Ribonucleotide Reductase Small Subunit p53R2 Facilitates p21 Induction of G1 Arrest under UV Irradiation

Lijun Xue, Bingsen Zhou, Xiyong Liu, Yvonne Heung, Jennifer Chau, Emilie Chu, Shan Li, Chung Lin Jiang, Frank Un, and Yun Yen

Department of Clinical and Molecular Pharmacology, City of Hope National Medical Center, Duarte, California

Abstract

p53R2, which is one of the two known ribonucleotide reductase small subunits (the other being M2), is suggested to play an important role in supplying deoxyribonucleotide triphosphates (dNTP) for DNA repair during the G1 or G2 phase of the cell cycle. The ability of p53R2 to supply dNTPs for repairing DNA damages requires the presence of a functional p53 tumor suppressor. Here, we report in vivo physical interaction and colocalization of p53R2 and p21 before DNA damage. Mammalian two-hybrid assay further indicates that the amino acids 1 to 113 of p53R2 are critical for interacting with the NH2-terminal region (amino acids 1–93) of p21. The binding between p21 and p53R2 decreases inside the nucleus in response to UV, the time point of which corresponds to the increased binding of p21 with cyclin-dependent kinase-2 (Cdk2), and the decreased Cdk2 activity in the nucleus of G1. Interestingly, p53R2 dissociates from p21 but facilitates the accumulation of p21 in the nucleus in response to UV. On the other hand, the ribonucleotide reductase activity increases at the corresponding time in response to UV. These data suggest a new function of p53R2 of cooperating with p21 during DNA repair at G1 arrest. [Cancer Res 2007;67(1):16–21]

Introduction

Ribonucleotide reductase is responsible for the reduction of nucleoside diphosphate to their corresponding deoxyribonucleoside diphosphate and plays a key role in DNA synthesis (1). p53R2, which is one of ribonucleotide reductase small subunits, is suggested to play an important role for supplying deoxyribonucleotide triphosphate (dNTP) to DNA damage repair system in G1 or G2 phase of the cell cycle (2). The ability of p53R2 to repair DNA damage requires the presence of a functional p53 tumor suppressor. p53R2 acts as a transcriptional activator of p21 (2) and, by binding with p53R2, also helps in supplying dNTP for DNA repair (3). p21 is an integral and critical component of p53-mediated cellular response to DNA damage (4). Both p21 and p53R2 have been shown to undergo induction in cells after p53 activation (2), p21 is a well-characterized cyclin-dependent kinase inhibitor that belongs to the Cip/Kip family of Cdk inhibitors. It mainly binds to cyclin/Cdk complexes to inhibit the activity of Cdk2, and the physiologic function of p21 gene is believed to be broadly involved in connecting various cellular pathways to cell cycle control (5). These functions of p21 could be achieved through direct p21-to-protein interactions, and the subcellular localization of p21 plays an important part in dictating the binding partners to which p21 is exposed (6). In our current study, we found that p53R2 physically interacts with p21, which mediates the cooperation of DNA repair signals from the ribonucleotide reductase metabolic pathway to the G1 cell cycle arrest.

Materials and Methods

Cell culture. Human oropharyngeal carcinoma KB (p21 wild-type) cells were purchased from American Type Culture Collection (Manassas, VA). Mouse embryonic fibroblasts (MEFs; p21 knockout and wild type) were generous gifts of Dr. James Roberts (Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Center, Seattle, WA). MEFs (p53R2 knockout and wild type) were generous gifts of Dr. Hirofumi Arakawa (Cancer Medicine and Biophysics Division, National Cancer Center Research Institute, Tokyo, Japan). Cells 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.

Antibodies. All antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal immunoglobulin G (IgG)-2b against p21, goat polyclonal antibodies against p53R2, goat polyclonal antibody against Cdk2, and mouse monoclonal IgG1 against SP-1 were used for Western blotting. Agarose-conjugated p21 mouse monoclonal IgG2b and p53R2 goat polyclonal IgGs were used for immunoprecipitation. FITC-conjugated goat anti-mouse and rhodamine-conjugated bovine anti-goat IgGs were used for the immunofluorescence microscopy studies.

