Structurally Dependent Redox Property of Ribonucleotide Reductase Subunit p53R2

Lijun Xue,1 Bingsen Zhou,1 Xiyong Liu,1 Tieli Wang,2 Jennifer Shih,1 Christina Qi,1 Yvonne Heung,1 and Yun Yen1

1Department of Medical Oncology and Therapeutic Research, City of Hope National Medical Center, Duarte and 2Department of Chemistry, California State University, Carson, California

Abstract

p53R2 is a newly identified small subunit of ribonucleotide reductase (RR) and plays a key role in supplying precursors for DNA repair in a p53-dependent manner. Currently, we are studying the redox property, structure, and function of p53R2. In cell-free systems, p53R2 did not oxidize a reactive oxygen species (ROS) indicator carboxy-H2DCFDA, but another class I RR small subunit, hRRM2, did. Further studies showed that purified recombinant p53R2 protein has catalase activity, which breaks down H2O2. Overexpression of p53R2 reduced intracellular ROS and protected the mitochondrial membrane potential against oxidative stress, whereas overexpression of hRRM2 did not and resulted in a collapse of mitochondrial membrane potential. In a site-directed mutagenesis study, antioxidant activity was abrogated in p53R2 mutants Y331F, Y285F, Y49F, and Y241H, but not Y164F or Y164C. The fluorescence intensity in mutants oxidizing carboxy-H2DCFDA, in order from highest to lowest, was Y331F > Y285F > Y49F > Y241H > wild-type p53R2. This indicates that Y331, Y285, Y49, and Y241 in p53R2 are critical residues involved in scavenging ROS. Of interest, the ability to oxidize carboxy-H2DCFDA indicated by fluorescence intensity was negatively correlated with RR activity from wild-type p53R2, mutants Y331F, Y285F, and Y49F. Our findings suggest that p53R2 may play a key role in defending oxidative stress by scavenging ROS, and this antioxidant property is also important for its fundamental enzymatic activity. (Cancer Res 2006; 66(4): 1900-5)

Introduction

It is well known that the basic structure and function of ribonucleotide reductase (RR) is closely linked to its redox state. In Escherichia coli, active B2 (RR small subunit) harbors the iron center and the radical in oxidized states (1). However, reduction and oxidation studies in E. coli and mouse showed the existence of a stable, fully reduced form of the B2 protein (2, 3). The B2 protein shows dynamic carboxylate, radical, and water shifts in different redox forms (4). Similarly, cytochrome c oxidase, which has redox active tyrosine in the binuclear center, participates in reducing oxygen in respiration (5, 6), suggesting that proteins which possess tyrosyl radicals with a binuclear center may be reducing or oxidizing reagents.

Materials and Methods

Cell culture and transfections. Human oropharyngeal carcinoma KB cells and their derivative clones were cultured on tissue culture plates in RPMI 1640, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a 5% CO2 atmosphere at 37°C. KB cells were transfected with pcDNA3.1 vector (KB-vector), p53R2 sense (KB-p53R2S) or antisense (KB-p53R2AS), hRRM2 sense (KB-M2S) or antisense (KB-M2AS) cDNA as described (10). Exponentially growing cells were treated with indicated doses of H2O2 and incubated for 24 hours.

Site-directed mutagenesis. A site-specific mutation in p53R2 and hRRM2 was made by PCR, using pET28a-p53R2 and pET28a-hRRM2 as templates, respectively (11). The PCR products were digested with restriction enzyme Dpol, using QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Protein expression and purification. Recombinant hRRM2, p53R2 or mutant p53R2, hRRM2 proteins were expressed and purified as described (11). Protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA), and purity was determined by densitometric scanning of the Coomassie-stained SDS-PAGE gels.

