Molecular Biology of the Cell
Vol. 10, 2119–2129, July 1999

The C-terminal Domain of p21 Inhibits Nucleotide Excision Repair In Vitro and In Vivo

Marcus P. Cooper, Adayabalam S. Balajee, and Vilhelm A. Bohr*

Laboratory of Molecular Genetics, National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224-6823

Submitted January 27, 1999; Accepted April 21, 1999
Monitoring Editor: Mark J. Solomon

The protein $p21^{Cip1,\ Waf1,\ Sdi1}$ is a potent inhibitor of cyclin-dependent kinases (CDKs). p21 can also block DNA replication through its interaction with the proliferating cell nuclear antigen (PCNA), which is an auxiliary factor for polymerase δ. PCNA is also implicated in the repair resynthesis step of nucleotide excision repair (NER). Previous studies have yielded contradictory results on whether p21 regulates NER through its interaction with PCNA. Resolution of this controversy is of interest because it would help understand how DNA repair and replication are regulated. Hence, we have investigated the effect of p21 on NER both in vitro and in vivo using purified fragments of p21 containing either the CDK-binding domain (N terminus) or the PCNA binding domain (C terminus) of the protein. In the in vitro studies, DNA repair synthesis was measured in extracts from normal human fibroblasts using plasmids damaged by UV irradiation. In the in vivo studies, we used intact and permeabilized cells. The results show that the C terminus of the p21 protein inhibits NER both in vitro and in vivo. These are the first in vivo studies in which this question has been examined, and we demonstrate that inhibition of NER by p21 is not merely an artificial in vitro effect. A 50% inhibition of in vitro NER occurred at a 50:1 molar ratio of p21 C-terminus fragment to PCNA monomer. p21 differentially regulates DNA repair and replication, with repair being much less sensitive to inhibition than replication. Our in vivo results suggest that the inhibition occurs at the resynthesis step of the repair process. It also appears that preassembly of PCNA at repair sites mitigates the inhibitory effect of p21. We further demonstrate that the inhibition of DNA repair is mediated via binding of p21 to PCNA. The N terminus of p21 had no effect on DNA repair, and the inhibition of DNA repair by the C terminus of p21 was relieved by the addition of purified PCNA protein.

INTRODUCTION

The protein $p21^{Cip1,\ Waf1,\ Sdi1}$ was initially identified as a component of quaternary complexes containing p21, proliferating cell nuclear antigen (PCNA), cyclin D, and a cyclin-dependent kinase (CDK) (Zhang *et al.*, 1993). p21 is a potent and universal inhibitor of cyclin-dependent kinases and causes G1 growth arrest (Harper *et al.*, 1993; Xiong *et al.*, 1993). It is up-regulated by p53 after DNA damage (Xiong *et al.*, 1993) and is overexpressed 10- to 20-fold in senescent and quiescent cells (Noda *et al.*, 1994). p21 was later shown to inhibit PCNA-dependent replication (Shivji *et al.*, 1994; Waga *et al.*, 1994; Chen *et al.*, 1995). Because PCNA is common to both replication (Prelich *et al.*, 1987; Prelich and Stillman, 1988; Tsurimoto *et al.*, 1990) and nucleotide excision repair (NER) (Shivji *et al.*, 1992), it has been speculated that p21 might play regulatory roles in several essential biological processes, including cell cycle progression, replication, and DNA repair.

PCNA is a 36-kDa protein that serves as a processivity factor for polymerase δ (reviewed in Kuriyan and O'Donnell, 1993) during replication (Prelich *et al.*, 1987; Prelich and Stillman, 1988; Tsurimoto *et al.*,

* Corresponding author. E-mail address: vbohr@nih.gov.
Abbreviations used: CDK, cyclin-dependent kinases; NER, nucleotide excision repair; PCNA, proliferating cell nuclear antigen.

1990). It is also essential for the resynthesis step of NER (Shivji *et al.*, 1992). The C terminus of p21 has been shown to bind PCNA (Chen *et al.*, 1995, 1996; Goubin and Ducommun, 1995; Warbrick *et al.*, 1995), and the binding domain is within amino acids 142–163 (Goubin and Ducommun, 1995; Warbrick *et al.*, 1995). The C terminus inhibits DNA replication (Chen *et al.*, 1995). Although p21/PCNA binding occurs in a 1:1 ratio, a 5- to 10-fold excess of p21 to PCNA is necessary to inhibit PCNA-dependent replication both in vivo and in vitro (Li *et al.*, 1996). p21 exerts it effect on PCNA-dependent replication by inhibiting the assembly of the trimeric PCNA clamp onto DNA. Additionally, p21 inhibits the loading of polymerase δ onto the preassembled PCNA clamp (Podust *et al.*, 1995). Interestingly, however, p21 does not affect translocation of the assembled clamp (Podust *et al.*, 1995). Thus, the regulatory role of p21 in replication through its interaction with PCNA has been firmly established. Its precise role in DNA repair, however, remains uncertain.

One in vitro DNA repair study showed no effect of p21 on repair of a UV-damaged plasmid even at 60- to 240-fold excess of p21 to PCNA monomer (Li *et al.*, 1994). p21 also had no effect on the filling of a 28-nucleotide gap by either polymerase δ or ε (Li *et al.*, 1994), suggesting that the PCNA-dependent short gap filling is independent of p21. Similar findings were reported by Shivji *et al.* (1994), who showed no effect of p21 on DNA repair of a UV-damaged plasmid in *Xenopus* egg extracts and fractionated human cell extracts at a p21 level as high as 0.8 μM. In contrast, Pan *et al.* (1995), using similar assays, demonstrated that full-length (renatured) p21 concentrations as low as 0.34 μM exhibited 50% repair inhibition and that concentrations approaching 1 μM showed nearly 100% inhibition of DNA repair. Interestingly, a mere threefold excess of p21 to PCNA inhibited nucleotide excision repair by 50%. This is surprising because the level of p21 required to inhibit PCNA-dependent replication is considerably higher, in the range of 5- to 10-fold excess of p21 to PCNA (Li *et al.*, 1996).

There have been no direct in vivo studies on the role of p21 in DNA repair. Indirectly, in vivo studies have suggested that increased p21 expression is associated with enhanced DNA repair by measuring survival. McDonald *et al.* (1996) showed that overexpression of p21 in p21 −/− HCT116 human colon cancer cells resulted in a twofold increase in reporter activity from an in vitro UV-damaged CMV-driven β-galactosidase plasmid. They also demonstrated that overexpression of p21 increased clonogenic survival after UV irradiation. Similar findings were noted using a reporter gene transfected into p53 null colorectal cells overexpressing p21 (Sheikh *et al.*, 1997). Both studies, however, did not quantitate the level of p21 overexpressed and did not directly measure DNA repair. Also, the cells used in these experiments were transformed and might respond differently compared with normal cells. Hence, the question of the role of p21 in vivo NER remains unclear.