Nuclear extraction. KB cells were harvested at 0 and 2 h after UV, and nuclear extraction was done with the Nuclear Extraction Kit (Chemicon International, Inc., Temecula, CA) following the manufacture’s protocol.

Immunoprecipitation and Western blot. Immunoprecipitation and Western blot were done as previously described (3).

Immunofluorescence microscopy analysis. This assay was done as previously described (3).

Plasmid constructions. Full-length human p21 and p53R2 cDNAs or truncated p21 (1–279 and 259–495 bp cDNAs) and p53R2 (319–1,056, 631–1,056, and 811–1,056 bp cDNAs) cDNA fragments were amplified by PCR using PCR Master Mix (Promega, Madison, WI). Primer sequences used in PCR were listed in Supplementary data. All resulting PCR products were BamHI/NotI double digested and inserted into BamHI/NotI–opened pAct-VP16 or pBIND-Gal4 plasmids (Promega), resulting in expression constructs pBIND-Gal4/p53R2 and pBIND-Gal4/p53R2-truncated. pBIND-Gal4/p53R2 811–1,056 bp BIND-Gal4/p53R2 319–1,056 bp pACT-VP16/p21, and p21-truncated pACT-VP16/p21 1–279 bp pACT-VP16/p21 259–495 bp All constructs were verified by sequencing.

Mammalian two-hybrid assay. KB cells were grown in the presence of 10 μg/mL hygromycin B in DMEM supplemented with 5% FCS and transfected with p53R2 and p21 expression vectors. Here, p53R2 was transfected into p53R2-291 wild-type and 811–1,056 bp pBIND-Gal4/p53R2. Transfected cells were then cultivated in media containing hygromycin B (10 μg/mL). Twenty-four hours posttransfection, cells were stained with fluorescein isothiocyanate (FITC)-conjugated antibody against Cdk2, and goat polyclonal antibodies against p53R2. After fixation, cells were stained with DAPI in order to visualize cell nuclei. p53R2 and p21 localization were determined by confocal microscopy.

Requests for reprints: Yun Yen, Department of Clinical and Molecular Pharmacology, City of Hope National Medical Center, Duarte, CA 91010. Phone: 626-359-3301; Fax: 626-359-3303; E-mail: yyen@coh.org. ©2007 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-06-3200

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Inc. Carlsbad, CA) according to the manufacturer’s protocol. Lysates were normalized for retinoblastoma content by parallel measurement of total retinoblastoma using the Total Rb ELISA kit (BioSource International).

**Cell cycle analysis.** KB cells were harvested at the indicated hour after UV at 20 J/m² and fixed with 70% ethanol for 2 h on ice. Fixed cells were washed in PBS, stained with 20 μg/mL propidium iodide, 200 μg/mL RNase A, and 0.1% Triton X-100 for 30 min at 37°C, and analyzed by flow cytometry (City of Hope Core Facility).

**Ribonucleotide reductase activity assay.** KB cells were harvested at the indicated hour after UV at 20 J/m², and ribonucleotide reductase activity assay was done as described (3).

**Results and Discussion**

**Protein-to-protein interaction between p53R2 and p21.** To determine whether p21 interacts with p53R2 in *vivo*, an immunoprecipitation-Western blot assay was done. The Western blot analysis of the input (1.5% of the lysate used for immunoprecipitation assay) indicated the presence of p53R2 in both KB (p21 wild-type) and MEF (p21 null) cell lysates (Fig. 1A-a). The cell lysate of KB or MEF cells was incubated with either anti-p21 or anti-p53R2 antibody to selectively immunoprecipitate intracellular p21 or p53R2, respectively. The immunoprecipitates were then subjected to immunoblot analysis with anti-p53R2 antibody. As shown in Fig. 1A-b, p21 could bind p53R2 in p21-containing KB cells (Fig. 1A-b, lane 1). However, p21 could not bind p53R2 in p21-deficient MEF cells (Fig. 1A-b, lane 3), although the positive control experiment showed that anti-p53R2 antibody could immunoprecipitate p53R2 (Fig. 1A-b, lane 2). When the input was analyzed with anti-p21 antibody, p21 was clearly detected in KB cells (Fig. 1A-c, lanes 1 and 2), but not in p21-deficient MEF cells (Fig. 1A-c, lane 3). Immunoprecipitation-Western blot assay showed that p53R2 could bind p21 (Fig. 1A-d, lane 2). Control experiments showed that anti-p21 antibody can immunoprecipitate p21 selectively in p21-containing cells (Fig. 1A-d, lane 1), but not in p21-deficient cells (Fig. 1A-d, lane 3). These findings indicate that p21 may interact with p53R2 in *vivo*.

