Mammalian p53R2 Protein Forms an Active Ribonucleotide Reductase in Vitro with the R1 Protein, Which Is Expressed Both in Resting Cells in Response to DNA Damage and in Proliferating Cells*

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Recently, a homologue of the small subunit of mammalian ribonucleotide reductase (RNR) was discovered, called p53R2. Unlike the well characterized S phase-specific RNR R2 protein, the new form was induced in response to DNA damage by the p53 protein. Because the R2 protein is specifically degraded in late mitosis and absent in G0/G1 cells, the induction of the p53R2 protein may explain how resting cells can obtain deoxyribonucleotides for DNA repair. However, no direct demonstration of RNR activity of the p53R2 protein was presented and furthermore, no corresponding RNR large subunit was identified. In this study we show that recombinant, highly purified human and mouse p53R2 proteins contain an iron-tyrosyl free radical center, and both proteins form an active RNR complex with the human and mouse R1 proteins. UV irradiation of serum-starved, G0/G1-enriched mouse fibroblasts, stably transformed with an R1 promoter-luciferase reporter gene construct, caused a 3-fold increase in luciferase activity 24 h after irradiation, paralleled by an increase in the levels of R1 protein. Taken together, our data indicate that the R1 protein can function as the normal partner of the p33R2 protein and that an R1-p53R2 complex can supply resting cells with deoxyribonucleotides for DNA repair.

Ribonucleotide reductase (RNR) catalyzes de novo synthesis of deoxyribonucleotides from the corresponding ribonucleotides. This is the rate-limiting step in DNA precursor synthesis, and it is regulated at many different levels in the mammalian cell: (i) by allosteric control of the activity and specificity of ribonucleotide reductase by nucleoside triphosphate effectors (2), (ii) by the S phase-dependent transcription of the ribonucleotide reductase genes (3), and (iii) by the rapid, proteasome-dependent proteolysis of the small subunit in late mitosis (4).

Errors in the allosteric control lead to unbalanced dNTP pools, misincorporation of deoxyribonucleotides in DNA, genetic abnormalities or cell death (5). The mammalian ribonucleotide reductase consists of a 1:1 complex of two homodimeric non-identical subunits called proteins R1 and R2. The 90-kDa R1 protein contains the catalytic site, the binding sites for the allosteric effectors, and redox active disulfides that participate in the reduction of the substrates, while the 45-kDa R2 protein contains an iron center-generated tyrosyl free radical essential for catalysis (6). Transcription of both the R1 and R2 genes is S-phase specific but only the R2 protein shows S-phase-specific expression (3, 7). The levels of the R1 protein are in excess and almost constant during the cell cycle in proliferating cells because of a long half-life of the protein (>24 h) (8, 9). Therefore, the cell cycle-dependent activity of ribonucleotide reductase is controlled by the synthesis and degradation of the R2 protein. This synthesis starts in early S phase, and then the R2 protein slowly accumulates in the cell up to late mitosis when it is rapidly degraded (4). DNA damage or replication blocks in S-phase cells lead to cell cycle arrest and stabilization of the R2 protein, which is effective until cells pass into mitosis. This mechanism ensures an adequate supply of dNTPs for replication and repair in S phase and during G2. The specific degradation of the R2 protein in late mitosis, which inactivates ribonucleotide reduction in G1 may contribute to preventing reduplication of DNA before the next S phase. However, DNA damage and replication blocks neither increase nor prolong the R2 promoter activity in S phase or in logarithmically growing cells (4).

