Glutathione-glutaredoxin is an efficient electron donor system for mammalian p53R2–R1-dependent ribonucleotide reductase

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Deoxyribonucleotides are DNA building blocks and are produced de novo by reduction of ribose to deoxyribose. This reduction is catalyzed by ribonucleotide reductase (RNR), a heterodimeric tetramer enzyme in mammalian cells, having one of two free radical-containing subunits called R2 and p53R2. R2 is S-phase specific and used for DNA replication, whereas p53R2 functions in DNA repair and mitochondrial DNA synthesis. The larger RNR subunit, R1, has catalytically active cysteine thiols in its buried active site and a C-terminal swinging arm, with a Cys-Leu-Met-Cys sequence suggested to act as a shuttle dithiol/disulfide for electron transport. After each catalytic cycle the active site contains a disulfide, which has to be reduced for turnover. Thioredoxin (Trx) and glutaredoxin (Grx) systems have been implicated as electron donors for the RNR disulfide reduction via the swinging arm. Using mouse R1–R2 and R1–p53R2 complexes, we found here that the catalytic efficiency of the GSH-Grx system is 4–6 times higher than that of the Trx1 system. For both complexes, the Vmax values for Grx are strongly dependent on GSH concentrations. The GSH disulfide resulting from the Grx reaction was reduced by NADPH and GSH reductase and this enzyme was essential because reaction with GSH alone yielded only little activity. These results indicate that C-terminal shuttle dithiols of mammalian R1 have a crucial catalytic role and that the GSH-Grx system favors the R1–p53R2 enzyme for DNA replication in hypoxic conditions, mitochondrial DNA synthesis, and in DNA repair outside the S-phase.

Ribonucleotide reductases (RNR)3 catalyze the rate-limiting step in deoxyribonucleotides synthesis required for DNA replication and repair (1, 2) (Fig. 1). The two main antioxidant systems in mammalian cells, thioredoxin and GSH-glutaredoxin, support RNR catalysis. The mammalian class Ia RNR enzyme is composed of two homodimeric subunits: R1 (87 kDa) and R2 (40 kDa), which together form a heterotetrameric active enzyme (3). The R1 subunit carries the substrate-binding site, the active site composed of three redox-active cysteine residues, allosteric effector binding sites, and two redox-active thiols in the C-terminal (4) (Fig. 1). The R2 subunit contains a diferric-tyrosyl radical cofactor required for enzyme catalysis. The p53R2, an additional mammalian RNR protein, functions as a catalytic partner of the R1 subunit (5–7). The mouse p53R2 (40 kDa) showed 81% sequence identity to the mouse R2 subunit at the amino acid level (8). Like R2, the p53R2 subunit also contains a diferric-tyrosyl radical cofactor. In contrast to the S-phase–specific R2 subunit, the p53R2 is induced by DNA damage and the expression is mediated by the tumor suppressor protein p53 (9).

The requirement of dNTPs is widely different during the cell cycle. The RNR concentration and composition change following the requirement of the cell cycle with the R2 subunit being degraded after S-phase. The concentration of R1 subunit instead remains constant also in resting cells. In fact, the R1–p53R2 complex has a crucial role in dNTP supply for basal DNA repair and also for mitochondrial DNA synthesis outside the S-phase (5, 10).

Inefficient RNR activity may lead to fork retardation, reduction of DNA replication, and blockage of cell cycle. Thus, RNR inhibitors, such as hydroxyurea, act as inducer of replication stress.

The RNR reaction cycle involves formation of a disulfide in the active site, with the reduction of a substrate ribonucleoside diphosphate (4, 11). In the R1 subunit, the narrow entrance to the catalytic site sterically prohibits a direct reduction of the active-site disulfide by thioredoxin (Trx) or glutaredoxin (Grx). The active-site thiols are regenerated by a pair of thiols in the swinging arm of R1 by a thiol-disulfide exchange reaction (1). Then, Trx and GSH-Grx systems are required to reduce the resulting C-terminal disulfide for the next cycle of RNR reaction (12, 13). Trx is reduced by thioredoxin reductase (TrxR), whereas Grx is reduced by glutathione (GSH) and GSH reductase (GR) (14, 15). Several reports have been published describing the catalytic mechanism of RNR (16–19), although the electron transfer flow is still not completely characterized.
Redox mechanism of mammalian RNR enzymes