To obtain further evidence for the interaction, immunofluorescence microscopy was done to determine if p21 colocalizes with p53R2. KB cells were costained with anti-p21 and anti-p53R2 antibodies and the results are shown in Fig. 1B. p21 (green) and p53R2 (red) seem to homogeneously distribute throughout the cytoplasm. The merged images show the colocalization of p21 with p53R2 as indicated by the yellow color.

We next used a mammalian two-hybrid system to confirm the physical interaction between p21 and p53R2 in *vivo*. In this experiment, we constructed the plasmids pAct-VP16/p21 and pBind-Gal4/p53R2, respectively. p21 and p53R2 were fused to the Gal4-binding domain. Eighty-percent confluent KB cells in 60-cm plates were transfected with 10 μg of pAct-VP16/p21, 10 μg of pBind-Gal4/p53R2, and 10 μg of pG5Luc. One day posttransfection, the cells were lysed and analyzed for firefly and Renilla luciferase expression using the dual luciferase kit. All firefly luciferase values were normalized in relation to their Renilla luciferase values. Shown is the relative value of normalized firefly luciferase values. Typical result of three independent experiments. Each experiment was carried out in duplicate.

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**Figure 1.** p21 interacts with p53R2. A, p21 and p53R2 physically interact in *vivo*. KB (p21 wild-type) and MEF (p21 null) cell lysates (5 mg) were immunoprecipitated with either anti-p21 (lanes 1 and 3) or anti-p53R2 (lane 2) antibody. Top, input (a) and immunoprecipitate (IP; b). Input indicates 1.5% of the lysates used for immunoprecipitation assay. p53R2 in input and coimmunoprecipitated lysate was probed with anti-p53R2 antibody. Input shows p53R2 in KB and MEF cells (a, lanes 1–3). p53R2 from the cell lysate of KB cells, but not MEF cells, was detected in p21 immunoprecipitates (b, lane 1 and 3). p53R2 from the cell lysate of KB cells was also detected in p53R2 immunoprecipitates (b, lane 2). Bottom, input (c) and immunoprecipitate (d), p21 in input and coimmunoprecipitated lysate was probed with anti-p21 antibody. Input shows p21 in KB (c, lanes 1 and 2) but not MEF (c, lane 3) cell lysate. p21 from the cell lysate of KB cells was detected in p53R2 immunoprecipitates (d, lane 2). p21 from the cell lysates of KB cells, but not MEF cells, was detected in p21 immunoprecipitates (d, lanes 1 and 3). B, p21 and p53R2 colocalize in KB cells. KB cells were stained and examined by immunofluorescence microscopy. p21 was visualized with FITC-conjugated secondary antibody and p53R2 was visualized with rhodamine-conjugated secondary antibody. The colocalization of p21 (green) with p53R2 (red) appears as a yellow color in the merged images. C, p21 interacts with p53R2 in the mammalian two-hybrid system. Mammalian two-hybrid analysis of the VP16/p21 interaction with full-length p53R2 fused to the Gal4-binding domain. Eighty-percent confluent KB cells in 60-cm plates were transfected with 10 μg of pAct-VP16/p21, 10 μg of pBind-Gal4/p53R2, and 10 μg of pG5Luc. One day posttransfection, the cells were lysed and analyzed for firefly and Renilla luciferase expression using the dual luciferase kit. All firefly luciferase values were normalized in relation to their Renilla luciferase values. Shown is the relative value of normalized firefly luciferase values. Typical result of three independent experiments. Each experiment was carried out in duplicate.
pBind-Gal4/p53R2 that encode the full-length p21 and p53R2 proteins, respectively. KB cells were cotransfected with the plasmids expressing VP16-p21 fusion protein, Gal4-p53R2 fusion protein, and the Gal4-luciferase reporter. Cotransfection of pAct-VP16/p21 with pBind-Gal4/p53R2 (full-length) led to an 3.5-fold increase in luciferase activity relative to negative controls (Fig. 1C). The results are consistent with our immunoprecipitation data and suggest that p21 physically interacts with p53R2 in vivo.