How then would proliferating cells in G1, which lack the R2 protein and consequently an active ribonucleotide reductase, obtain deoxyribonucleotides for DNA repair? Even more importantly, how would nonproliferating cells with no detectable R1 or R2 proteins and no ribonucleotide reductase activity obtain dNTPs for repair? This question recently received an elegant answer by the discovery of a new, p53-induced R2 protein called p53R2 (10). Similar results were later published from another laboratory (11). The p53R2 gene is located on chromosome 8 while the R2 gene is located on chromosome 2 in humans (6, 10). The finding of the DNA damage-inducible p53R2 gene demonstrates the direct involvement of the p53 protein in DNA repair. Cells that cannot make the p53R2 protein were reported to be more sensitive to killing by DNA-damaging agents. However, the assay used to demonstrate ribonucleotide reductase activity of the p53R2 protein was indirect, and its validity has been questioned (10, 12, 13). Fur-
Moreover, an R2 protein by itself would be completely inactive and up to now, no new DNA damage inducible form of the R1 protein has been reported. In this study, we express and purify recombinant human and mouse p53R2 proteins and demonstrate by EPR spectroscopy that they both can form a tyrosyl free radical very similar to the tyrosyl radical in the R2 proteins. In vitro ribonucleotide reductase assays with highly purified proteins, the human and mouse p53R2 proteins form fully active ribonucleotide reductase complexes with the human and mouse R1 proteins. Finally, UV irradiation of serum-synchronized Go/G1 mouse fibroblast cells results in activation of the R1 promoter and expression of the R1 protein, indicating that this protein can function as a partner of the p53R2 protein in DNA repair in G1 cells.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The coding sequence of the human p53R2 protein was amplified by polymerase chain reaction from a pET21a plasmid containing the human p53R2 coding sequence (10) cloned in-frame with an N-terminal T7 tag and a C-terminal histidine tag using the 5′/H11032 oligonucleotide primer CCGATCCCATATGGGCGACCCGGAAAGGCCG and the 3′/H11032 oligonucleotide primer GTGGTGGTGGATCCCTTA AAAATCTGCATCCAAGGTGAA. After cleaving the PCR product with the restriction endonucleases NdeI and BamHI, the fragment was inserted into a pET3a vector (Novagen) opened with the same enzymes. The coding sequence of the human R2 protein was amplified by PCR from a Okayama-Berg cDNA clone isolated from a human fibroblast cDNA library (14) using mouse R2 cDNA as a probe. The 5′ oligonucleotide primer was CTGCGCCGCCCATATGCTCTCCCTCCGTGTC and the 3′ oligonucleotide primer was GCCAAGTAAGGGATCCTCTTCAGTTTATTTAGAAG. The cloning of the PCR fragment into a pET3a vector was made as described for the human p53R2 cDNA. Finally, most of the coding sequence of the human R1 protein was isolated by cleaving a human R1 cDNA clone in a Bluescript SK+/H11001 vector (15) with the restriction endonucleases NsiI and XbaI. The fragment was then cloned into a pET3a plasmid containing a linker oligonucleotide with restriction endonuclease sites for NsiI and XbaI and in addition the first 5 nucleotides of the human R1 coding sequence. All constructs were verified by dideoxyribonucleotide sequencing.

**Expression and Purification of Recombinant Proteins**—Recombinant mouse R1 and R2 proteins were prepared from BL21(DE3)plysS bac-
Mammalian p53R2 Proteins and Ribonucleotide Reductase

**Table 1**

| Small subunit | Assay No. | Total protein | Formation of dCDP/30 min at 37°C | Specific activity |
|---------------|-----------|---------------|----------------------------------|-------------------|
| Mouse R2 protein | 1 | 0.5 µg | 2.1 nmoles | 139 nmoles/mg/min |
| Mouse R2 protein | 2 | 1 µg | 1.2 nmoles | 140 nmoles/mg/min |
| Mouse p53R2 protein | 3 | 0.7 µg | 1.2 nmoles | 59 nmoles/mg/min |
| Mouse p53R2 protein | 4 | 1.3 µg | 2.4 nmoles | 60 nmoles/mg/min |
| Human p53R2 protein | 5 | 2.6 µg | 4.1 nmoles | 52 nmoles/mg/min |
| Human R2 protein | 6 | 0.8 µg | 3.8 nmoles | 158 nmoles/mg/min |
| Mouse p53R2 protein | 7 | 0.8 µg | 2.3 nmoles | 95 nmoles/mg/min |
| Mouse R2 protein | 8 | 1.1 µg | 2.1 nmoles | 63 nmoles/mg/min |

*Assays No. 1–5 were made in the presence of 18 µg of mouse R1 protein and assays No. 6–8 were made in the presence of 20 µg of human R1 protein. No activity was observed with R1 protein alone, p53R2 protein alone, or R2 protein alone.

teria containing the plasmids pETM1 or pETM2 and purified to homogeneity according to Refs. 16 and 17. Expression and purification of the human R1 and R2 proteins were made in essentially the same way as the mouse proteins except that the human R1 protein was expressed at 30°C instead of 15°C. For the expression of p53R2 proteins, an overnight culture of BL21CodonPlus(DE3)-RIL or RP bacteria (Stratagene) transformed with the human or mouse pET p53R2 plasmids was diluted 200 times in 4.8 liters of Luria-Bertani medium containing 100 µg/ml of carbenicillin and 34 µg/ml of chloramphenicol. The bacteria were grown at 37°C until they reached an A600 of 0.6 and then induced by 400 µM isopropyl-1-thio-β-D-galactopyranoside and grown for an additional 2.5 h at 37°C before harvesting. The cells were disrupted by bead beating with 0.1-mm glass beads (Biospec Products) or by using an X-press (AB BIOD, Gothenburg). Precipitation of nucleic acids with streptomycin, and ammonium sulfate fractionation were performed as described for the purification of mouse R2 protein (17). The human p53R2 protein was eluted with 10 mM potassium phosphate, pH 7.0, 200 mM KCl in the final DEAE-cellulose chromatography. Instead of the DEAE-cellulose column, the mouse p53R2 protein was chromatographed on a 6-ml Resource Q column (Amersham Pharmacia Biotech) in 25 mM Tris-Cl, pH 7.6 using a 120-ml linear gradient of 150–270 mM KCl. The mouse p53R2 protein eluted around 180 mM KCl.

