Ribonucleotide Reductase: In-vitro S-glutathionylation of R2 and p53R2 Subunits of Mammalian Class I Ribonucleotide Reductase Protein

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Short Report

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Abstract

Ribonucleotide reductases (RNR) catalyze the rate-limiting step in DNA synthesis during the S-phase of the cell cycle. Its constant activity in order to maintain dNTP homeostasis is a fascinating area of research and an attractive candidate for cancer research and antiviral drugs. Redox modification such as S-glutathionylation of the R1 subunit of mammalian RNR protein has been presumed to regulate the activity of RNR during catalytic cycles. Herein, we report S-glutathionylation of the R2 subunit. We have also shown Grx1 system can efficiently deglutathionylate the S-glutathionylated R2 subunit. Additionally, our data also showed for the very first time S-glutathionylation of mammalian p53R2 subunit that regulates DNA synthesis outside S-phase during DNA damage and repair. Taken together, these data will open new avenues for future research relating to exact physiological significance, target thiols, and/or overall RNR activity due to S-glutathionylation of R2 and p53R2 subunits and provide valuable insights for effective treatment regimes.

1. Introduction:

Ribonucleotide reductase (RNR) is an essential enzyme that catalyzes the rate-limiting step in the conversion of ribonucleotides (rNTP) to concomitant deoxyribonucleotides (dNTP) during *de novo* DNA synthesis [1, 2]. RNR is a heterodimeric tetramer consisting of $\alpha_2\beta_2$ heterodimers, where α_2 denote the larger R1 subunit which serves as the catalytic site for rNTP reduction and β_2 denote the R2 subunit which serves a regulatory role in conjunction with the R1 catalytic counterpart and function in S-phase DNA replication. Mammals also have additional regulatory RNR protein p53R2 (encoded by RRM2B gene) that function during DNA repair, hypoxia, and mitochondrial DNA synthesis [3, 13]. The R1 and R2/p53R2 subunits together form the active functional enzyme and these subunits are endowed with reactive thiols which are susceptible to redox modifications. R1 subunit has catalytically active cysteine residues at the active site and a C-terminal swinging arm bearing Cys-Leu-Met-Cys sequence that behaves as shuttle dithiol/disulfide for electron transport during enzyme turn over and reduction cycles. R2/p53R2 subunit also bears tyrosyl free radical which allows them to participate in the free radical mechanism of reduction [4]. RNRs are evolutionarily conserved across all species and play an essential role in maintaining dNTP pools for the proper regulation of the rate of DNA synthesis to cell volume during cell division and repair. It is due to this fundamental role, RNRs are attractive targets for anti-cancer and anti-viral drugs. During rNTP reduction, the R1 active site cysteine forms a disulfide after each cycle which is reduced further by shuttle dithiols of the C-terminal swinging arm *via* accepting electrons from Thioredoxin (Trx) or glutathione (GSH)-glutaredoxin (Grx) systems. The disulfide formed in turn at the shuttle dithiol is further reduced by GSH-Grx or Trx systems [4, 5]. The mechanism of RNR mediated catalysis is largely aided by redox modification S-glutathionylation which depends upon the availability of GSH-Grx electron donor systems. S-glutathionylation is the covalent addition of glutathione to thiols of proteins leading to the formation of disulfide bonds. Avval et al. reported that Trx and GSH-Grx systems are hydrogen donors for RNR catalysis [6]. The C-terminal tail of the R1 subunit reportedly underwent S-glutathionylation which facilitated the RNR catalysis. Recently, we have reported that GSH-Grx system is a more efficient electron

donor for RNR function with catalytic efficiency 4–6 fold higher than Trx, and GSH-Grx complex favors R1-p53R2 systems during DNA repair and replicative stress [7]. GSH formed adduct with the R1 subunit in a concentration-dependent manner presumably at a higher concentration (5–10 mM) with R1-R2 or R1-p53R2 complex during the catalysis and provided electrons to the shuttle dithiols for efficient reduction of active site thiols, which would otherwise not be reduced by redoxins themselves due to sterically narrow active site pocket of R1 [8, 4]. This novel mechanism allowed RNR to maintain its active site thiols in reduced state. This also facilitated RNR to maintain its activity in hypoxic and/or even in low Trx environment as occurs in the tumor microenvironment and prolonged DNA synthesis [7, 13]. GSH concentration however gets up-regulated in cancer cells due to metabolic reprogramming which has been well documented to support tumor progression and tumor resistance [9]. S-glutathionylation has also been reported to contribute to cancer progression and treatment resistance [10]. This can also provide a reasonable explanation why the GSH-Grx system is a more efficient electron donor for RNR disulfide reduction and sustain high RNR activity. However, no such data relating to R2 or mammalian p53R2 subunit S-glutathionylation has been made so far. Herein, we report for the first time S-glutathionylation of the R2 subunit of RNR in cell free assay system and we have also shown that Grx1 can efficiently deglutathionylate the S-glutathionylated R2 subunit (R2-SSG). Additionally, we also report S-glutathionylation of p53R2 subunit similar to R2 subunit.