Different concentrations of Trx (0.01–10 μM) (Fig. 3, A and B) and Grx1 (0.1–1 μM) (Fig. 3, C and D) were used to measure RNR activity. The result of the kinetic parameters are summarized in Table 2. The Trx system as electron donor showed a five times higher value of $V_{\text{max}}$ for the R1–R2 complex and a typical apparent $K_m$ value of 1.4 μM (Fig. 3A), whereas the R1–p53R2 complex had a $K_m$ equal to 0.7 μM (Fig. 3B).

The $K_m$ value for the R1–R2 complex with varying concentrations of human Grx1 in combination with 5 or 10 mM GSH, NADPH, and excess GR (Fig. 3C) was equal to 0.15 μM, in agreement with previously reported data (24). However, we obtained a 1.9-fold higher value of $V_{\text{max}}$ compared with the previous report (24). The same experiment was performed with the R1–p53R2 complex (Fig. 3D) and it displayed an apparent $K_m$ value of 0.08 μM for Grx1.

The specific activities, apparent $V_{\text{max}}$, and corresponding $k_{\text{cat}}$ were found to be lower for the GSH-Grx1 system compared with the Trx1 system for both of the RNR complexes (Table 1 and 2). However, the catalytic efficiency ($k_{\text{cat}}/K_m$) was higher for GSH-Grx1, having lower $K_m$ values for both RNR complexes (Table 2).

Mammalian cells also express a cytosolic isoform of Grx1, named Grx2, with similar enzymatic properties but a different active-site motif (25, 26). We wanted to test if R1–p53R2 is a substrate also of this other Grx isoform. We performed experiments with different concentrations of Grx2 and also with the active-site mutant Grx2C40S that has been shown to operate via a monothiol mechanism with the R1–R2 complex (24) (Fig. 4). Grx2 was able to support the activity of both mammalian RNR complexes. The $K_m$ values (Table 2) were higher for Grx2 in the R1–R2 complex, 0.3 μM, compared with R1–p53R2 complex, 0.21 μM. The C40S mutant of Grx2 was found to act with similar efficiency for both RNR complexes ($K_m$ values of 0.36 and 0.38 μM, respectively).

Results

Characterization of mammalian R1–p53R2 compared with the R1–R2 complex

A common way to get active RNR is to use DTT as electron donor (1, 2). To investigate the effect of DTT for mammalian RNR catalysis, experiments were carried out with R1–R2 and R1–p53R2 complexes (Fig. 2, A and B). The active RNR hetero-tetramer was formed by mixing 200 μg/ml of R1 with 100 μg/ml of R2 (Fig. 2A) or p53R2 (Fig. 2B) in the presence of Mg$^{2+}$ and ATP as cofactors. RNR reduced $[^{3}H]$dCDP to $[^{3}H]$dCDP with maximum activity at 10 mM DTT for the R1–R2 complex as previously described (24) and 2.5 mM DTT for the R1–p53R2 complex. Inhibition by higher concentrations of DTT was observed for both the RNR complexes but was much more pronounced for R1–p53R2; in fact this complex lost half of the activity at 15 mM, whereas the corresponding R1–R2 complex has 50% of maximal activity at 40 mM DTT.

We titrated the concentrations of R2 and p53R2 using the physiological electron donors for RNR, such as Trx and Grx-GSH systems to check if increasing concentrations of p53R2 were leading to a similar activity as the complex of R1–R2. In Fig. 2, C and D, it is possible to see that both R2 and p53R2 reached saturation at 200 μg/ml concentrations (1:2 molar ratio R1:R2; R1:p53R2) but the R1–p53R2 complex had a lower enzymatic catalytic efficiency.