**Electron Paramagnetic Resonance Spectroscopy**—EPR spectra were measured on a Bruker ESP 300 spectrometer as described earlier (18).

Ribonucleotide Reductase Assay—The ability of the different R2 and p53R2 proteins to catalyze the reduction of [3H]CDP in the presence of the R1 protein was determined as described earlier (19).

**Cell Cultures**—Balb/3T3 cells (ATCC CCL 163) were grown as monolayer cultures in Dulbecco’s modified Eagle medium (Sigma) supplemented with 10% heat-inactivated horse serum (Life Technologies, Inc.). Serum synchronization and UV irradiation of cells were performed as described earlier (20). Flow cytometry was carried out on a CCA-1 flow cytometer, according to the instructions of the manufacturer (Partec, GMBH). The construction of the pGL3R1 5.7 and 287 mouse R1 promoter-luciferase reporter constructs, the 1.5 kilobase pair mouse R2 promoter-luciferase reporter construct, transfection by electroporation, and selection of stably transformed clones were described earlier (4, 21).

Luciferase assays were performed on cell lysates obtained by incubating cells with passive lysis buffer (Promega) and luciferase activity was measured in a TD-20/20 Luminometer (Turner designs) as described (4).

**Immunoblotting**—For immunoblotting, cells were lysed, and equal amounts of protein from the lysates were electrophoresed on 8% SDS-polyacrylamide gels, blotted to membranes, and analyzed (4) using the anti-R1 protein monoclonal antibody AD203 (22). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, detection was done using the ECL-plus system (Amersham Pharmacia Biotech). As an independent control for equal loading of the gel lanes, a monoclonal anti-β-actin antibody (Sigma) was used.

**RESULTS**

Comparison of the Amino Acid Sequence between Human and Mouse R2 and p53R2 Proteins—The nucleotide sequence of the human and mouse p53R2 genes differs enough from the corresponding R2 genes to explain why they were not detected in Southern blot analyses or by in situ hybridization using R2 cDNA probes. In contrast, the amino acid sequence homology between the p53R2 and R2 proteins is very high (Fig. 1). The mouse p53R2 protein shows 81% identity to the mouse R2 protein and 82% identity to the human R2 protein while the human p53R2 protein shows 90% identity to the mouse p53R2 protein. The major difference between the p53R2 proteins and the R2 proteins is that the former lacks 33 amino acid residues in the N terminus. All the iron ligands (23), the tyrosyl free radical (23), the amino acid residues involved in long range radical transfer (18), and the C-terminal heptapeptide, essential for binding to the R1 protein (18), are conserved (Fig. 1).

Expression of Recombinant Human R1, R2, and p53R2 Proteins and Mouse p53R2 Protein—Human R1 and R2 proteins were expressed in Escherichia coli using the T7 polymerase pET vector system and purified by dATP-Sepharose affinity chromatography and chromatography on DEAE-cellulose, respectively, as described earlier for the corresponding recombinant mouse proteins (16, 17). The purity of the final preparations was demonstrated by SDS-polyacrylamide gel electrophoresis (Fig. 2, lanes 1 and 2). Initial attempts to use the same pET vector system in E. coli BL21(DE3)pLysS bacteria for expression of the p53R2 proteins were unsuccessful, and no expression was detected. However, changing to BL21-CodonPlus(DE3) strains containing extra copies of rare E. coli tRNA genes including argU, resulted in good expression of the p53R2 proteins. The purity of the final preparations of the human and mouse p53R2 proteins is shown in Fig. 2, lanes 3 and 4.

Characterization of the p53R2 Proteins—A minimal requirement of an active R2 protein is that it can form an iron-tyrosyl...
free radical center. The presence of a tyrosyl free radical is best studied by EPR spectroscopy, and therefore we analyzed our p53R2 proteins by this technique. Fig. 3 shows the EPR spectra at 30 K of the mouse R2 and p53R2 proteins and the human p53R2 protein. All three spectra show very similar hyperfine patterns indicating similar iron-center-tyrosyl free radical structures. Also the microwave saturation at 20 K is very similar (data not shown). Like the mouse R2 protein, the mouse p53R2 protein rapidly lost its tyrosyl free radical on incubation with hydroxyurea (T1/2 = 2 min at 20° and 4 min hydroxyurea, see Ref. 24).

