* Δ*trx2* strains.

α-Factor-synchronized Yeast Lacking Thioredoxin Fail to Accumulate dNTPs at Late G$_1$/Early S Phase—Given that thioredoxin can serve as the electron donor for ribonucleotide reduction by RNR *in vitro* (23) and that yeast must maintain dNTPs above a critical threshold to prevent replication arrest (5), one explanation for S phase protraction in Δ*trx1* Δ*trx2* yeast would be that dNTP synthesis was impaired. Muller (2) investigated this explanation by measuring dNTP levels in asynchronously growing wild-type and Δ*trx1* Δ*trx2* cells and found that dNTP pools were not repressed and in fact were ~40% higher in Δ*trx1* Δ*trx2* cells. The result suggested that S phase protraction in Δ*trx1* Δ*trx2* cells was not due to a deficiency in dNTP synthesis. However, having shown that dNTP levels oscillate during the cell cycle, reaching maximal levels at late G$_1$/early S (3), we reasoned that a deficiency in dNTP synthesis in Δ*trx1* Δ*trx2* cells may be observed only during S phase, when the demand for dNTPs was highest. To investigate this possibility, we measured dNTP levels during the cell cycle in yeast synchronized by α-factor block and release.

Fig. 2*A* shows DNA profiles of wild-type and Δ*trx1* Δ*trx2* cells synchronized by α-factor block and release. Both cell types arrested with a 1N complement of DNA when incubated for 3 h with α-factor, and both cell types began to accumulate DNA about 40 min after α-factor was removed. Microscopy confirmed that >95% of the cells were unbudded and had acquired the characteristic "schmooed" morphology during the 3-h incubation with α-factor and that bud emergence began about 40 min after α-factor release (data not shown). The flow cytometry and microscopy results indicated that wild-type and Δ*trx1* Δ*trx2* cells collected in G$_1$ during the α-factor block and began entering S phase about 40 min after α-factor release. Although wild-type and Δ*trx1* Δ*trx2* cells entered S phase with the same kinetics, Δ*trx1* Δ*trx2* cells traversed S phase more slowly. As a result, emergence of a new G$_1$ peak was not observed until 140 min after pheromone removal in Δ*trx1* Δ*trx2* cells.

Fig. 2*B* shows dNTP levels in α-factor-synchronized wild-type yeast (*black histograms*) and Δ*trx1* Δ*trx2* yeast (*gray histograms*). Consistent with previous findings (2), dNTP levels in asynchronous Δ*trx1* Δ*trx2* cells (84 μM for dTTP, 48 μM for dATP, 25 μM for dCTP, and 12 μM for dGTP) were not lower and in fact were about 60% higher than in wild type cells (53 μM for dTTP, 24 μM for dATP, 17 μM for dCTP, and 8 μM for dGTP) (Fig. 2*B*, compare *black* and *gray* "*a*" time point *histograms*). Also consistent with previous findings (3), the dNTP pools decreased to less than half of asynchronous cell levels when wild-type cells were arrested in G$_1$ with α-factor. A much smaller decrease in dNTP levels was observed when Δ*trx1* Δ*trx2* cells were arrested in G$_1$ with α-factor (Fig. 2*B*, compare *black* and *gray* "*0*" time point *histograms*). As a result of higher initial asynchronous levels and the smaller decrease after incubation with α-factor, the dNTP pools in α-factor-arrested Δ*trx1* Δ*trx2* cells (65 μM for dTTP, 21 μM for dATP, 22 μM for dCTP, and 10 μM for dGTP) were about 3-fold higher than in α-factor-arrested wild-type cells (24 μM for dTTP, 8 μM for dATP, 8 μM for dCTP, and 3 μM for dGTP). Consistent with previous findings (3), when wild-type cells were released from α-factor, dNTP levels increased about 5-fold as cells approached and entered S phase. In contrast, when Δ*trx1* Δ*trx2* cells were released from α-factor, dNTP levels increased very little as cells approached S phase and in fact decreased as cells entered S phase. Thus, at 80 min after α-factor release, when flow cytometry indicated that most cells were in S phase, dNTP levels in Δ*trx1* Δ*trx2* cells (63 μM for dTTP, 14 μM for dATP, 19 μM for dCTP, and 11 μM for dGTP) were on average about 60% of wild-type levels (108 μM for dTTP, 28 μM for dATP, 45 μM for dCTP, and 14 μM for dGTP). The results suggested that in the absence