Fluorescence assays of carboxy-H2DCFDA oxidation. Carboxy-H2DCFDA (Molecular Probes, Eugene, OR) is an indicator for reactive oxygen species (ROS) that do not fluoresce until hydrolyzed by esterases and oxidation occurs within the reaction system. For functional assays in a cell-free system, esterases inherent in mitochondrial extracts or commercial esterases (esterase from porcine liver, Sigma, St. Louis, MO) were added. All incubations contained 10 μmol/L carboxy-H2DCFDA and 240 μg/mL of mitochondrial lysate or 75 μg/mL of esterase enzyme, and with wild-type p53R2, hRRM2, or mutant p53R2, hRRM2 proteins at 37°C for 30 minutes. For in vitro studies, 100 μL of 107 cells/mL KB-vector and KB-p53R2S cells were plated in a 96-well cell culture plate and incubated at 37°C for 24 hours. Following incubation, cells were treated with H2O2 for another 24 hours. Cells were washed with HBSS without phenol red. Carboxy-H2DCFDA (10 μmol/L) was added and cells were further incubated for 30 minutes. The fluorescence of the oxidized form of carboxy-H2DCFDA was...
measured using a JMax microplate reader (Molecular Devices, Sunnyvale, CA) with a fluorescence excitation of 485 nm and emission at 538 nm.

**Electron paramagnetic resonance spectra.** X-band electron paramagnetic resonance (EPR) spectra of purified hRRM2 and p53R2 proteins were measured as described previously (11).

**Determination of catalase activity.** Catalase activities were detected using the Amplex reagent-based assay kit (Molecular Probes) according to manufacturer protocols. We prepared catalase standard curve samples and recombinant purified p53R2 protein sample in 1× reaction buffer (0.1 mol/L Tris-HCl pH 7.5; 25 μL of the above samples were delivered to separate wells of a 96-well microplate. The reaction was initiated in 20 μmol/L H2O2 in 1× reaction buffer and incubated for 30 minutes. The final reaction contained 50 μmol/L Amplex reagent and 0.2 units/mL horseradish peroxidase and was incubated at 37°C. After 30 minutes, absorbance was measured in a microplate reader at 550 nm. Change in absorbance is reported as the observed absorbance intensity subtracted from a negative control. The absorbance of p53R2 was measured, and catalase-specific activity was calculated using the catalase standard curve.

**Inner mitochondrial membrane potential analysis.** JC-1 dye (Molecular Probes) was dissolved in DMSO to produce a 1 mg/mL stock solution in our study. Cells were loaded with 1 μg/mL JC-1 for 30 minutes at 37°C, trypsinized, resuspended in medium at a density of ~10^6/mL, and transferred on ice to a flow cytometer (MoFlo MLS, DakoCytomation, Inc., Carpinteria, CA). JC-1 was excited at 488 nm and the monomer signal (green) was analyzed at 525 nm. Simultaneously, the aggregate signal (red) was analyzed at 590 nm. The distribution of red and green fluorescence from JC-1 was displayed in two-color contour plots. The ratios between red and green signals were measured.

**Results and Discussion**

Recombinant purified p53R2 protein lacked the ability to oxidize carboxy-H2DCFDA. The recombinant human p53R2 and hRRM2 proteins were purified and immediately used to evaluate their ROS-generating activity in a cell-free system containing mitochondrial extract. Mitochondrial extract was used to supply esterase; without esterase, carboxy-H2DCFDA is not sensitive to oxidants (9). Therefore, the fluorescence intensity (FI) of oxidized carboxy-H2DCF of both p53R2 and hRRM2 without mitochondrial extract was considered background (Fig. 1A). After mitochondrial extract was added, FI of p53R2 was unchanged at 4 μg/mL to 40 μg/mL concentrations whereas the FI of hRRM2 significantly increased. Furthermore, FI of p53R2 was much lower than hRRM2, at 6% and 20% of hRRM2 at 8 and 40 μg/mL, respectively (Fig. 1A). These results suggest that hRRM2 is able to generate ROS in the presence of mitochondrial extract (designated as assay 1), but p53R2 is not. Similarly, when mitochondrial extract was replaced with pure esterase (designated as assay 2), FI of p53R2 was consistently close to background and lower than hRRM2 (Fig. 1B), suggesting that the inability of p53R2 to oxidize carboxy-H2DCF was inherent to p53R2. However, larger differences in FI were observed between hRRM2 and p53R2 in assay 1 than in assay 2, suggesting the mitochondrial environment may influence redox properties of p53R2 and hRRM2. This may be because mitochondria contain oxidants and antioxidants, whereas the tyrosyl radicals in B2 proteins are known to react with a large number of radical scavengers, antioxidants, and oxidants (1). Because p53R2 and hRRM2 have an identical tyrosyl radical, as shown in the EPR spectra (Fig. 1C), the difference in their ability to oxidize carboxy-H2DCFDA cannot be attributed to the tyrosyl radical itself.