Thioredoxin and GSH-glutaredoxin systems as electron donors for RNR complexes

Our results show that both Trx and the GSH-Grx system are able to sustain the RNR activity for both complexes. However, at the physiological concentration of GSH, the Grx system seems more efficient. The role of GSH as a key mediator of cell proliferation has been shown by several groups of researchers. Co-localization of GSH with nuclear DNA was observed in proliferating cells (20). In highly purified normal human T cells, GSH modulated proliferation and regulated DNA synthesis (21). Depletion of GSH blocks DNA synthesis in mammary carcinoma cells (22). In a similar study done in 3T3 fibroblast cells, the relationship between the distribution of nuclear GSH and cell cycle progression was observed (23). Thus, these data emphasize the role of the GSH-Grx system in RNR activity. Considering also that the R1–p53R2 complex has higher affinity for the GSH-Grx system, these data may be used to better understand replication stress, tumor cell growth, and treatment.

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Importance of GSH concentration in mammalian RNR catalysis

From the results of Fig. 3, C and D, we saw that increased concentrations of GSH lead to an increase of RNR activity. The importance of reduced GSH was confirmed by running the RNR assay with 5 mM GSH but in the absence of GR (Table 3). Moreover, we titrated GSH (0.5–20 mM) in the presence of 1 μM Grx1 plus excess GR and 1 mM NADPH (Fig. 5, A and B).
p53R2 complex showed again a lower $K_m$ value compared with R1–R2 complex (Fig. 5B).

To further verify the role of GSH for RNR catalysis, the R1–R2 complex was incubated with Trx and GSH in the presence of GR and NADPH (Fig. 5C). RNR activity increased with increasing concentrations of GSH. The result suggests that GSH could maintain the level of reduced Trx to support the RNR catalysis. Altogether, the results clearly show that GSH is necessary for the reduction of both mammalian RNR complexes.

To study glutathionylation of the R1 subunit, pre-reduced R1 was incubated with eosin-labeled GSSG called Di-E-GSSG and the release of E-GSH in the reaction mixture was monitored by measuring fluorescence at 545 nm after excitation at 520 nm (Fig. 5D). Di-E-GSSG has almost no fluorescence in its disulfide form due to self-quenching, whereas the reduced form (E-GSH) has a large fluorescence emission at 545 nm after excitation at 520 nm. The fluorescence values increased over time, suggesting a reaction between R1 and Di-E-GSSG with release of the highly fluorescent E-GSSH molecule.

We were able to visualize the glutathionylated R1 in SDS-PAGE because the eosin group can be detected when exposed to UV light (27). The glutathionylated R1 (R1-SG-E) was detected only in the Di-E-GSSG–treated samples and a subsequent incubation with either DTT or Grx1 systems was able to deglutathionylate R1-SG-E (Fig. 5D, inset). Also addition of the Grx2 system showed a similar effect (data not shown).

Considering that the R1 subunit has Cys residues exposed not only in the swinging arm, we wanted to test if the thiols in the C-terminal part, Cys-787 and Cys-790, which are active to reduce the R1 active site, can be glutathionylated. We used the peptide corresponding to the C-terminal part of R1, which had mutations of Cys-787 to Cys-790 to Ser. The C-terminal C787S peptide was treated with Di-E-GSSG, and the release of E-GSH in the reaction mixture was monitored by measuring an increase in fluorescence (Fig. 5E). The increase in fluorescence over time suggests the glutathionylation of Cys-790 with the concomitant release of free E-GSH. Similar results were obtained when the same reaction was performed with the C790S peptide (data not shown) suggesting the glutathionylation of Cys-787.

**Discussion**

A balanced reduced cellular redox environment is essential to maintain normal physiological functions. Within cells, enzymes containing redox-active cysteines play a crucial role in regulating different signaling events. The regulatory and catalytic subunits (R1) of RNR complexes have two different classes of redox-active cysteines that control the enzyme activity. Trx and Grx systems play crucial roles in keeping the swinging arm cysteines of R1 reduced to maintain a balanced supply of dNTPs for proper DNA replication and repair. Unbalanced dNTP pools result in replication stress, misincorporation of nucleotides into DNA, mutations, and cell death. As cells enter S-phase of the cell cycle, the dNTP pools increase several fold. This event is possible because of increased synthesis of R1 and induction of R2 (3). In contrast, the p53R2 protein is present constantly at low levels in both nonproliferating and proliferating cells (5, 17).