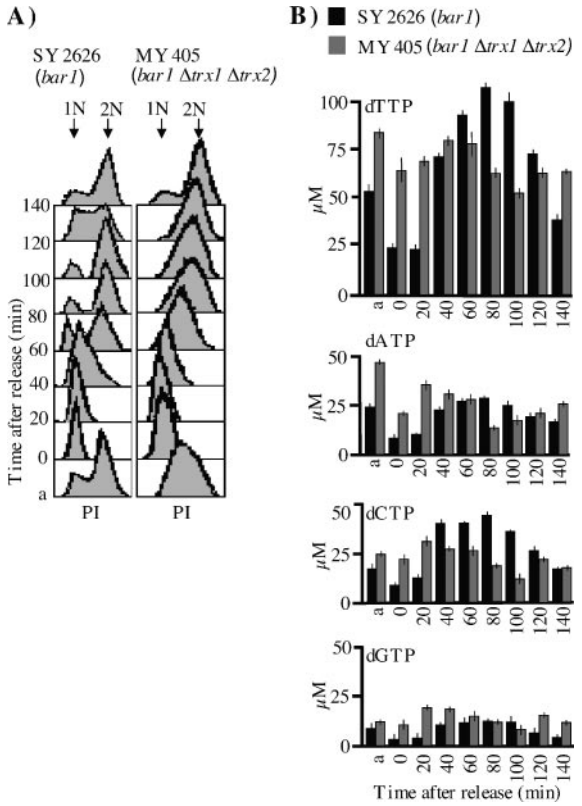

FIGURE 2. **Deoxyribonucleotide levels in α-factor-synchronized wild-type and Δ*trx1* Δ*trx2* yeast.** α-Factor-sensitive *bar1* yeast that were wild type for thioredoxin (strain SY2626) or Δ*trx1* Δ*trx2* deletion mutants (strain MY405) were used. Yeast that were growing asynchronously (*a*), were arrested in G$_1$ by a 3-h incubation in 100 ng/ml α-factor (*0*), or were released from α-factor for the indicated number of minutes were fixed for flow cytometric analysis of DNA content (*A*) or extracted by the trichloroacetic acid-Freon-amine method for analysis of dNTP levels (*B*). *Histograms* and *error bars* represent mean ± range of duplicate determinations. *PI*, propidium iodide.

of thioredoxin, cells were not able to accumulate dNTPs in late G$_1$ and were not able to maintain dNTPs once S phase began. Previous hydroxyurea experiments have shown that a relatively small perturbation of S phase dNTP levels can elicit a complete replication block (5). Thus, the 40% decrease in S phase dNTP levels observed in Δ*trx1* Δ*trx2* cells likely would have an inhibitory effect on replication efficiency. Given that increased dNTP synthesis would require increased flux through RNR and that thioredoxin is capable of donating electrons to RNR *in vitro* (23), the simplest explanation for the results shown in Fig. 2*B* is that thioredoxin is the physiological electron donor for RNR, and in the absence of thioredoxin, alternative electron donors were not able to keep pace with the demands of the replication forks for activated precursors.

cdc15-synchronized Yeast Lacking Thioredoxin Fail to Maintain dNTPs during S Phase—To confirm the α-factor results suggesting that thioredoxin is required to maintain dNTP levels in S phase cells, we determined dNTP levels in wild-type and Δ*trx1* Δ*trx2* cells that were synchronized by an alternative method. Temperature-sensitive *cdc15* mutants that arrest at M/G$_1$ at the nonpermissive temperature have been used for cell synchrony studies in the past because they reenter the cell cycle with relatively homogeneous kinetics when returned to the permissive temperature (17). Thus, we introduced the Δ*trx1* Δ*trx2* mutations into a *cdc15* genetic background. The resulting *cdc15* Δ*trx1* Δ*trx2* strain, MY445, was used to study the effect of thioredoxin deletion on dNTP metabolism during the cell cycle in cells synchronized by temperature-sensitive block and release.

Flow cytometric analysis of DNA content in *cdc15*-synchronized cells is shown in Fig. 3*A*. The DNA profiles were complicated by the fact that cell separation did not immediately follow mitosis when *cdc15*-arrested cells were released to the permissive temperature. In fact, cell separation did not occur until about the time cells initiated a new cycle and began to synthesize DNA. Up to that time, binucleated doublets in the population registered as having a 2N DNA content, when in fact they consisted of two G_1 cells. Microscopy showed that cell separation occurred about 60 min after release in the Δ*trx1* Δ*trx2* population and 75 min after release in the wild-type population. DNA synthesis, as evidenced by the appearance of cells with an apparent DNA content greater than 2N or a DNA content between 1N and 2N, began about 60 min after release in both populations.