**p53R2 scavenged H2O2.** The inability of p53R2 to oxidize carboxy-H2DCFDA indicates that p53R2 might be an antioxidant. To explore this hypothesis, experiments were designed to determine the catalase activity of purified p53R2 in breaking down H2O2. The specific catalase activity of p53R2 was derived from the standard curve of catalase activity and increased in a dose-dependent manner (Fig. 2A). These results suggest that p53R2 can directly break down H2O2.

To further evaluate the efficacy of p53R2 as a defense against H2O2-induced oxidative stress, intracellular ROS were compared between KB-p53R2S (p53R2 overexpressed) and KB-vector (vector transfected) clones. p53R2 protein levels in these clones are described below. Both clones were treated with H2O2 for 24 hours.

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**Figure 1.** Recombinant purified p53R2 lacked the ability to oxidize carboxy-H2DCFDA. Carboxy-H2DCFDA (10 μmol/L) was incubated at 37°C with mitochondrial lysate of KB cells (A) or esterases purchased (B) for 30 minutes in the presence of purified proteins hRRM2 or p53R2. Control incubations contained hRRM2 or p53R2 but no mitochondrial lysate (A) or no commercial esterase enzyme (B). Carboxy-DCF generation was monitored fluorometrically. Points, mean of three independent experiments; each carried out in triplicate. MIT, mitochondrial extract; ES, esterase enzyme purchased. C, EPR spectra of the tyrosyl radical in human p53R2 and hRRM2 proteins.
To exclude the discrepancy of cell number from each sample, the oxidative stress. Dp53R2 enhances the antioxidant capacity for scavenging H2O2. In the KB-wt cells, treatment with 250 μmol/L H2O2 resulted in a shift of ratios toward lower values compared with untreated cells (Fig. 3C, KB-wt). A threshold ratio of 100 clearly divides the cells into two populations, one representing the untreated cells with a ratio >100 and the other representing cells with injured mitochondria with a ratio <100. On the basis of this clear distinction, we designated a threshold ratio of 100 to distinguish between cells with intact (high ratio, designated as R2 cells) and injured mitochondria (low ratio, designated as R1 cells). Treatment with 250 μmol/L of H2O2 resulted in a cell shift of R2 to R1 compared with the respective untreated clones, except KB-p53R2S clones, which shifted from R1 to R2 (Fig. 3C). To clearly show the effect of p53R2 and hRRM2 on Δψmt induced by H2O2, the mean fluorescence intensity (MFI) ratio of the red/green ratio was plotted as a function of H2O2 concentration (Fig. 3D). KB-p53R2S and KB-M2AS cells were more resistant to H2O2 attack than KB-wt cells, shown by MFI increase, whereas KB-p53R2AS and KB-M2S cells were more sensitive to H2O2 attack than KB-wt cells with MFI decrease. These results clearly show that p53R2 protected the mitochondrial membrane against oxidative stress. This result is consistent with a study using p53R2 knockout mouse embryonic fibroblasts (MEF) in which MEFs containing p53R2 (p53R2-wt) had higher viability in response to H2O2 attack than mutant MEFs (p53R2-ko; ref. 14), although the difference of viability between p53R2-wt and p53R2-ko cells was much higher than the difference of their DNA repair ability. Our current results may explain this variance, because p53R2 may protect cells against the loss of Δψ from H2O2 stress by decreasing ROS. This idea is supported by our results of catalase activity displayed by recombinant p53R2 protein (Fig. 2A) and intracellular ROS was reduced in p53R2-overexpressing cells compared with control cells in response to H2O2 (Fig. 2B). These findings are in agreement with another study demonstrating the catalase activity of B2 protein from E. coli (15).