The active-site cleft in R1 between the barrel and N-terminal domain does not allow Trx or Grx to enter to the active-site pocket to reduce the disulfide bond between Cys-218 and Cys-444, which is formed after substrate reduction (Fig. 6). Two thiols located at the C-terminal tail (Cys-787 and Cys-790 in mouse R1) reduce that disulfide bond formed in the active site (Fig. 6). The critical role for the C-terminal cysteine pair (in a CXXXC or CXXC motif) of R1 subunit in regeneration of its active site have been suggested by in vitro mutagenesis and kinetic studies (1, 28, 29). Moreover, interallelic complementation studies with R1 from Saccharomyces cerevisiae demonstrated that the C terminus of one monomer acts in trans to regenerate the active site of its neighboring monomeric subunit (30). The last 24 residues at the C-terminal tail, including the shuttle thiols, are not visible in the electron density map. For the Escherichia coli R1 subunit, the distance from the last visible residue, 737 to Cys-225 is about 30 Å. This unique arrangement of the flexible C-terminal tail allows the enzyme to retain a buried active site for the substrate reduction.

The efficiency of the Trx1 and Grx1 system as electron donor for RNR activity is summarized (Tables 1 and 2) for both of the mammalian RNR complexes. In mammalian cells, the R1–R2 complex supports the S-phase–specific DNA replication, whereas the R1–p53R2 complex performs the RNR activity in G0/G1 phase cells. Our data shows that the R1–p53R2 complex has 3–5 times lower activity compared with R1–R2 complex (Table 1). Thus, low constitutive levels of p53R2 with low levels of R1 subunit in mammalian cells provide the supply of dNTPs for basal levels of DNA repair and mitochondrial DNA synthesis during the G0/G1 phase. Our study suggests that the Trx1 system, having a higher $k_{cat}$, has the advantage to act as an electron donor for the RNR complexes. However, Grx1 has a
Redox mechanism of mammalian RNR enzymes

Figure 4. RNR activity using different reducing systems. Samples with 200 μg/ml of R1 and 100 μg/ml of R2 (A) or 100 μg/ml of p53R2 (B) were used for the assays with different reductants as indicated in each column. The GSH-Grx system contained 1 μM Grx1 or Grx2 or Grx2C40S, 5 mM GSH, 1 mM NADPH, and 0.1 μM GR. The Trx system contained 3.6 μM Trx1 with 1 mM NADPH and 0.1 μM TrxR. The results are expressed as the mean ± S.E. of two independent experiments performed in triplicates.

Table 3
Specific activities of human R1–p53R2 complex in presence and absence glutathione reductase

| Specific activity | unit [dCDP]/min/mg of R1 | (2019) 294(34) 12708 –12716 |
|------------------|---------------------------|-------------------------------|
| R1–p53R2 + GSH-Grx system without human GR | 0.71 ± 0.44 | 0.71 ± 0.44 |
| R1–p53R2 + GSH-Grx system with1 μM human GR | 5.7 ± 0.56 | 5.7 ± 0.56 |

higher catalytic efficiency ($k_{cat}/K_m$) compared with Trx1 with both RNR complexes. Interestingly, the two main cytosolic Grx isoforms showed activity with both RNR complexes. The C40S mutant of Grx2 that act with a monothiol mechanism was found to act as an efficient mediator also for R1–p53R2 activity (Fig. 4B). Here, it is noteworthy to mention that the bacterial monothiol mutant of Grx1 showed no activity with E. coli RNR. Recently, it has been shown that human monothiol Grx5 is active as a GSH-dependent electron donor for mammalian ribonucleotide reductase (31). However, the apparent $V_{max}$ and specific activity with the RNR complexes were found to be much lower for Grx5 compared with Grx1 or Grx2. Our results confirm the glutathionylation mechanism for Grx catalysis in contrast to the dithiol mechanism for Trx (Fig. 6). The treatment with Di-E-GSSG resulted in glutathionylation of the R1 subunit, which could be detected by the increase in fluorescence values (Fig. 5, D and E). Furthermore, the glutathionylated R1 (R1-SG-E) was also detected in SDS-PAGE with the help of a UV transilluminator and the R1-SG-E was found to be deglutathionylated in the presence of either DTT or the complete Grx1 system (Fig. 5D, insets). Moreover, the C787S peptide of the R1 subunit was found to be glutathionylated by Di-E-GSSG, which suggests the glutathionylation of Cys-790 (Fig. 5E). In a similar way, the C790S peptide of the R1 subunit was also found to be glutathionylated (data not shown), suggesting the glutathionylation of Cys-787. Thus, both C-terminal dithiols of the R1 subunit can be glutathionylated, which could be regulated by the Grx-GSH system.