To confirm the kinetics of cell cycle reentry in *cdc15*-synchronized cells, "budding index" was determined (Fig. 3*B*). In yeast, bud emergence occurs contemporaneously with replication initiation and is frequently used as a morphological marker of S phase cells (17). In both wild-type yeast (strain SSC18) and Δ*trx1* Δ*trx2* yeast (strain MY445), budding was first detected at 60 min after release and was maximal by 80 min after release. Although very small buds may have escaped detection at earlier times, the results in Fig. 3 suggest that *cdc15*-arrested cells began entering S phase about 60 min after release to the permissive temperature and that most cells had entered S phase by 80 min after release.

Fig. 3*C* shows dNTP levels in cells synchronized by *cdc15* block and release. As observed previously in *bar1* strains, deletion of thioredoxin in the *cdc15* strains resulted in about a 50% increase in asynchronous cell dNTP levels (Fig. 3*C*, compare *black* and *gray* "*a*" time point *histograms*). In contrast to the results obtained when *bar1* cells were arrested with α-factor (see Fig. 2*B*), no decrease in dNTPs was observed when *cdc15* cells were arrested at the nonpermissive temperature, regardless whether the cells contained or lacked thioredoxin. In fact, for dTTP and dATP, incubation at the nonpermissive temperature resulted in higher dNTP levels. Apparently, the balance between dNTP synthesis and turnover results in higher dNTP levels in *cdc15*-arrrested cells than in α-factor-arrested cells. When *cdc15*-arrested cells were released to the permissive temperature, no transient dip in dNTP levels was observed at early time points, even though microscopy showed that the cells completed mitosis and entered early G_1 phase. The result suggested either that the released cells traversed early G_1 too rapidly to affect dNTP levels or that the decrease in dNTPs previously observed in α-factor-treated cells was not because of arrest in early G_1 *per se* but rather was due to some other effect of α-factor. Finally, and most significantly, the results in Fig. 3*C* show that beginning about 45 min after release, dNTP levels decreased precipitously in Δ*trx1* Δ*trx2* cells but remained constant or increased slightly in wild-type cells. The results suggested that from 45 min onward, the rate of dNTP synthesis in Δ*trx1* Δ*trx2* cells could no longer keep pace with the rate of dNTP consumption. Although budding was not evident until 60 min, we suspect that DNA synthesis began before buds were visible and that dNTP consumption at replication forks created greater demands on the dNTP-synthesizing machinery. Wild-type cells were able to meet the greater demand for dNTPs, but cells lacking thioredoxin were not. Given that thioredoxin functions as an electron donor for ribonucleotide reduction by RNR *in vitro* (23), the simplest explanation for impaired dNTP synthesis in Δ*trx1* Δ*trx2* cells is that thioredoxin serves as the preferred electron donor for ribonucleotide reduction by RNR *in vivo*.