Y331, Y285, Y49, and Y241 are critical residues for p53R2 in a lack of oxidative function and maintaining enzymatic activity. To clarify whether there are structural requirements for the inability of p53R2 oxidation, we determined FI of site-directed mutants of p53R2 and hRRM2. Primers for p53R2 and hRRM2 mutants are shown in Fig. 4A. The proteins used in this study were expressed and purified to 90% purity (Fig. 4B). As shown in Fig. 4C, when Y49, Y285, and Y331 residues in p53R2 were individually replaced with phenylalanine, a nonredoxable amino acid, FI was enhanced five to eight times that of p53R2-wt at a concentration of 32 μg/mL, in order of highest to lowest: Y331F > Y285F > Y49F > p53R2-wt. The FI induced by mutants Y285F and Y49F was similar to the level of hRRM2-wt. Of interest, the FI of Y331F was about double that of hRRM2-wt within the investigated protein concentration range. However, replacement of Y164 on p53R2 by phenylalanine (Y164F) or cysteine (Y164C) did not affect FI, suggesting that Y49 and Y285, and especially, Y331 on p53R2 are critical residues for the lack of oxidative function of p53R2. Unexpectedly, when Y87, Y323, and Y369 in hRRM2 (Y87, Y323, and Y369 residues in hRRM2 are analogous to Y49, Y285, and Y331 in p53R2, respectively) were also replaced with phenylalanine, the FI is unchanged compared with hRRM2-wt (data not shown), indicating that Y49, Y285, and Y331 are critical, but not exclusive residues for the inability of p53R2 oxidation. Further site-directed mutagenesis showed that when Y241 in p53R2 was replaced with histidine, FI was enhanced four times that of p53R2-wt at a concentration of 32 mg/mL. Correspondingly, when H279, which

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**Figure 2.** p53R2 scavenged H2O2. A, p53R2 displayed catalase activity. Catalase activity was detected using the Amplex Red reagent-based assay. Initially, each reaction contained the catalase enzyme (for standard curve) or p53R2 protein and 20 μmol/L H2O2 in 1× reaction buffer and was incubated for 30 minutes. The final reaction contained 50 μmol/L Amplex Red reagent and 0.2 units/mL of horseradish peroxidase and was incubated at 37 °C. After 30 minutes, absorbance was measured in a microplate reader at 550 nm. Change in absorbance is reported as the observed absorbance intensity subtracted from that of a no-catalase control. The absorbance of p53R2 was then changed to catalase-specific activity according to the catalase standard curve. Catalase activity, where 1 unit is defined as the amount of enzyme that will decompose 1.0 μmol of H2O2 per minute (pH 7.0) at 25 °C. B, p53R2 reduced intracellular ROS induced by H2O2. KB-vector and KB-p53R2S cells were treated with indicated concentrations of H2O2 for 24 hours before staining with 10 μmol/L carboxy-H2DCFDA. Carboxy-DCF generation (FI) was monitored fluorometrically. Points, mean of three independent experiments, each carried out in quadruplicate; bars, ± SE.

Overall, the FI of KB-p53R2S cells was 2-fold lower than KB-vector clones in response to H2O2 attack (Fig. 2B), further indicating that p53R2 enhances the antioxidant capacity for scavenging H2O2.

**p53R2 protected the mitochondrial membrane against oxidative stress.** Because mitochondria are particularly susceptible to oxidative damage, leading to mitochondrial membrane potential (Δψmt) decrease (12), we investigated the influence of both p53R2 and hRRM2 on Δψmt in response to H2O2 in KB-wt, KB-p53R2S, KB-M2S, KB-p53R2AS, and KB-M2AS cells with JC-1. Expression of p53R2 increased 3-fold with KB-p53R2S compared with the KB-wt or KB-vector, whereas expression of p53R2 decreased with KB-p53R2AS compared with the control (Fig. 3A). Expression of p53R2 was then changed to catalase-specific activity according to the catalase standard curve. Catalase activity, where 1 unit is defined as the amount of enzyme that will decompose 1.0 μmol of H2O2 per minute (pH 7.0) at 25 °C. B, p53R2 reduced intracellular ROS induced by H2O2. KB-vector and KB-p53R2S cells were treated with indicated concentrations of H2O2 for 24 hours before staining with 10 μmol/L carboxy-H2DCFDA. Carboxy-DCF generation (FI) was monitored fluorometrically. Points, mean of three independent experiments, each carried out in quadruplicate; bars, ± SE.

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is aligned with Y241 in p53R2, in hRRM2 was replaced with tyrosine, FI was decreased three times that of hRRM2-wt at a concentration of 32 mg/mL. However, FI from F183Y of p53R2 and Y221F of hRRM2 (F183 in p53R2 is aligned with Y221 in hRRM2) are unchanged compared with their wild-type protein, respectively (data not shown). These data suggest that a geometric structure composed of Y49, Y285, Y331, and Y241 in p53R2 is responsible for the inability of p53R2 oxidation. Y241 is the most important among these four tyrosine residues, whereas H279 in hRRM2 is a key residue responsible for the oxidation by hRRM2.