Our study also shows that GSH can reduce Trx1 directly to support RNR catalysis and the RNR activity was very much dependent on the concentrations of GSH (Fig. 5C). This is in agreement with a recent study where it has been shown that both GSH and Grx1 directly and effectively reduced Trx1 (32). Interestingly, it was found that normal mice and mice having Trx1-deficient hepatocytes showed similar liver growth rates during development and regeneration (33). Thus, in mouse hepatocytes, a TrxR1-independent electron transfer pathway to RNR is most likely dependent on GSH, and can support DNA replication. Similarly, it was shown that the TrxR1-lacking lymphomas can initiate and progress normally in mice (34). A critical experiment demonstrating that the thioredoxin system is not essential for DNA replication has recently been published by Prigge et al. (35). The authors used a conditional knockout of Trx, TrxR, and GR in hepatocytes and the resulting mice with co-disruption of all three proteins surprisingly showed a 2–3-fold larger liver per body mass with hyper-proliferation. When GSH synthesis was inhibited by addition of BSO the mice died from liver necrosis. These results demonstrate the importance of the glutaredoxin system for DNA synthesis. Another important result particularly regarding RNR in tumor tissues comes from results presented in an article by Hammond and co-workers (36). These authors showed that in hypoxic conditions the R1–p53R2 is the predominant subunit during DNA replication because p53R2 is better suited to retain activity during hypoxia. A further insight in the role of Grx is shown by results that Grx-1 silencing induces cell senescence via the p53/p21/p16b signaling axis (37). In conclusion our results demonstrate the importance of the glutaredoxin system for DNA synthesis.
Experimental procedures

Materials

[^3]H]CMP and[^3]H]CDP were obtained from Moravek Biochemicals and Radiochemicals, respectively. The cation exchanger resin AG-50W+H23041 was purchased from Bio-Rad. Yeast GSH reductase, GSH, DTT, NADPH, and insulin were from Sigma. Di-Eosin-GSSG was purchased form IMCO, Sweden. Human Trx1, Grx1, Grx2, and Grx2C40S were prepared as described previously (25, 38, 40). Rat recombinant TrxR was a kind gift from Dr. Elias Arner (41).

Conversion of[^3]H]CMP to[^3]H]CDP

E. coli CMP kinase plasmid was a kind gift from Prof. Anne-Marie Gilles (Laboratoire d’Enzymologie et de Biochimie Structurales, France). E. coli CMP kinase was expressed, purified, and used to convert[^3]H]CMP to[^3]H]CDP as described by Bucurenci et al. (42). The[^3]H]CDP was separated from the reaction mixture by a C18 HPLC column using a mobile phase comprising 20 mM ammonium acetate buffer, pH 5, and 5% acetonitrile (data not shown).

Expression and purification of mouse glutaredoxin

Expression and purification of Grx1, Grx2, and Grx2C40S were done as previously described (25).

Expression and purification of RNR subunits

The E. coli strains expressing mouse R1, R2, and p53R2 were kindly provided by Dr. Lars Thelander (Umeå University, Umeå, Sweden). Recombinant proteins were expressed and purified as previously described (17, 24, 43, 44).

C-terminal peptides of R1

C-terminal peptides of R1 subunit were purchased from Caslo Laboratory, Denmark. The peptide represents the last 10 amino acids (mutation at Cys-787 or Cys-790) of the C-terminal tail of mouse R1 subunit. The two monothiol mutant peptides (C790S and C787S) have sequences of NREECLMSGS (1125.61 Da) and NREESLMCGS (1124.70 Da), respectively.