There are two major domains of the p21 protein. The C-terminal domain has been shown to bind to PCNA (Chen *et al.*, 1995, 1996; Goubin and Ducommun, 1995; Luo *et al.*, 1995; Warbrick *et al.*, 1995), whereas the N-terminal end of the molecule binds to cdk kinase (Chen *et al.*, 1995; Goubin and Ducommun, 1995). We have investigated the effect of p21 on NER both in vitro and in vivo using these two separate domains of the p21 protein. Although the C terminus of p21 might play a regulatory role in NER via its interaction with PCNA, the role of the CDK-binding domain of p21 in DNA repair has not been examined previously. We purified the p21 C-terminal and p21 N-terminal fragments of the p21 protein and tested them in the DNA repair resynthesis assay. This approach offers the advantage of being able to assess each of the two functional domains of the p21 protein. In addition, these fragments of p21 are highly soluble, whereas the whole protein often aggregates, which might complicate biochemical assays. Other investigators have also noted that aggregation is a major problem in the purification of full-length p21 (Pan *et al.*, 1995).

The effect of p21 on DNA repair in vivo was studied in intact or permeabilized cells. The protein was added directly or by electroporation. Both methods have the advantage of introducing the proteins uniformly into the cells. In the permeabilized cell system, we specifically measured the resynthesis step of the DNA repair process as reported previously by Jackson *et al.* (1994). This assay measures repair incorporation as does the in vitro study. Using both this assay and an electroporation approach, we observed a substantial inhibitory effect of the C-terminal fragment of the p21 protein. These results thus suggest that the p21 protein plays a role in DNA repair.

MATERIALS AND METHODS

Cell Lines and Extracts

Human primary fibroblast and lymphoblast cell lines (Normal GM38A and Normal 1310B, respectively) were obtained from the Corriel Cell Repository (Camden, NJ). All of the fibroblast cells were maintained in 2× MEM supplemented with 15% FBS and antibiotics. All lymphoblast cells were maintained in RPM I 1640 medium supplemented with 10% FBS, 2 mM glutamine, and penicillin/streptomycin. Nuclear extracts were prepared according to the method of Dignam *et al.* (1983).

Preparation of p21 Protein Derivatives

The GST-tagged human p21 (the construct was kindly provided by Dr. Dutta, Brigham and Women's Hospital, Boston, MA) was overproduced in *Escherichia coli* (BL21-DE3 pLysS). Freshly transformed bacteria were grown for 8 h in Luria-Bertani medium containing 0.1 mg/ml ampicillin and 0.034 mg/ml chloramphenicol at 37°C. The medium was replaced with fresh medium and stored at 4°C over-

night. A 250-ml culture was inoculated with a 1:50 dilution of the 8-h culture. The culture was grown until A_{600} was 0.5. The culture was then induced for 2 h in the presence of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. The culture flasks were then stored on ice for 10 min, and the cells were pelleted. The pellet was frozen in liquid nitrogen and stored at −80°C overnight.

The bacterial pellet was lysed and resuspended in 75 ml of cold buffer containing 25 mM HEPES-NaOH, pH 8.0, 0.1% Triton X-100, 0.2 mg/ml lysozyme, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin for 10 min. The lysate was sonicated by two 10-s repetitive cycles on ice. The lysate, containing soluble p21 N terminus or p21 C terminus, was then clarified by centrifugation at 100,000 × *g* for 1 h at 4°C and filtered through a 0.45 μM filter. It was then loaded onto a 20-ml DEAE Sepharose Fast Flow column (Pharmacia, Piscataway, NJ). The protein was eluted with a 50- to 1000-mM KCl gradient in buffer containing 25 mM HEPES-NaOH, pH 7.3, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin. The fractions containing p21 were pooled and loaded onto a 2.5-ml glutathione sepharose 4B column (Pharmacia). The column was then washed with 25 ml buffer containing PBS, pH 7.4, containing 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin. A 50-ml wash with buffer containing 25 mM HEPES, pH 7.5, 50 mM KCl, 20 mM $MgCl_2$, 5 mM ATP, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin followed. The protein was eluted with 7.5 ml of buffer containing 25 mM HEPES, pH 8.0, 10 mM glutathione, and 2 mM EDTA.

The sample was concentrated to a final volume of 0.6 ml by membrane filtration (Microcon, Amicon, Beverly, MA). It was then dialyzed against 25 mM HEPES, pH 7.5, 2 mM EDTA, 0.15 M KCl, and 1 mM DTT for 1 h at 4°C. Dialysis was repeated once, and the protein was further concentrated in a Microcon 10 (Amicon) by centrifugation at 14,000 × *g* for 35 min at 4°C. The final volume was brought to 0.1 ml using dialysis buffer. The samples were frozen in liquid nitrogen and stored at −80°C. The typical yield of protein from a 250-ml culture was 2.5 mg.

Purification of Histidine-tagged PCNA

The gene encoding human PCNA (kindly provided by Dr. T. Kunkel, NIEHS, Chapel Hill, NC) was subcloned into pET 15b (Novagen, Madison, WI) and overexpressed in *E. coli* (BL21-DE3 pLys S). Freshly transformed bacteria were grown for 8 h in Luria-Bertani medium containing 0.1 mg/ml ampicillin and 0.034 mg/ml chloramphenicol at 37°C. The medium was replaced with fresh medium and stored at 4°C overnight. A 100-ml culture was inoculated with a 1:50 dilution of the 8-h culture. The culture was grown until A_{600} was 0.5. The culture was then induced 2 h in the presence of 0.4 mM isopropyl-1-thio-β-galactopyranoside. The culture flasks were stored on ice for 10 min, and the cells were pelleted. The pellet was frozen on liquid nitrogen and stored at −80°C overnight.

The pellet was resuspended in 4 ml ice cold buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9) from Novagen supplemented with protease inhibitors and lysozyme (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pestatin A, 10 μg/ml aprotinin, 0.2 mg/ml lysozyme). The sample was subjected to two 10-s cycles of sonication on ice. The lysate containing PCNA was clarified at 100,000 × *g* for 1 h at 4°C and filtered through a 0.45-μm filter. The lysate was loaded onto a 1-ml Ni^{2+} resin column (Novagen) charged per manufacturer's instructions. The sample was washed sequentially with 10 vol of resuspension buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and 6 vol of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The bound PCNA protein was then eluted with 1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, and dialyzed two times against 25 mM HEPES, pH 7.5, 0.15 M KCl for 1 h at 4°C. The purified PCNA was concentrated using Centriprep 10 and Microcon 10 (Amicon) micro concentrators. The protein was then dialyzed against 25 mM HEPES, pH 7.5, 0.15 M KCl, 2 mM EDTA, and 1 mM DTT.

Preparation of Substrates for In Vitro DNA Repair Studies

UV-damaged DNA substrate devoid of pyrimidine hydrates and apurinic sites was prepared as described by Wood *et al.* (1988). Briefly, pUC18 plasmid DNA was irradiated with 450 J/m^2 UV, treated with *E. coli* Nth protein to remove pyrimidine hydrates and AP sites, and purified once on a CsCl/ethidium bromide gradient followed by a final purification over a 5–25% sucrose gradient. Undamaged DNA substrate was prepared similarly using pUC18 plasmid DNA containing a 1.5-kbp insert.