The Human and Mouse p53R2 Proteins Form an Enzymatically Active Ribonucleotide Reductase Complex in Vitro Together with the R1 Protein—The specific activity of the mouse p53R2 protein was about 43% of the specific activity of the mouse R2 protein when both were assayed for ribonucleotide reductase activity in the presence of an excess of mouse R1 protein (Table I). Similarly, the specific activity of the human p53R2 protein was 60% of the specific activity of the human R2 protein when assayed in the presence of an excess of human R1 protein. We know from earlier experiments that the human and mouse R1 and R2 proteins give fully active ribonucleotide reductase complexes in all combinations, and in Table I we also show that the mouse p53R2 protein gives the same activity when assayed with the mouse R1 protein as with the human R1 protein.

Expression of the Mouse R1 Protein Is Induced in Resting Cells after UV Irradiation—In resting, nonproliferating cells neither of the R1 and R2 mRNA transcripts nor the corresponding proteins or ribonucleotide reductase activity can be detected. We now wanted to study if the R1 gene promoter and R1 protein expression can be induced in resting cells after DNA damage. Mouse fibroblast cells stably transformed with the pGL3R1 5.7 plasmid (21), containing 5.7 kilobase pairs of the mouse R1 promoter in front of the luciferase gene, were synchronized by serum starvation for 48 h, irradiated by UV light (10 J/m²), and harvested at the indicated time points. A, luciferase activity in UV-irradiated (black circles) and non-irradiated cells (open circles). B, flow cytometry profiles of Balb 3T3 cells at 0 and 48 h after UV irradiation. C, detection of the R1 protein and β-actin levels by immunoblotting in non-irradiated (left) and UV-irradiated cells (right).

DISCUSSION

Our results clearly demonstrate that the human and mouse p53R2 proteins can form active iron-tyrosyl free radical centers like the R2 proteins. The specific activities of the p53R2 proteins assayed in the presence of an excess R1 protein were almost as high as the specific activities of the R2 proteins. We believe that the small differences can be explained by a different purity of the preparations or lower content of iron-radical center in the p53R2 proteins as a result of the different expression systems.

By itself, the ability of the p53R2 proteins to form an active ribonucleotide reductase complex with the R1 protein does not prove that this is what happens in a resting cell after DNA damage. However, our demonstration that the mouse R1 promoter is activated in serum-synchronized G0/G1-enriched fibroblast cells after UV irradiation and that this activation results in increased R1 protein levels strongly indicates that the R1 protein is the physiological partner of the p53R2 protein. Interestingly, the rather slow R1 promoter activation and increase in R1 protein levels after UV irradiation correlate well to
the slow induction of the p53R2 transcript in DNA-damaged cells (10). As seen in Fig. 4C, the long half-life of the R1 protein makes it still detectable in cells after 48 h of serum starvation (8, 9) but the increase in the irradiated cells compared with the non-irradiated control cells after 18 h is quite clear.

The DNA damage-induced activation of the R1 promoter is in strong contrast to the R2 promoter, which is not affected by DNA damage or replication blocks. This observation has been repeatedly made both by us and in other laboratories (4, 10). Our earlier report that cells stably transformed with an older version of an R1/R2 promoter-luciferase reporter construct showed a very rapid increase in luciferase activity after UV irradiation most probably was an artifact caused by the p19luc reporter plasmid (20). Furthermore, in previous studies we were never able to record a parallel increase in promoter activity and protein levels as we now show in Fig. 4.

The levels of dNTP in a cell appear to be critical for DNA repair and replication. The DNA damage response has been best studied in yeast where it activates the MEC1 and RAD53 protein kinases pathway, which activates the transcription of the ribonucleotide reductase genes (25) and in parallel leads to the degradation of the Sml1 protein, a specific inhibitor of the yeast ribonucleotide reductase large subunit (26). The mammalian homologues of MEC1 and RAD53 are the ATM/ATR and CHK2 (27), which activate and stabilize p53, which in turn activates the p53R2 gene (10, 11). Interestingly, unlike the mammalian enzyme, the major control of yeast ribonucleotide reductase appears to focus on the large subunit. The RNR1 transcript fluctuates more than the RNR2 transcript during the cell cycle (28). In addition, DNA damage in yeast specifically induces a homologue of RNR1 called RNR3 (28). In mammalian cells, ribonucleotide reductase activity is controlled during the cell cycle by the synthesis and breakdown of the small subunit and DNA damage induces a new form of this subunit, the p53R2 protein. Once an active ribonucleotide reductase is formed in mammalian cells, the fine-tuning of the overall size of the dNTP pools is controlled at every moment by the feedback inhibition of the enzyme by the negative allosteric effector dATP. This regulation functions independently of the amounts of R1 and R2 proteins (4).

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