Ribonucleotide reductase activity assay

Determination of RNR activities were carried out as described previously (24). Briefly, the RNR system was reconstituted by mixing recombinant R1 and R2 or p53R2 proteins. Activity was assayed following the conversion of[^3]H]CDP into[^3]H]dCDP. R1 and R2 or p53R2 proteins were pre-incubated with 2 mM ATP and 10 mM MgCl2 at 37 °C for 15 min and the reaction was initiated by adding a reaction mixture containing 40 mM Tris-HCl buffer, pH 7.6, 2 mM ATP, 10 mM MgCl2, 200 mM KCl, 20 μM FeCl3, and 0.5 mM[^3]H]CDP (10,000 cpm/nmol) in a final volume of 50 μl. Incubation was carried out at 37 °C for...
Figure 6. Structure of human R1 dimer. A, each R1 subunit contains one catalytic site and two allosteric regulatory sites. The allosteric specificity site regulates the substrate preference and the allosteric activity site regulates the ribonucleotide reductase activity. Adapted and modified from Ref. 39. B, model of reduction of RNR by the Trx and GSH-Grx system: for simplicity, interaction of only one active site and one CXXC motif are shown. The active-site pocket of each R1 subunit is shown in a circle. The cysteine residue (–SH) in the active site of R1 is converted to a transient thyl radical through electron transfer from the tyrosyl radical located in R2. At the completion of each turnover cycle, a disulfide bond is formed between the conserved cysteine pair at the active site (1). Reduction of the R1 active site is mediated by the C-terminal CXXC motif of the neighboring subunit (2). The resulting disulfide bond is reduced by Trx and Grx systems through disulfide-exchange resulting in an active R1 (3 → 4). The reaction leads the RNR reaction cycle to continue (4 → 1). The role of the Grx system for a monothiol mechanism involving glutathionylation is also shown (3 → 5 and 5 → 4). Adapted from Ref. 11.
30 min. The reaction was stopped by the addition of 1 M perchloric acid. 100 μl of 10 mM dCMP was added to determine the recovery of the product. To hydrolyze CDP and dCDP into CMP and dCMP, respectively, the samples were incubated in boiling water for 10 min. The amount of radioactivity derived recovered product. To hydrolyze CDP and dCDP into SKF-525A system was used together with RNR, the samples contained 1 μM Grx1 (or Grx2 or Grx2C40S), 0.15 μM GR, 1 mM NADPH, and 5 mM GSH. To avoid oxidation of GSH to GSSG, each time a freshly prepared stock of GSH was titrated to pH 7.0. The enzyme activity was measured as nanomole of dCDP produced after 30 min of reaction. The activity data were analyzed with Prism 8 (GraphPad Software, Inc.) and fitted with Michaelis-Menten kinetics for calculation of catalytically efficiency of the RNR complex with various reducing system.

Glutathionylation of R1 subunit and peptides

To study the glutathionylation of R1, Di-E-GSSG was used to glutathionylate R1 protein and C-terminal peptides of R1 (27, 45). Di-E-GSSG has quenched fluorescence in the disulfide form that increases up to 20-fold on reduction of its disulfide bond and formation of E-GSH. Black 96-well plates were used in a PerkinElmer Victor3 multilabel counter containing a final well volume of 200 μl in 0.1 M potassium phosphate buffer (pH 7.5), 1 mM EDTA. The reaction was started by addition of 20 μM Di-E-GSSG to R1 or C-terminal peptide solutions, followed by recording the fluorescence emission at 545 nm after excitation at 520 nm. Controls where no R1 protein or peptide was added were used as fluorescent background.

To confirm the glutathionylation of R1 with a different method, R1 subunit was incubated with Di-E-GSSG. Aliquots of S-glutathionylated R1 (R1-SG-E) were further treated with 10 mM DTT or with the complete GSH-Grx system. All samples were run on a nonreducing SDS-PAGE 4–12% acrylamide. The gel was exposed to UV transilluminator to visualize eosin-tagged glutathionylated protein. The same gels were later stained with Coomassie Blue staining.

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