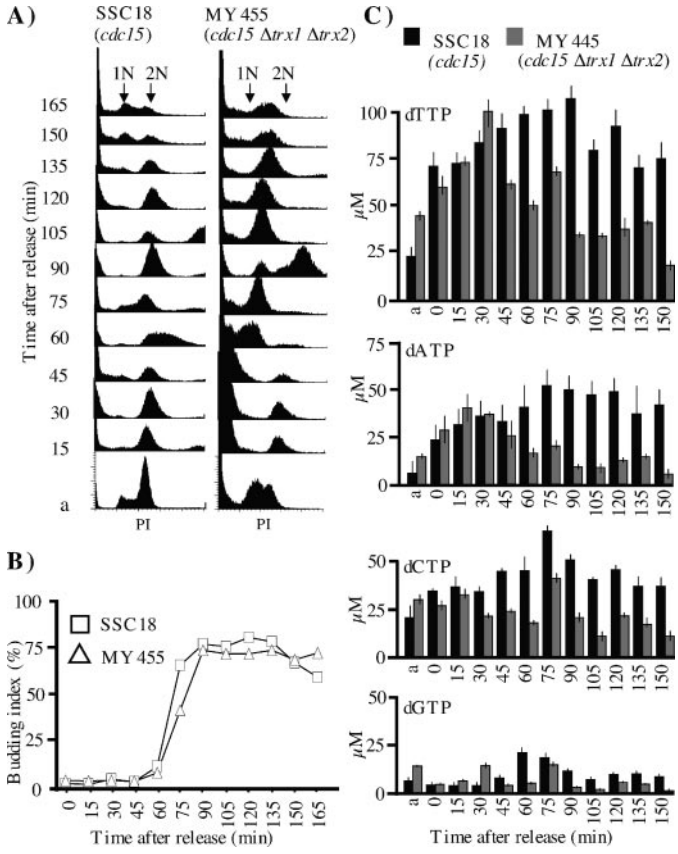

FIGURE 3. **Deoxyribonucleotide levels in *cdc15*-synchronized wild-type and Δ*trx1* Δ*trx2* yeast.** Temperature-sensitive *cdc15* yeast that were wild-type for thioredoxin (strain SSC18) or were Δ*trx1* Δ*trx2* deletion mutants (strain MY445) were used. Yeast that grew asynchronously at 25 °C (*a*), were arrested at M/G_1 by a 3.7-h incubation at the nonpermissive temperature of 37 °C (*0*), or were released at 25 °C for the indicated number of minutes were fixed for flow cytometry and budding index determination or were extracted by the boiling methanol method for dNTP analysis. *A*, flow cytometric analysis of DNA content of cells harvested at the indicated times after release to the permissive temperature. *PI*, propidium iodide. *B*, budding index of wild-type yeast (*squares*) and Δ*trx1* Δ*trx2* yeast (*triangles*) at the indicated times after release to the permissive temperature. At least 50 cells were scored in triplicate for each time point. *C*, dNTP levels in wild-type (*black*) or Δ*trx1* Δ*trx2* (*gray*) yeast at the indicated times after release to the permissive temperature. *Histograms* and *error bars* represent mean ± range of duplicate determinations.

DISCUSSION

Subsequent to its discovery as a hydrogen donor for *Escherichia coli* ribonucleotide reductase *in vitro* (23), few experiments investigated whether this defining activity of thioredoxin plays a significant role in dNTP synthesis *in vivo*. The viability of *E. coli* mutants lacking thioredoxin or thioredoxin reductase indicated that alternative pathways for ribonucleotide reduction existed and led to the discovery of glutaredoxins (24). *In vitro* ribonucleotide reductase assays showed that glutaredoxin was almost 10-fold more active than thioredoxin on a molar basis (25). However, thioredoxin is 10-fold more abundant than glutaredoxin in the *E. coli* cytosol (25). An extract from an *E. coli* mutant lacking thioredoxin showed high levels of NADPH-dependent ribonucleotide reductase activity (26). However, deletion of thioredoxin may lead to compensatory changes in the expression of RNR protein and glutathione system proteins, which would tend to mask a reduction in ribonucleotide reductase catalytic efficiency. Indeed, *E. coli* mutants lacking thioredoxin (TrxA) and glutaredoxin 1 (Grx1), which presumably survive via Grx3, have 20-fold higher levels of RNR enzyme (26). In prokaryotes, proteins functioning in a common pathway are frequently encoded by genes located in the same operon. For example, the *NrdA* and *NrdB* genes encoding the large and small RNR subunits are frequently located in the same operon. We examined the genomic context of NrdA and NrdB orthologs in 25 evolutionarily distant prokaryotic species and noted close linkage to a glutaredoxin-like gene in three cases

(*Agrobacterium tumefaciens*, *Corynebacterium efficiens*, and *Salmonella typhimurium*) and close linkage to a thioredoxin system gene in three cases (*Clostridium perfringens*, *Legionella pneumophila*, and *Rickettsia prowazekii*). The genomic context of the *NrdA* and *NrdB* genes thus suggests that the primary reductant used by RNR may differ between species. Deoxyribonucleotide measurements in mutant cells lacking glutaredoxin or thioredoxin could potentially shed light on the identity of the physiological RNR reductant. The finding that dNTP levels were higher in asynchronously growing Δ*trx1* Δ*trx2* yeast than in wild-type yeast (2) argued against the thioredoxin system playing a primary role in supplying reducing equivalents to RNR *in vivo*.