In Vitro DNA Repair

The in vitro nucleotide excision repair assay was performed as described previously by Wood *et al.* (1988). Reaction mixtures (20 μl) contained 45 mM HEPES, pH 7.7, 70 mM KCl, 7 mM $MgCl_2$, 0.4 mM EDTA, 1 mM DTT, 2 mM ATP, 8 μM [α-^{32}P]dCTP (specific radioactivity of 3000 Ci/mmol), 20 μM remaining dNTPs, 40 mM phosphocreatine, 50 μg/ml creatine phosphokinase, 0.36 mg/ml BSA, 4.4% glycerol, 6 ng/μl each of the UV-damaged and control plasmids, and 1.07 mg/ml GM1310B nuclear extract. The reaction was incubated for 1 h for 30°C. After incubation the reaction was treated with 0.18 mg/ml proteinase K and 0.06 mg/ml RNaseA in 0.68% SDS and 12 mM EDTA for 30 min at 37°C. After proteinase K digestion, the proteins were removed by phenol/chloroform extraction, ethanol-precipitated, and linearized by *Eco*RI digestion by incubation at 37°C for l h. The plasmids were resolved by electrophoresis on 0.75% agarose and visualized by ethidium bromide staining. After drying under vacuum, the gel was exposed to a PhosphorImager screen for 3 h. Quantification was performed using Image Quant Software on a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

DNA Repair in Permeabilized Cells: Encapsulation of Cells

The cells were prelabeled with ^{3}H-thymidine (0.1 μCi/ml) to uniformly label the DNA. The procedure for encapsulation was essentially the same as described by Jackson and Cook (1988). Briefly, 2.5% agarose (Sigma Type VII, Sigma, St. Louis, MO) in PBS was melted and cooled to 39°C. Five milliliters of cells (2–4 × 10^6 cells/ml) in complete medium were mixed with 1.25 ml of molten agarose in a conical flask at 39°C. After the addition of 15 ml liquid paraffin oil (Fisher Scientific, Houston, TX) to the cells in molten agarose, the mixture was vortexed for 30 s and kept on ice with constant swirling of the flask for 2 min. Fifteen milliliters of complete medium were added to the flask, and the contents were transferred to a 50-ml centrifuge tube. The agarose beads were pelleted (500 × *g*, 5 min). After the removal of paraffin and excess aqueous phase, the agarose beads were thoroughly washed in PBS.

Permeabilization of Encapsulated Cells

The beads were irradiated with UV (20 J/m^2) in PBS before permeabilization with lysolecithin. The beads containing the cells were permeabilized with lysolecithin (60 μg/ml) in a modified physiological buffer for 15 min on ice. This buffer contained 10 mM Na_2HPO_4, 2.5 mM $MgCl_2$, 65 mM KCl, 65 mM $KC_2H_3O_2$, 1 mM Na_2ATP, 1 mM DTT, and 0.2 mM PMSF. After permeabilization, the cells were washed in PBS, and the repair reaction was performed at 37° using 250 μM dATP, dGTP, dTTP, and 40 μCi/ml ^{32}P-α-dCTP.

Electroporation

Electroporation of proteins into cells was performed according to method of Winegar and Lutze (1990). Briefly, ^{14}C-labeled GM38A fibroblast cells were grown to confluency, trypsinized, and harvested. The cells were washed in PBS devoid of magnesium and calcium. Approximately 3 × 10^5 cells were transferred to a cuvette

prechilled on ice in a total volume of 800 μl. GST, p21 N (amino acids 1–90), and p21 C (amino acids 87–164) were added to final concentration of 0.2 μM. The cells were then electroporated at 250 V and 960 F. The cells were immediately chilled on ice, seeded onto culture dishes containing fresh media, and allowed to reattach for 2–4 h. After reattachment, the cells were washed and irradiated with 254 nm UV radiation (30J/m^2). The cells were incubated with medium containing 10 μCi/ml ^{3}H-thymidine for 2 h. The cells were lysed in 0.5% SDS containing proteinase K, precipitated with 5% TCA, spotted on filters, and washed with ethanol and acetone. The samples were then scintillation-counted.

RESULTS

Purification of Proteins

The use of full-length GST-p21 protein was avoided because it invariably formed aggregates during the purification. Precipitation of the GST-p21 full-length protein routinely occurred when the eluted product was subjected to high-speed centrifugation. This problem was circumvented by using highly soluble p21 fragments, which were previously shown to be fully functional by Chen *et al.* (1995). Both the C- and N-terminal domains of the p21 protein were overexpressed as GST-fusion proteins and affinity-purified using glutathione 4B Sepharose column chromatography as soluble monomers to >95% purity. All experiments were conducted with p21 protein fragments designated hereafter as p21 N (amino acids 1–90) and p21 C (amino acids 87–164). PCNA was overexpressed as a histidine tagged-fusion protein. It was purified as soluble monomers to >95% purity using nickel column chromatography (our unpublished results)

In Vitro DNA Repair

In vitro NER was measured using the repair resynthesis assay initially described by Wood *et al.* (1988). Repair of a UV-damaged plasmid devoid of pyrimidine hydrates and apurinic sites was measured in an in vitro assay using nuclear extracts prepared from normal human fibroblast cells (GM1310B). A control plasmid of different molecular weight was used to assess DNA repair specificity. UV damage induces both cyclobutane pyrimidine dimers and 6–4 photoproducts in DNA that are repaired by NER. To optimize conditions for high repair specificity, we conducted experiments at different extract concentrations and incubation times (Figure 1). Our results indicated that the repair incorporation was linear at a concentration of 1.07 mg/ml nuclear extract and an incubation time of 1 h. We also tested the repair incorporation in XPA cell extracts and found no incorporation (our unpublished results). This suggests that the repair incorporation that we measure is specific to NER of UV-induced photoproducts.

Effect of p21 N and p21 C on In Vitro DNA Repair

Increasing concentrations of p21 N and p21 C were used in the in vitro repair assays. The samples were normalized to a GST protein control devoid of purified proteins, and the percentage inhibition was calculated (Figure 2A). The use of purified GST protein alone showed no difference compared with the control without protein (our unpublished results). Hence, GST protein was omitted in subsequent experiments as a control. Our results demonstrate that p21 N, the CDK-binding domain of p21, had no effect on NER even at concentrations as high as 3.6 μM (Figure 2). p21 C, however, had a marked inhibitory effect on NER. Concentrations as low as 0.2 μM exhibit 20% repair inhibition. Concentrations >0.5 μM resulted in >50% inhibition and those near or >1 μM inhibited NER by ~80% (Figure 2B). These data demonstrate that the PCNA binding domain of p21 specifically inhibits in vitro NER. Similar results were also obtained with HeLa cell extracts and with two other human cell extracts.