2. Materials And Methods:

1. **2.1 Materials and reagents-** All reagents and chemicals were purchased from Sigma Chemical Co. Di-Eosin-GSSG were purchased from IMCO, Sweden.
2. **2.2 Expression and purification of RNR subunits-** Mouse R2, and p53R2 were cloned in *E.coli* strains and plasmids were a kind gift from Dr. Lars Thelander (Umeå University, Umeå, Sweden). These recombinant (6-His tagged) mouse proteins R2 and p53R2 were expressed in *E. coli* BL21(DE3) strain and purified as previously described [11, 12, 7]
3. **2.3 Expression and purification of mouse Glutaredoxin-** Expression and purification of mouse Glutaredoxin system (Grx1) was also done similar to above as previously described [12].
4. **2.4 S-glutathionylation of R2 and p53R2 proteins-** In order to study glutathionylation, Di-E-GSSG (two eosin tagged GSSG) was used to glutathionylate purified R2 and p53R2 proteins. Di-E-GSSG has quenched fluorescence in its disulfide form however, upon reduction of the disulfide bond and formation of E-GSH, fluorescence increases up to 20-fold which was measured. Black 96-well-plates were used in a PerkinElmer Victor3 multilabel counter containing a final well volume of 200µl in 0.1 M potassium phosphate buffer (pH 7.5) and 1 mM EDTA. The reaction was started by addition of 20µM Di-E-GSSG to R2 or p53R2, followed by recording the fluorescence emission at 545 nm after excitation at 520 nm. Controls where no R2 protein or p53R2 protein was added were used as fluorescent background.
5. **2.5 S-(de) glutathionylation of R2 subunit-** In order to study S-(de) glutathionylation of R2 subunit, S-glutathionylated R2 protein (R2-SGE) was purified by several cycles of centrifugation using 10 kDa membrane filters. Following purification, 5 or 10µM R2-SGE was incubated with 5mM synthetic

reducing agent DTT or with purified mouse Grx1 system (1 μ M Grx1, 1 mM NADPH, 5 mM GSH, and 0.1 μ M GR).

3. Results

3.1 R2 subunit undergoes S-glutathionylation with GSH-Grx1 system-

In our previous study, the S-glutathionylation of R1 subunit was assayed in presence of eosin-tagged glutathione (Di-E-GSSG) [7]. Here, we have performed the same assay to study the S-glutathionylation of the R2 subunit. R2 subunit was treated with DTT and incubated for 15 min. Then, the excess of DTT was removed by several cycles of centrifugation using 10 kDa MW cut-off filters. In the experiment, pre-reduced R2 was found to be S-glutathionylated in presence of Di-E-GSSG that led to the formation of R2-SSG (Fig. 1). The release of eosin-tagged reduced glutathione (E-GSH) was monitored using Fluorescence measurement with an emission at 545 nm after excitation at 520 nm. Fluorescence intensity from Di-E-GSSG alone was taken as control with which relative changes in fluorescence emission with time was compared. This data clearly implicates S-glutathionylation of R2 subunit.

3.2 S-(de) glutathionylation of R2-SSG-

Next, we attempted to check if the S-glutathionylated R2 subunit (R2-SSG) can be deglutathionylated (remove the glutathione moiety) by Grx-1 and synthetic reducing agent DTT treatment. R2-SGE was treated with Grx-1 system (1 μ M Grx1, 1 mM NADPH, 5 mM GSH, and 0.1 μ M GR) or 5 mM DTT independently and the release of E-GSH was monitored (excitation at 520 nm, emission at 545 nm) and plotted against time (Fig. 2). Fluorescence background intensity of untreated R2-SGE (not treated with either Grx-1 or DTT) was used as a control. The relative changes in fluorescence emission with control were plotted with time. This data clearly denotes S-deglutathionylation of R2-SGE by physiological antioxidant Grx1 as well as DTT, where DTT has been found to be more potent.

3.3 S-glutathionylation of p53R2 RNR protein-

The p53R2 RNR protein of mammals specifically allows DNA synthesis during DNA damage, repair, and mitochondrial DNA synthesis outside S-phase. We next wanted to identify if this subunit is also susceptible to S-glutathionylation similar to R2. Similar to R2 subunit, purified mouse p53R2 protein was reduced with DTT. Then, pre-reduced p53R2 was treated with Di-E-GSSG and the release of E-GSH was monitored with an emission at 545 nm after excitation at 520 nm (Fig. 3). Fluorescent background intensity obtained from Di-E-GSSG alone was used as control. The graph shows an increase in release of E-GSH emission for 10 μ M p53R2 over time, showing higher S-glutathionylation of p53R2. Herein, we are providing with a first-hand report that p53R2 subunit undergoes S-glutathionylation, whose exact significance in biology is not known and would be of huge interest in the field for future investigation.