The NH2 termini of p53R2 and p21 are required for the interaction between p21 and p53R2. To map the p21 binding site of p53R2 using the two-hybrid system as mentioned above, various terminal deletions were introduced to p53R2 of pBind-Gal4/p53R2 (Fig. 2A). As shown in Fig. 2B, the NH2-terminal truncations P2, P3, and P4 of p53R2 disrupted the interaction between p21 and p53R2. Consistent with the above, the NH2-terminal p53R2 segment composed of amino acids 1 to 113 exhibited a similar level of luciferase activity as the full-length p53R2 (Fig. 2B), suggesting that this region of p53R2 contains essential features for interacting with p21.

To map the p53R2 binding site of p21, pAct-VP16/p21 full-length (W1) and NH2 and COOH termini (W2 and W3, respectively) were constructed (Fig. 2C). In mammalian two-hybrid experiments using KB cells, the NH2-terminal deletion of W2 disrupted the interaction between p21 and p53R2 whereas the W3 mutant exhibited a similar luciferase activity as the full-length p21 (Fig. 2D), suggesting that W3 retains essential sequences for interacting with p53R2. W3 contains the Cdk-cyclin binding domain of p21 (7). The NH2-terminal residues 1 to 82 of p21 bind the Cdk-cyclin complex and inhibit Cdk2 activity in vitro (7). As W3 contains the NH2-terminal residues 1 to 93 of p21, it suggests that p53R2 may regulate p21 function with regard to Cdk2. This is in agreement with the notion that p53R2 is involved in G1 and G2 cell cycle arrests for the repair of damaged DNA (2). The more detailed p53R2 binding site on p21 or vice versa will be further clarified in our future study.

Throughout our experiments, we noted that the affinity between p21 and p53R2 seems to be much lower when compared with that for p21-Cdk2 or p53R2-hRRM1 interaction (data not shown), suggesting that the interaction between p21 and p53R2 might be disrupted under an environment wherein cell needs p21 binding with Cdk2 or p53R2 binding with hRRM1.

The p21-p53R2 complex dissociates in the nucleus in response to UV-induced DNA damage. To clarify the functional significance of p21 binding with p53R2, KB cells were UV irradiated at 20 J/m2. At 2 h after UV exposure, cytoplasmic and nuclear lysates were immunoprecipitated with anti-p21 antibody, electrophoresed, and immunoblotted with anti-p53R2 antibody. As shown in Fig. 3, the p21 remained associated with p53R2 in the cytoplasm after the UV treatment (Fig. 3A, top, lanes 1 and 2). However, p53R2 dissociated from p21 in the nucleus after the exposure to UV (Fig. 3A, top, lanes 3 and 4) despite no significant reduction in the

![Figure 2](https://cancerres.aacrjournals.org)
amount of nuclear p53R2 (Fig. 3B, p53R2, lanes 2 and 4). At the same time, an increase in the binding of nuclear p21 with unphosphorylated Cdk2 occurred (Fig. 3A, bottom, lanes 3 and 4) although the level of nuclear Cdk2 decreased (Fig. 3B, Cdk2, lanes 2 and 4). The efficient separation of cytoplasmic and nuclear functions is indicated by the Sp-1 data shown in Fig. 3B. The decrease in binding of p21 with p53R2 after the exposure to UV was confirmed via mammalian two-hybrid assay (Fig. 3C). A similar binding pattern was found in Adriamycin-treated KB cells via mammalian two-hybrid assay (data not shown), suggesting that this phenomenon might also apply to double-strand DNA break. However, the exact scope of this phenomenon needs further investigation in detail. Simultaneously, the nuclear (Fig. 3D, top) and total (Fig. 3D, bottom) Cdk2 activities declined at 2 h after UV treatment.