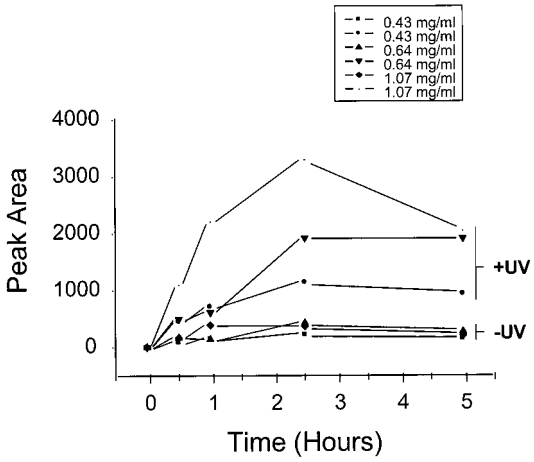

Figure 1. Optimization of in vitro repair assay. Reaction mixtures (20 μl) containing control and damaged DNA substrates were incubated with increasing amounts of nuclear extract prepared from normal human fibroblast cells (GM1310B). The reactions were incubated at 30°C for 1 h. The samples were then subjected to RNAse A and proteinase K digestion. After extraction with organic solvents and ethanol precipitation, the plasmid DNA samples were linearized with *Eco*RI and resolved on a 0.75% agarose gel. The agarose gel was dried under heated vacuum, and the bands containing radioactively labeled DNA were quantified on a PhosphorImager (Molecular Dynamics).

PCNA Competitively Reverses the Inhibitory Effect of p21 C on NER

If p21 C inhibits NER by binding to PCNA, its inhibitory effect should be reversed by the addition of PCNA. Such an assessment would first require the precise determination of the amount of PCNA present in the extract. Using Western blot analysis, we determined that the concentration of native PCNA present in the extract used for NER was 10 nM (Figure 3). By

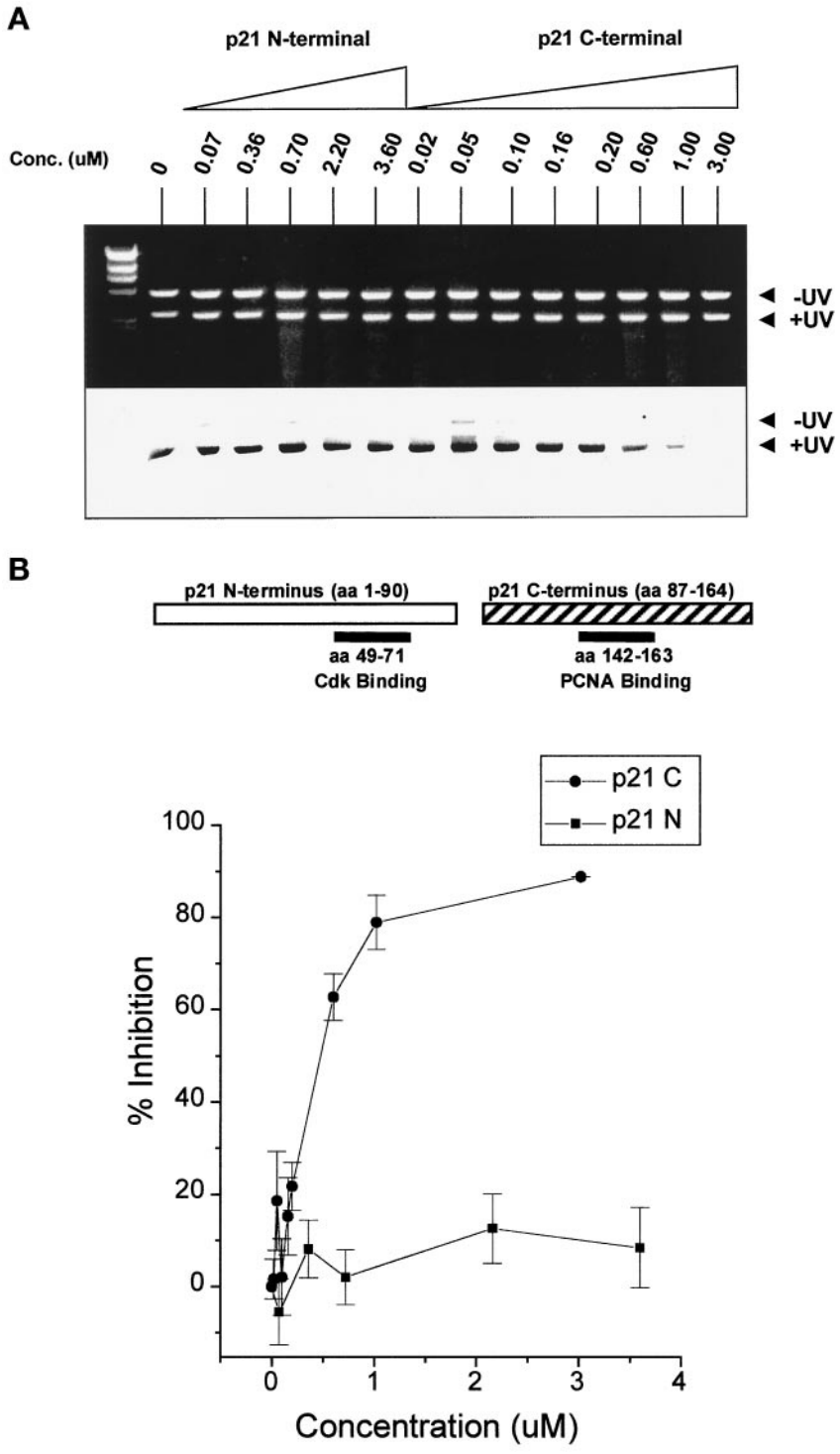

Figure 2. The effect of p21 N and p21 C on in vitro NER. (A) Ethidium bromide gel (top panel) and phosphorimage of dried ethidium bromide gel (bottom panel). Increasing levels of p21 N and p21 C were preincubated with GM1310B nuclear extract at 30°C for 10 min. Reaction buffers and UV-damaged and undamaged DNA substrates were added. The reactions were incubated at 30°C for 1 h. The samples were then subjected to RNAse A and proteinase K digestion. After extraction of organic solvents and ethanol precipitation, the plasmid DNA samples were linearized with *Eco*RI and resolved on a 0.75% agarose gel. The agarose gel was dried under heated vacuum, and the bands containing radioactively labeled DNA were quantified on a PhosphorImager (Molecular Dynamics). (B) Plot of the samples shown in A (n = 2 or greater). Error bars represent SEM. Inset shows a map with locations of the C and N fragments of p21.

titrating with increasing amounts of p21 C we could then determine the ratio of p21:PCNA under conditions of repair inhibition. At 50% inhibition of the repair reaction the p21 concentration was 500 nM (Figure 2). Thus, under these conditions the p21:PCNA ratio is 50:1. Increasing amounts of PCNA were then added to the repair reactions containing 2 μM p21 C (Figure 4, A and B). The results demonstrated that at near equimolar amounts of PCNA to p21 (0.5 molecule PCNA:1 molecule p21 C) the inhibitory effect of p21 C

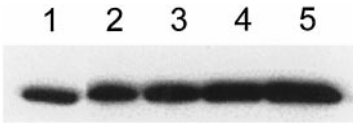

Figure 3. Determination of the amount of PCNA in the nuclear extract (GM1310B) used for the in vitro repair assays. Nuclear extract (75 μg) (lane 1) and different amounts of histidine-tagged PCNA protein (lane 2, 25 μg; lane 3, 50 μg; lane 4, 100 μg; lane 5, 200 μg) were electrophoresed on 4–12% SDS-PAGE and transferred to PVDF membrane per manufacturer instructions. PCNA was immunologically detected using an anti-human mouse monoclonal antibody to PCNA. The values for the standards were linear.

was reversed. This suggests that p21 C is effecting DNA repair inhibition by binding to PCNA.