Although earlier work in asynchronously growing yeast did not support a role for thioredoxin in ribonucleotide reduction, our results using synchronized yeast suggest otherwise. Deoxyribonucleotide pool measurements in α-factor-synchronized wild-type and Δ*trx1* Δ*trx2* yeast revealed that thioredoxin was required for dNTP accumulation in late G_1/early S phase of the cell cycle. In yeast lacking thioredoxin, none of the four dNTP pools increased as cells approached S phase, and in fact, the pools decreased as cells entered S phase. A failure to maintain the dNTP pools during S phase was also observed in *cdc15*-synchronized cells. Inhibition of dNTP accumulation in Δ*trx1* Δ*trx2* cells was consistent with the idea that thioredoxin is the primary electron donor for ribonucleotide reductase *in vivo*. Previous dNTP pool measurements in asynchronous Δ*trx1* Δ*trx2* cells (2) were accurate but misleading. Undiminished dNTP levels in asynchronously growing Δ*trx1* Δ*trx2* cells do not mean that the mutant cells have normal rates of dNTP synthesis throughout the cell cycle. In asynchronous populations, apparent dNTP pool sizes are dependent on the percentage of cells in each phase of the cell cycle and on the rates of dNTP synthesis and consumption in each phase of the cell cycle. When we synchronized cells and checked their dNTP pools during the cell cycle, we found that Δ*trx1* Δ*trx2* cells were unable to accumulate dNTPs as they approached S phase and were unable to maintain dNTP levels once they entered S phase. Our results suggest that impaired synthesis of dNTPs was the basis for S phase protraction in cells lacking thioredoxin.

Clearly, thioredoxin is not the sole hydrogen donor to ribonucleotide reductase in yeast, as Δ*trx1* Δ*trx2* cells are viable. Mutants lacking thioredoxin are inviable in the absence of glutathione reductase (*GLR1*), indicating that thioredoxin mutants need reduced glutathione to survive (11). We speculate that in the absence of an intact thioredoxin system, the glutaredoxin system can provide the reducing power for ribonucleotide reductase but does so inefficiently. It is important to note that participation of the glutaredoxin system in ribonucleotide reduction may be dependent on epigenetic changes induced by ablation of the thioredoxin system (11, 13, 14, 27). The yeast glutathione reductase gene (*GLR1*) and two glutaredoxin genes (*GRX1* and *GRX2*) are induced 2-, 8-, and 170-fold, respectively, in Δ*trr1* yeast lacking thioredoxin reductase (14). Furthermore, the ribonucleotide reductase subunit genes *RNR1*, *RNR2*, and *RNR3* are induced 4.3-, 6.8-, and 3.7-fold, respectively, in Δ*trx1* Δ*trx2* yeast lacking thioredoxin (2). Compensatory increases in ribonucleotide reductase and glutaredoxin system proteins in Δ*trx1* Δ*trx2* cells may be responsible for the higher levels of dNTPs observed in nonreplicating thioredoxin null cells (Fig. 2*B*). According to this view, the induced RNR and glutaredoxin system proteins may result in higher than normal dNTP levels in α-factor-arrested or *cdc15*-arrested cells when the demand for DNA precursors is low, but they cannot maintain elevated dNTP levels in S phase cells when the demand for DNA precursors is high.

The failure of Δ*trx1* Δ*trx2* cells to maintain normal dNTP pools during S phase suggests that S phase protraction in cells lacking thioredoxin is due to a primary effect on the ability of cells to make dNTPs. Alternatively, it is possible that thioredoxin deletion has a primary effect on the replication apparatus and only secondarily affects dNTP levels. We consider this alternative explanation unlikely, however. The observation that dNTP pools increase when replication origin firing is blocked by a *dbf4* mutation (5) argues against the existence of a complex mechanism that reduces dNTP synthesis when replication is inhibited. Thus, the simplest explanation for S phase protraction in cells lacking thioredoxin is that DNA elongation at replication forks is slower because dNTP levels are suboptimal. Given that thioredoxin can function as a ribonucleotide reductase reductant *in vitro*, the simplest explanation for suboptimal dNTP levels in Δ*trx1* Δ*trx2* cells is that thioredoxin is the primary ribonucleotide reductase reductant *in vivo*.

Acknowledgments—We thank Julie Oughton and Castle Funatake for help with flow cytometry, Tamara Fraley for help with microscopy, and Eric Muller for supplying yeast strain EMY-56D.

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Thioredoxin Is Required for Deoxyribonucleotide Pool Maintenance during S Phase

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J. Biol. Chem. 2006, 281:15058-15063.
doi: 10.1074/jbc.M601968200 originally published online March 29, 2006