Nevertheless, the p21 nuclear accumulation in response to UV seems to necessitate p53R2. As shown in Fig. 4A, considerably less p21 accumulates in the nucleus in MEF p53R2−/− cells compared with MEF p53R2+/+ cells in response to UV at 20 J/m2 (Fig. 4A, top), suggesting that p53R2 may signal p21 to move into the nucleus or increase the nuclear p21 stability in response to UV. The details of the mechanism will be further clarified in our ongoing project. An amplified view of a representative cell at 2 h after UV is shown in Fig. 4A (bottom). After UV irradiation, both p21 (Fig. 4A, bottom, green color indicated by arrow) and p53R2 (Fig. 4A, bottom, red color indicated by arrow) accumulated at the nucleus. Yet, the majority of them were not colocalized inside the nucleus when compared with those at the periphery of the nuclear membrane inside the cytoplasm (Fig. 4A, bottom, yellow color indicated by arrow). The identical phenomenon was observed with KB cells (data not shown). This lack of colocalization is consistent with the dissociation of p53R2 from p21 in the nucleus in response to UV shown in Fig. 3A (top).

Flow cytometry based on DNA content showed that KB cells became arrested in G1 at 2 h after UV treatment (Fig. 4B). Correspondingly, the ribonucleotide reductase activity increased at 2 h (Fig. 4C) and 3 h (data not shown) after UV treatment. These data suggest that p21 may dissociate from p53R2 and accumulate in the nucleus to bind and inhibit Cdk2 to arrest cells at G1. Likewise, p53R2 enters the nucleus to form a holoenzyme by binding to the subunit hRRM1 to supply dNTP for DNA repair. The above is consistent with the report that p53R2 accumulates in the nucleus upon DNA damage and plays a crucial role in supplying dNTP for DNA repair in a p53-dependent manner (2, 3). Of novelty here is that p53R2 nuclear accumulation and its role in DNA repair are directly linked to p21, p21 mostly executes cell cycle arrest, whereas p53R2 represents ribonucleotide reductase metabolic pathway. Why are cell cycle arrest and nucleotide mobilization linked in response to UV? UV-induced DNA damage triggers complex cellular responses in eukaryotic cells, including induction of cell cycle arrest and initiation of DNA repair. When cell cycle arrest is accomplished, the DNA repair machinery can become effective (9–11). Exposure of mammalian cells to UV irradiation leads to the introduction of a number of photo-products in cellular DNA, which are removed by the nucleotide excision repair pathway (12). Damaged nucleotides are excised together with a number of adjacent nucleotides. The resulting gaps are filled in by DNA polymerase and sealed by ligase. To fill these gaps, cells need precursors for DNA synthesis. Ribonucleotide reductase is a unique enzyme that can supply these precursors (13, 14). Thus, there is a biological requirement for the coordination of nucleotide mobilization and cell cycle arrest after DNA damage. p21 and p53R2 are canonical transcriptional targets of p53; a currently found regulatory relationship between them potentially adds a new level of understanding that downstream

![Figure 3](https://example.com/figure3.png)

**Figure 3.** p21 dissociates from p53R2 to bind Cdk2 and reduce Cdk2 activity in the nucleus in response to UV. A, reduced binding of p21 with p53R2 in the nucleus at 2 h after UV. B, protein expression of p53R2 and Cdk2 in cytosol and nucleus at 2 h after UV. C, mammalian two-hybrid system assay of p21 with p53R2 binding. D, Cdk2 activity of KB cells in response to UV. Top, Cdk2 activity of cytosol and nucleus lysate after UV. Bottom, Cdk2 activity of total cell lysate after UV (20 J/m2). C, cytosol; N, nucleus. Sp-1 is indicative of purity of separation of cytoplasmic and nuclear lysate.
events of p53 are functionally linked. Before this report, it has been difficult to integrate the UV-induced cell cycle arrest by p21 in the context of the repair of UV-damaged DNA because there have been conflicting reports on whether p21 can inhibit the proliferating cell nuclear antigen function in nucleotide excision repair (15, 16). Our findings may potentially help to understand p21 involvement in DNA repair. Because p21 and p53R2 play crucial roles in p53-induced cell cycle arrest and p53-induced DNA repair, respectively, our results suggest that the interaction of p21 with p53R2 may represent one of the mechanisms on how cell cycle arrest and DNA repair cooperate in response to UV-induced DNA damage.

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References


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