Synchronization of Cells for In Vivo Studies

To determine whether p21 C inhibited NER in vivo, protein electroporation studies and a permeabilized cell system were used. Both systems allow measurement of efficient UV-induced NER activity in human fibroblast cells. UV-induced NER synthesis is difficult to measure in exponentially growing cells because of DNA replication of S-phase cells. To measure repair synthesis efficiently, cells were synchronized at G1 by growing them to a confluent state. In addition, the cells were treated with 10 mM hydroxyurea to block semiconservative replication. Flow cytometric analysis of the synchronized cells is shown in Figure 5. The results demonstrate that well over 95% of the cells are in G1 or G2 phase. There was no difference at 48 h between control and UV-treated cells. These findings were confirmed by autoradiography of cells grown on coverslips in the presence of ^{14}C-thymidine (our unpublished results).

Effect of p21 N and p21 C on In Vivo Repair

The effect of p21 N and p21 C on DNA repair under in vivo conditions was studied. Electroporation served as a simple and rapid technique to introduce and distribute protein uniformly within cells, including their nuclei. This is necessary, because the p21 N protein fragment lacked a nuclear localization sequence. After electroporation the cells were allowed to reattach and then were UV-irradiated. After UV-treatment the cells were incubated with tritiated thymidine to monitor DNA repair as measured by unscheduled DNA synthesis. GST protein was used as a control. The results show no effect of p21 N on unscheduled DNA synthesis relative to GST (Figure 6). p21 C, however, shows an inhibition of 28%.

We then used a permeabilized cell system as described by Jackson *et al.* (1994). Once the cells are permeabilized they no longer undergo initiation or

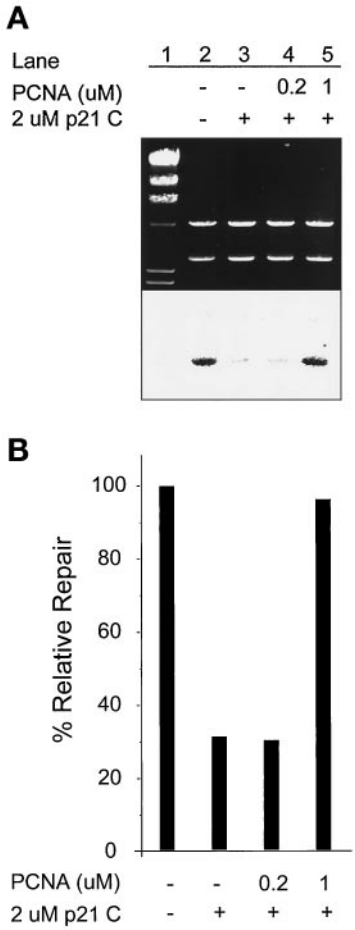

Figure 4. The effect of PCNA on p21 C inhibition of NER. (A) Ethidium bromide gel (top) and phosphorimage of dried ethidium bromide gel (bottom). Increasing amounts of PCNA (lane 1, molecular weight marker; lane 2, control; lane 3, 0 μM PCNA; lane 4, 0.2 μM PCNA; lane 5, 1 μM PCNA) were incubated with nuclear extract containing p21 C at 2 μM for 10 min at 30°C. Reaction buffers, UV-damaged plasmid DNA, and undamaged plasmid DNA were then added. The reactions were incubated at 30°C for 1 h. The samples were then subjected to RNAse A and proteinase K digestion. After extraction with organic solvents and ethanol precipitation, the plasmid DNA samples were linearized with *Eco*RI and resolved on a 0.75% agarose gel. The agarose gel was dried under heated vacuum, and the bands containing radioactively labeled DNA were quantified on a PhosphorImager (Molecular Dynamics). (B) Histogram of the results shown in A.

incision. Hence, the assay specifically measures the resynthesis and ligation steps of NER (Jackson *et al.*, 1994; Balajee *et al.*, 1998). Normal human fibroblast cells were encapsulated in agarose beads and permeabilized with lysolecithin. It was shown previously by Dent *et al.* (1989) that lysolecithin permeabilization permits the entry of high molecular weight proteins into the nucleus. The permeabilized cells were incubated with reaction buffer containing P^{32}-labeled dCTP. Unscheduled DNA synthesis was