RR activity was assayed using the proteins above. Five micrograms of protein were used to determine FI and RR activity of p53R2-wt and mutants Y331F, Y285F, and Y49F. FI was negatively correlated with RR activity (Fig. 4D), indicating that a lack of oxidative function may be important for maintaining the enzymatic activity of p53R2.

In investigations of the crystal structure of B2 protein from E. coli and mouse, the tyrosyl radical was buried in a hydrophobic pocket, away from the B1 protein active site (4). Therefore, the RR enzymatic reaction may involve a coupled electron/proton transfer pathway from the tyrosyl radical in B2 to cysteine in B1. The COOH-terminal conserved tyrosine residues (356 in the E. coli B2 protein, and 370 in the mouse B2 protein) may be the connecting link between B1 and B2 (16–18). In human p53R2, this conserved residue corresponds to Y331. Substituting the p53R2 protein Y331 with phenylalanine dramatically increased FI (Fig. 4C), however, as in the B2 protein from E. coli and mouse, RR enzymatic activity was abolished (RRac, Fig. 4D). These results indicate that Y331 in p53R2 is an important residue that bridges p53R2 and B1 protein in the electron/proton transfer pathway. Abolishment of RR activity and increased FI occurred when Y331 was mutated to F331 in p53R2 (Fig. 4D), suggesting that efficient electron transfer through Y331 required the reduction potential of Y331. This is in agreement with the idea that the rate of radical transfer through position Y356 in E. coli is affected mainly by the reduction potential of the residue at that position (19). The Y285 residue is close to Y331. Y258 and Y49 residues in p53R2 have not yet been reported to involve electron/proton transfer, but according to the computer model, Y331, Y285, and Y49 residues localize on the surface of p53R2. RR activity was not abolished but decreased significantly after the Y49 or Y285 residues were replaced by phenylalanine (RRac, Fig. 4D), indicating that Y49 and Y285 residues may be partially involved in this transfer pathway.

ROS are potentially dangerous by-products of cellular metabolism that have direct effects on cell growth and development and...
cell survival, and have a significant role in the pathogenesis of cancer. To intercept toxic ROS and prevent the damage they may trigger, a goal for cancer prevention may be genetic mutation (20). The antioxidant ability of p53R2 implies that p53R2 plays an important role in preventing cancer because, not only can p53R2 supply deoxyribonucleotides to the DNA repair system (9), but it can also scavenge ROS (this report) to maintain genomic integrity in response to oxidative stress.

Figure 4. Y331, Y285, Y49, and Y241 are critical residues for p53R2’s lack of ability to oxidize carboxy-H2DCFDA. A, the primer sequences used in site-directed mutagenesis for p53R2 and hRRM2 mutants. B, in vitro expression and purification of wild-type p53R2, wild-type hRRM2, and mutant p53R2, hRRM2. 10% SDS-PAGE analysis of the purified recombinant proteins by one-step Ni-resin affinity chromatography. C, mutations of Y331F, Y285F, Y49F, and Y241H rather than Y164F or Y164C of p53R2 resulted in p53R2 obtaining the ability to oxidize carboxy-H2DCFDA. Carboxy-H2DCFDA (10 μmol/L) was incubated at 37°C with mitochondrial lysate of KB cells for 30 minutes in the presence of purified proteins wild-type p53R2, wild-type hRRM2, and mutant p53R2, hRRM2. Carboxy-DCF generation was monitored fluorometrically as in Fig. 1A. Points, mean of three independent experiments, each carried out in triplicate. D, the negative relationship of FI and RR activity produced by wild-type p53R2 and its mutants Y331F, Y285F, and Y49F. The FI was measured as in (C). The RR activity of the protein was measured as relative RR activity of dC / (C + dC)%, formation of percentage of dCDP at 37°C (see Materials and Methods). Data was normalized against the wild-type proteins, which are considered to give 100% conversion of CDP to dCDP. Each value is the average of two determinations with deviations <0.3. The enzyme activity was measured in the presence of 5 μg of B1 protein and 5 μg of p53R2 wild-type or mutant proteins with a total sample volume of 100 μL. Correlation coefficient between FI and RR activity is −0.91.

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References


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