4. Discussion:

S-glutathionylation, the covalent addition of glutathione moiety to redox-sensitive cysteine residues of enzymes serves as a vital regulation of cellular processes. S-glutathionylation plays an intriguing role in modifying RNR protein that serve in maintaining constant dNTP pools. Trx/GSH-Grx system serves as electron donor for RNR during each catalytic cycle [7]. As previously reported R1 subunit undergoes S-glutathionylation to aid in providing electrons to the shuttle dithiols for catalysis, our recent data show S-glutathionylation of the R2 subunit. It has been found that the R2 subunit underwent S-glutathionylation effectively (Fig. 1). The significance of R2 S-glutathionylation remains to be elucidated and would open new avenues for several investigations. It has been well documented that S-glutathionylation contributed to tumor progression and tumor resistance [10]. We intended to check if physiological antioxidants such as Grx1 can potentially reverse this modification to deglutathionylate R2-SSG and our data showed mouse Grx1 could catalyze the deglutathionylation reaction efficiently (Fig. 2). We have compared the deglutathionylation profiles using both physiological as well as synthetic reducing agent DTT, in which the latter was found to be more potent as compared to the Grx1 system. This was presumably due to several factors such as Grx1, being an enzymatic physiological antioxidant imparted its own kinetics of reduction displaying a sigmoid curve, however, DTT being a non-enzymatic strong reducing agent showed a partial hyperbolic curve and a higher reduction efficiency. Further, the size of DTT is smaller as compared to Grx1, making it accessible to structurally hindered thiols within the R2 subunit. **Figure-4** the scheme diagram shows a summary of our findings.

It has been well established that GSH concentration elevated in cancer cells due to metabolic reprogramming to reduce ROS generation from accelerated metabolic activities [9]. This elevated GSH favored the cancer cells and turned the fate towards cell survival. Increased GSH could result in the S-glutathionylation of RNR subunits. Therefore, it also intensifies the potential role of Grx1 in preventing oxidative stress-induced redox modification of the R2 subunit that might prove an effective intervention in preventing cancer survival. Our data has also shown S-glutathionylation of the p53R2 subunit. However, its exact significance remains to be elucidated. It would be of tremendous interest to further study the physiological significance of R2 and p53R2 S-glutathionylation (in an *in-vivo* system) which might underpin a novel strategy utilized by cancer cells and bridge an existing gap to understand the complexities of redox modifications and translate effective therapeutic strategies.

Declarations

5. Author Contributions:

A.C. and R.S. wrote the manuscript. A.H. provided resources, funding acquisition and oversight of the experiments. R.S. contributed to the experimental design, performed the experiments. A. C. performed the experiments and provided critical discussion of the manuscript. A.C. and R.S. reviewed the results and approved the final version of the manuscript (A.H. deceased on Jan 2020).

6. Conflict of Interest Disclosure:

The authors declare no competing interest.

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Figures

Figure 1

S-glutathionylation of R2 subunit. 5 M pre-reduced R2 was treated with 50 M Di-E-GSSG and the release of E-GSH was monitored with an emission at 545 nm after excitation at 520 nm (black circles). Fluorescent background intensity from Di-E-GSSG was used as control (white circles). The relative changes in fluorescence emission with time are compared. Results are representative of three independent experiments.

Figure 2

Deglutathionylation of R2-SGE. Pre-reduced R2 subunit was incubated with EGSSGE and after the incubation period, the R2-SGE was purified by several cycles of centrifugation using 10 kDa cut-off filters. 5 M R2-SGE was treated either with 5 mM DTT (Δ) or with Grx1 system ($\bullet$) and the release of E-GSH was monitored (excitation at 520 nm, emission at 545 nm). Fluorescent background intensity from 5 M R2-SGE (untreated) was used as control (white circles). The relative changes in fluorescence emission with time are compared. The Grx1 system contained 1 M Grx1, 1 mM NADPH, 5 mM GSH, and 0.1 M GR. Results are representative of three independent experiments.

Figure 3

S-glutathionylation of p53R2 RNR protein. 5 M (□) and 10 M (Δ) pre-reduced p53R2 was treated with 50 M Di-E-GSSG and the release of E-GSH was monitored with an emission at 545 nm after excitation at 520 nm (black circles). Fluorescent background intensity from Di-E-GSSG was used as control (○). The relative changes in fluorescence emission with time are compared. Results are representative of three independent experiments.

Figure 4

Summary of the experimental findings. In order to study glutathionylation, Di-E-GSSG was used to glutathionylate purified R2 and p53R2 proteins. Di-E-GSSG has quenched fluorescence in its disulfide form however, upon reduction of the disulfide bond and formation of E-GSH. The EGSH was washed out by several cycles of centrifugation using MW cut-off filters. The S-glutathionylated R2 subunit was further used to check the Grx-catalyzed deglutathionylation reaction in which Grx1 could efficiently deglutathionylate R2-SGE to R2-SH.