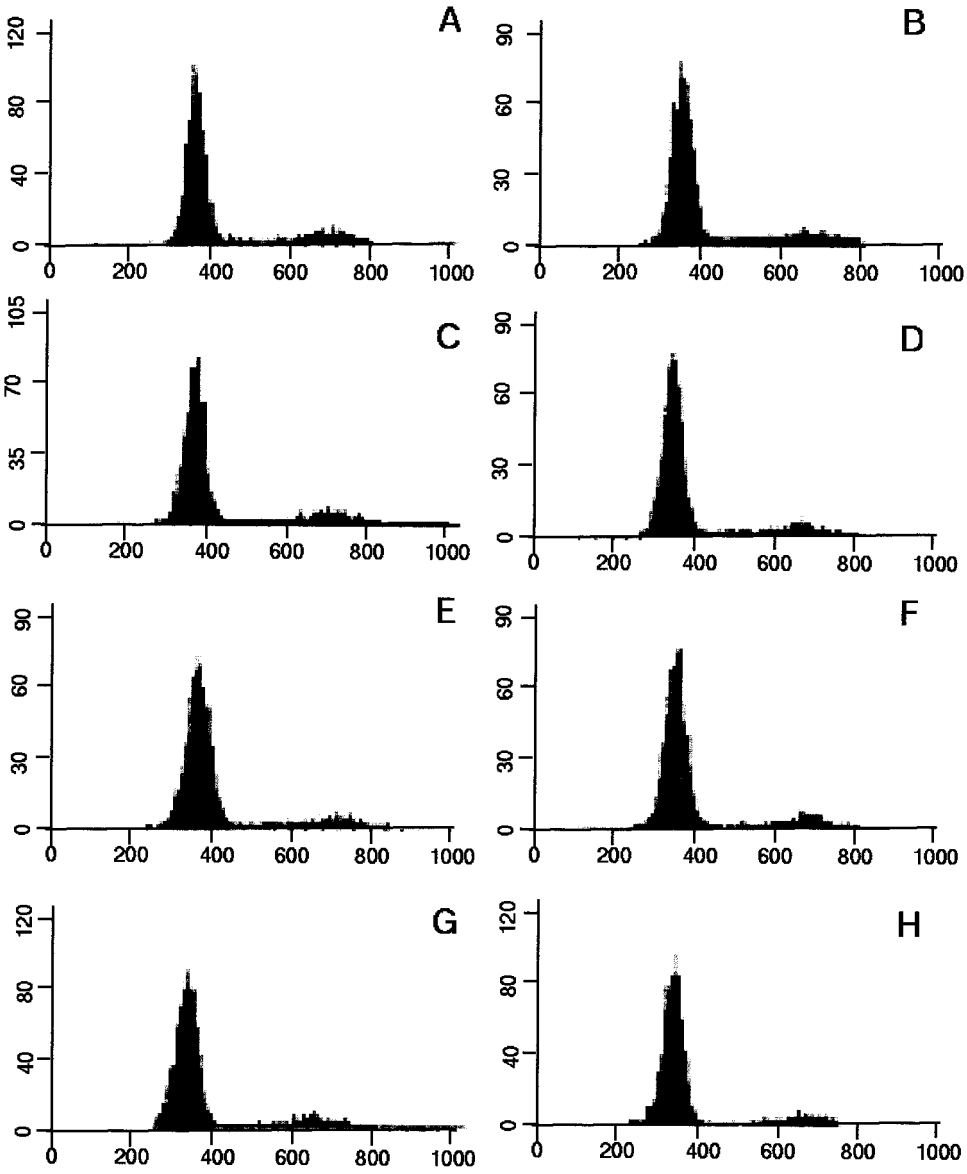

Figure 5. Synchronization of cells for in vivo repair assays. Normal human fibroblasts (GM 38A) were grown to confluency in media containing 10 mM hydroxyurea and incubated for 0 h (A, +UV; B, −UV), 8 h (C, +UV; D, −UV), 24 h (E, +UV; F, −UV), and 48 h (G, +UV; H, −UV) at 37°C in 5% CO_2 incubators. The cells were then harvested and treated with 70% ethanol and 100 μg/ml RNAse A. The DNA content of the cells was measured by flow cytometry.

measured by the incorporation of radioactive dCTP. Again, the results showed that p21 N had no effect on repair, suggesting that the CDK-binding domain of p21 plays no role in NER (Table 1). p21 C, however, exhibited an inhibition of 25% at 1.5 μM and 52% at 3.7 μM (Table 1). Similar experiments investigating replication alone showed an inhibition of 33% at 0.02 μM p21 C and of 57% at 0.2 μM p21 C. As expected, its effect on replication was much stronger than that on repair. We thus find that only the PCNA binding domain of p21 inhibited NER in vivo. Moreover, our findings suggest that the PCNA binding domain of p21 blocks the resynthesis step of NER in vivo.

DISCUSSION

A number of studies have demonstrated a regulatory role of p21 in replication and in the cell cycle; however, its role in DNA repair has been controversial. This study, using purified C- and N-terminus p21 protein, demonstrates that the C terminus, which contains the PCNA binding domain of p21, inhibits in vitro NER. Inhibition occurs at a high p21 to PCNA ratio. This conclusion differs significantly from previous studies that reported either no effect of p21 on NER even at high concentrations or that p21 inhibited NER at concentrations similar to those required for inhibition of replication. In contrast, this study con-

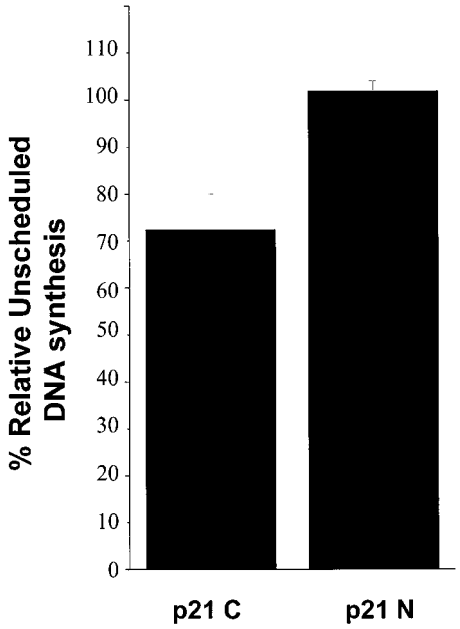

Figure 6. The effect of p21 N and C on in vivo DNA repair using electroporation. Histogram showing the role of p21 N and p21 C on DNA repair in normal human fibroblasts GM38A. ^{14}C-labeled cells were grown to confluence, trypsinized, and harvested. The cells were washed in PBS devoid of magnesium and calcium. Approximately 3×10^5 cells were transferred to a cuvette prechilled on ice in a total volume of 800 μl. GST, p21 N, and p21 C were added to a final concentration of 0.2 μM. The cells were then electroporated at 250 V and 960 F. The cells were immediately chilled on ice, seeded onto culture dishes containing fresh media, and allowed to reattach for 2–4 h. After reattachment, the cells were washed and irradiated with 254 nm UV radiation (30 J/m^2). The cells were incubated with medium containing 10 μCi/ml ^{3}H-thymidine for 2 h. The cells were lysed in 0.5% SDS containing proteinase K, precipitated with 5% TCA, spotted on filters, and washed with ethanol and acetone. The samples were then counted in a scintillation counter (n = 2). Error bars represent SEM.

Table 1. The effect of p21 N and p21 C on in vivo DNA repair using permeabilized cells

Treatment	% Incorporation
Control (0 J/m^2)	14.6 ± 12.4
UV (20 J/m^2)	100
UV + p21 N	
0.037 μM	97.7 ± 5.3
1.5 μM	101 ± 10.3
3.7 μM	103.8 ± 7.48
UV + p21 C	
0.037 μM	92.4 ± 9.29
1.5 μM	75.1 ± 12.8
3.7 μM	52.0 ± 14.8

Normal human fibroblasts (GM38A) were encapsulated in 2.5% (Sigma type III) agarose at 39°C (see Materials and Methods). The encapsulated cells were irradiated with 254 nm UV radiation (20 J/m^2). The cells were permeabilized in physiological buffer containing lysolecithin. p21 N and p21 C were added to the permeabilized encapsulated cells, and the reaction was incubated at 37°C with ATP and dNTPs. The samples were washed in ice-cold PBS and then lysed in 0.1% SDS. After lysis the samples were precipitated on filters with 5% TCA, washed, dried, and scintillation counted. Excluding p21 N at a concentration of 0.037 μM, all of the results are the mean of three independent biological experiments. Values are mean ± SD.

cludes that p21 differentially regulates repair and replication. NER is much less sensitive to inhibition by p21 than is replication. Our study provides a more physiologically relevant model for understanding the protective role of p21 because we show the effect of p21 both in vivo and in vitro. Thus, the inhibition of DNA repair by p21 is not merely an in vitro phenomenon. We show that p21 inhibits the resynthesis step of NER in cells. It appears that the inhibitory effect of p21 is significantly mitigated by preassembly of PCNA at repair sites before p21 addition. The failure of the N terminus to inhibit NER strongly supports our hypothesis that the inhibition of NER by p21 is mediated primarily through the C-terminal PCNA binding domain of p21. It also indirectly suggests that cdk-cyclins probably have no significant regulatory role in NER.

There have been several studies on the effect of p21 on DNA repair. Most of these have shown no inhibitory effect of p21 (Li *et al.*, 1994; Shivji *et al.*, 1994; McDonald *et al.*, 1996), whereas one found inhibition (Pan *et al.*, 1995). Although our data in general terms agree with those of Pan *et al.* (1995), their stoichiometry differed significantly from ours with respect to the ratio of p21 to PCNA required for inhibition of NER. We find that a minimal ratio of 50:1 p21 to PCNA monomer is necessary for 50% inhibition of in vitro NER and a ratio of 100:1 is required for 80% inhibition. This is in contrast to Pan *et al.* (1995) who reported that a ratio as low as 3:1 full-length p21 to PCNA monomer was necessary for 50% inhibition of in vitro DNA repair. This discrepancy is relevant, because the ratio of p21 to PCNA monomer required for inhibition of PCNA-dependent replication using both in vitro and in vivo studies has been shown to be 5–10:1 p21 to PCNA monomer (Li *et al.*, 1996). On the basis of the p21:PCNA ratio reported by Pan *et al.* (1995), it would be inferred that a cellular increase in p21 would inhibit DNA repair more efficiently than DNA replication. Our p21:PCNA ratio suggests that replication is more sensitive to the inhibitory effect of p21 than DNA repair. From a biological perspective, the latter scenario seems more compatible with cell survival. Thus, we view this notion as supportive of our observations. The in vitro repair assay used is quite sensitive to nicks and pyrimidine hydrates in the plasmid DNA. If such lesions exist, nick translation will falsely mimic DNA repair activity. To avoid such a problem, we routinely test our substrates with an XPA extract, which is defective in NER and thus detects the extent

of nonspecific nick translation. In our experiments there was no measurable repair incorporation in the XPA extracts. Perhaps the discrepancy observed between this study and that of Pan *et al.* (1995) is related to their use of renatured, full-length protein, whereas we have used soluble proteins. Nevertheless, the observation that a high ratio of p21 to PCNA is necessary to inhibit NER might explain the conflicting in vitro results reported by others who may have used cell extracts with saturating levels of PCNA (Li *et al.*, 1994; Shivji *et al.*, 1994). We argue that our observation of an inhibition by p21 C is not due just to high levels of the protein. Addition of p21 N to the in vitro DNA repair assay does not affect the reaction, even at much higher concentration than those at which the p21 C terminal effectively inhibits the repair.

Very recently, at the time of completion of this work, Shivji *et al.* (1998) reported some in vitro effects of p21. Their study used both histidine-tagged and GST-tagged full-length protein. They demonstrated inhibition of DNA repair by p21 at a high p21 to PCNA ratio. In agreement with our findings, they observed a much stronger effect of p21 on replication than on DNA repair; however, they did not examine the function of the N terminus of the protein and did not study the effect of the p21 protein in vivo.

Several studies have clearly implicated the role of PCNA in the repair resynthesis step of NER (Wood, 1996). Because the inhibition of repair by p21 is PCNA dependent, this suggests that the p21 effect on DNA repair is at the resynthesis step. To determine whether p21 also inhibits the repair process in vivo, we used a permeabilized cell system. The method of cell permeablization constitutes a valid model system for studying molecular functions. The encapsulation of cells in agarose microbeads prevents the shearing and aggregation of chromatin on isolation under isotonic conditions. This system has been used successfully to label the UV-induced repair sites and transcription sites in chromatin (Jackson *et al.*, 1994; Balajee *et al.*, 1998). One distinct advantage of this system is that the chromatin of permeabilized cells replicate and transcribe at rates comparable to those of intact cells (Jackson *et al.*, 1993). The cells remain viable for several days in tissue culture and also replicate at 85% of the rate of nonpermeabilized cells in a cell cycle-specific manner and efficiently perform transcription (Jackson and Cook, 1986). We exploited the inherent ability of permeabilized cells to perform only the resynthesis step and ligation after UV exposure. It has been shown that initiation of incision activity precedes the permeabilization step (Jackson *et al.*, 1994). Using this system we find that the C terminus of p21 effectively blocks the resynthesis step of NER through its interaction with PCNA.

In the electroporation experiments we also observed an in vivo inhibitory effect of p21 on NER. Although we observed the same inhibitory effect of p21 C terminus on NER activity in both systems, the concentration of p21 required for inhibition was lower in electroporation experiments. This may be related to the timing of p21 addition. Addition of p21 before DNA damage in electroporation experiments is likely to inhibit both loading of the PCNA clamp and the polymerase. In contrast, p21 protein was added after DNA damage in the permeabilized cell system. Studies have shown that PCNA is rapidly recruited to DNA repair sites within seconds after UV irradiation (Li *et al.*, 1996; Savio *et al.*, 1996). It is conceivable, therefore, that p21 mediates repair inhibition in permeabilized cells primarily by inhibiting the initial polymerase loading as well as any reloading of the polymerase after dissociation of polymerase from the PCNA clamp. Once the PCNA clamp is assembled its translocation is immune to p21; however, the loading and reloading of the polymerase remains prone to inhibition by p21 (Podust *et al.*, 1995). Hence, p21 inhibition of both clamp assembly and polymerase loading could take place in electroporated cells compared with only inhibition of polymerase loading in the permeabilized cells. Thus, preassembly of PCNA at repair sites may account for the increased level of p21 required for inhibition of NER.

Although p21 inhibits the resynthesis step of both replication and repair, the differential effect of p21 on PCNA-dependent synthesis is most likely due to the high processivity of the DNA polymerase δ or ϵ holoenzymes. Thus, long tracts of replicating DNA are more prone to inhibition of DNA synthesis at a low concentration of p21 compared with the short tracts of DNA synthesis in NER. It is possible, therefore, that actively dividing cells that undergo up-regulation of p21 after DNA damage use a p21 concentration-dependent repair threshold. Below this threshold replication is inhibited, whereas repair remains permissive.

It has been shown in several biological systems that p21 is up-regulated with aging, but it is not clear whether senescence or stress situations can effect a level of overexpression sufficient to inhibit DNA repair. Some experiments have suggested that the increase of expression of p21 with senescence is on the order of 10- to 20-fold (Noda *et al.*, 1994), which may not be high enough to affect DNA repair; however, this could be a potential mechanism to account for the observations that DNA repair capacity declines with age (reviewed in Bohr and Anson, 1995). The decrease in DNA repair could then explain the increased levels of DNA damage and mutation in senescent cells (reviewed in Bohr and Anson, 1995). Thus it is possible that the level of p21 plays a critical role in attaining the senescent phenotype and the associated high susceptibility for the development of malignancies. Also, several aggressive tumors overexpress p21 (Erber *et al.*, 1997; Trotter *et al.*, 1997), and some head and neck

squamous cell carcinomas, which maintain very high levels of p21, have decreased survival and malignant phenotypes (Erber *et al.*, 1997).

In conclusion, p21 regulates three crucial biological processes: cell cycle progression, replication, and DNA repair. p21 inhibits NER both in vitro and in vivo; however, the biological significance of this inhibition requires further study. It appears that the PCNA binding domain of p21 regulates both replication and NER. This regulation occurs at the resynthesis step and most likely proceeds through the inhibition of PCNA clamp assembly and polymerase loading. The impact of p21 on NER is dependent on its intracellular concentration at the onset of repair relative to the PCNA level. As a result, actively growing cells that undergo a p21-mediated growth arrest may be protected from p21-induced repair inhibition by using a concentration-dependent repair threshold.

ACKNOWLEDGMENTS

We thank Dr. Dutta for the p21 constructs. We thank the Danish Center of Molecular Gerontology for interactions. We thank Drs. Patricia Gearhart and Leonora Lipinski for critical reading and Dr. Jiuping Ji for advice.

REFERENCES

Balajee, A.S., May, A., and Bohr, V.A. (1998). Fine structure analysis of DNA repair in mammalian cells. Mutat. Res. *404*, 3–11.

Bohr, V.A., and Anson, R.M. (1995). DNA damage, mutation and fine structure DNA repair in aging. Mutat. Res. *338*, 25–34.

Chen, J., Jackson, P.K., Kirschner, M.W., and Dutta, A. (1995). Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. Nature *374*, 386–388.

Chen, J., Peters, R., Saha, P., Lee, P., Theodoras, A., Pagano, M., Wagner, G., and Dutta, A. (1996). A 39 amino acid fragment of the cell cycle regulator p21 is sufficient to bind PCNA and partially inhibit DNA replication in vivo. Nucleic Acids Res. *24*, 1727–1733.

Dent, G.A., Leglise, M.C., Pryzwansky, K.B., and Ross, D.W. (1989). Simultaneous paired analysis by flow cytometry of surface markers, cytoplasmic antigens, or oncogene expression with DNA content. Cytometry *10*, 192–198.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from mammalian nuclei. Nucleic Acids Res. *11*, 1475–1489.

Erber, R., Klein, W., Andl, T., Enders, C., Born, A.I., Conradt, C., Bartek, J., and Bosch, F.X. (1997). Aberrant $p21^{CIP/WAF1}$ protein accumulation in head-and-neck cancer. Int. J. Cancer *74*, 383–389.

Goubin, F., and Ducommun, B. (1995). Identification of binding domains on the p21Cip1 cyclin-dependent kinase inhibitor. Oncogene *10*, 2281–2287.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805–816.

Jackson, D.A., Balajee, A.S., Mullenders, L., and Cook, P.R. (1994). Sites in human nuclei where DNA damage by UV light is repaired: visualization and localization relative to the nucleoskeleton. J. Cell Sci. *107*(Pt 7), 1745–1752.

Jackson, D.A., and Cook, P.R. (1986). A cell-cycle-dependent DNA polymerase activity that replicates intact DNA in chromatin. J. Mol. Biol. *192*, 65–76.

Jackson, D.A., and Cook, P.R. (1988). A gentle method for preparing cyto- and nucleoskeletons and associated chromatin. J. Cell Sci. *90*, 365–378.

Jackson, D.A., Hassan, A.B., and Cook, P.R. (1993). Visualization of focal sites of transcription within human nuclei. EMBO J. *12*, 1059–1065.

Kuriyan, J., and O'Donnell, M. (1993). Sliding clamps of DNA polymerases. J. Mol. Biol. *234*, 915–925.

Li, R., Hannon, G.J., Beach, D., and Stillman, B. (1996). Subcellular distribution of p21 and PCNA in normal and repair-deficient cells following DNA damage. Curr. Biol. *6*, 189–199.

Li, R., Waga, S., Hannon, G.J., Beach, D., and Stillman, B. (1994). Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. Nature *371*, 534–537.

Luo, Y., Hurwitz, J., and Massague, J. (1995). Cell-cycle inhibition by independent CDK and PCNA binding domains of $p21^{Cip1}$. Nature *375*, 159–161.

McDonald, R.E., Wu, G.S., Waldman, T., and El-Deiry, W.S. (1996). Repair defect in $p21^{WAF/CIP1}$ −/−human cells. Cancer Res. *56*, 2250–2255.

Noda, A., Ning, Y., Venable, S.F., Pereira-Smith, O.M., and Smith, J.R. (1994). Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp. Cell Res. *211*, 90–98.

Pan, Z.-Q., Reardons, J.T., Li, L., Flores-Rozas, H., Legerski, R., Sancar, A., and Hurwitz, J. (1995). Inhibition of nucleotide excision repair by the cyclin-dependent kinase inhibitor p21. J. Biol. Chem. *270*, 22008–22016.

Podust, V.N., Podust, L.M., Goubin, F., Ducommun, B., and Hubscher, U. (1995). Mechanism of inhibition of proliferating cell nuclear antigen-dependent DNA synthesis by the cyclin-dependent kinase inhibitor p21. Biochemistry *34*, 8869–8875.

Prelich, G., Kostura, M., Maushak, D.R., Mathews, M.B., and Stillman, B. (1987). The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication in vitro. Nature *326*, 471–475.

Prelich, G., and Stillman, B. (1988). Coordinated leading and lagging strand synthesis during SV40 DNA replication in vitro requires PCNA. Cell *53*, 117–126.

Savio, M., Stivala, L.A., Scovassi, A.I., Bianchi, L., and Prosperi, E. (1996). $p21^{waf1/cip1}$ protein associates with the detergent-insoluble form of PCNA concomitantly with disassembly of PCNA at nucleotide excision repair sites. Oncogene *13*, 1591–1598.

Sheikh, M.S., Carrier, F., Papathanasiou, M.A., Hollander, M.C., Zhan, Q., Yu, K., and Fornace, A.J.J. (1997). Identification of several human homologs of hamster DNA damage-inducible transcripts. Cloning and characterization of a novel UV-inducible cDNA that codes for a putative RNA-binding protein. J. Biol. Chem. *272*, 26720–26726.

Shivji, M.K.K., Ferrari, E., Ball, K., Hubscher, U., and Wood, R.D. (1998). Resistance of human nucleotide excision repair synthesis in vitro to p21(Cdn1). Oncogene *17*, 2827–2838.

Shivji, M.K.K., Gray, S.J., Strausfeld, U.P., Wood, R.D., and Blow, J.J. (1994). Cip1 inhibits DNA replication but not PCNA-dependent nucleotide excision-repair. Curr. Biol. *4*, 1062–1068.

Shivji, M.K.K., Kenny, M.K., and Wood, R.D. (1992). Proliferating cell nuclear antigen is required for DNA excision repair. Cell *69*, 367–374.

Trotter, M.J., Tang, L., and Tron, V.A. (1997). Overexpression of the cyclin-dependent kinase inhibitor p21 (WAF1/CIP1) in human cutaneous malignant melanoma. J Cutaneous Pathol. *24*, 265–271.

Tsurimoto, T., Melendy, T., and Stillman, B. (1990). Sequential initiation of lagging and leading strand synthesis by two different polymerase complexes at the SV40 DNA replication origin. Nature *346*, 534–539.

Waga, S., Hannon, G.J., Beach, D., and Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature *369*, 574–578.

Warbrick, E., Lane, D.P., Glover, D.M., and Cox, L.S. (1995). A small peptide inhibitor of DNA replication defines the site of interaction between the cyclin-dependent kinase inhibitor p21WAF1 and proliferating cell nuclear antigen. Curr. Biol. *5*, 275–282.

Winegar, R., and Lutze, L.H. (1990). Introduction of biologically active proteins into viable cells by electroporation. Focus *12*, 34–37.

Wood, R.D. (1996). DNA repair in eukaryotes. Annu. Rev. Biochem. *65*, 135–167.

Wood, R.D., Robins, P., and Lindahl, T. (1988). Complementation of the *Xeroderma pigmentosum* DNA repair in cell-free extracts. Cell *53*, 97–106.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. Nature *366*, 701–704.

Zhang, H., Xiong, Y., and Beach, D. (1993). Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes. Mol. Biol. Cell *4*, 897–906.