Subcellular distribution of p21 and PCNA in normal and repair-deficient cells following DNA damage

Rong Li, Gregory J. Hannon*, David Beach* and Bruce Stillman

Background: The p21 protein binds to both cyclin-dependent kinases (Cdks) and the proliferating cell nuclear antigen (PCNA). In mammalian cells, DNA damage results in an increase in the level of p53 protein, which stimulates expression of the gene encoding p21, which in turn leads to an inhibition of Cdk activity. Biochemical studies have shown that the direct interaction between p21 and PCNA blocks the latter's function in DNA replication but not in DNA repair. In addition to the p53-dependent damage response, the stimulation of quiescent cells with serum can also cause a p53-independent elevation in *p21* gene expression. It is not clear, however, whether the induction of p21 protein under these two circumstances serves the same purpose. In this study, we have investigated the kinetics of p21 induction by DNA damage and serum stimulation and the consequent effects on cell-cycle progression. Using both normal and repair-deficient human cells, we have also analyzed the nuclear distribution of p21 in relation to that of PCNA.

Results: *In vivo* immunofluorescence staining experiments indicate that, following UV damage, DNA repair is not inhibited by the presence of a large amount of p21 protein in the nucleus; in contrast, cells undergoing DNA replication during S phase contain very low amounts of p21. The addition of serum induced a transitory elevation of p21 levels, whereas UV damage to cells resulted in a sustained, high level of p21 that was more tightly associated with the nuclear structure. Interestingly, cells deficient in global nucleotide excision–repair displayed a distinct pattern of detergent-insoluble p21 that co-localized with PCNA.

Conclusions: The *in vivo* studies presented here, which are consistent with our previous findings *in vitro,* indicate that p21 has a differential effect on DNA replication and DNA repair, and that the induction of p21 by serum and DNA damage may have different consequences. Furthermore, the co-localization of p21 and PCNA in the nucleus of normal and repair-deficient human cells indicates that p21 and PCNA interact during post-damage events.

Address: Cold Spring Harbor Laboratory and *Howard Hughes Medical Institute, Cold Spring Harbor, New York 11724, USA.

Correspondence to: Bruce Stillman

Received: **27 September 1995**
Revised: **23 November 1995**
Accepted: **12 December 1995**

Current Biology 1996, Vol 6 No 2:189–199

© Current Biology Ltd ISSN 0960-9822

Background

The major transition points in eukaryotic cell-cycle progression are controlled by multiple cyclin-dependent kinases (Cdks) [1–3]. One mechanism by which Cdk function is modulated is through the action of a group of Cdk inhibitors [4], whose activities are affected by various external cues [5]. One of these inhibitors, p21 (CIP/WAF1), may be responsible for controlling the G1–S-phase checkpoint in the event of DNA damage [6–10]. Expression of the gene encoding p21 is stimulated by the tumor suppressor protein p53, the induction of which is triggered by the presence of DNA damage. In addition to the p53-dependent DNA-damage pathway, p21 has also been shown to respond to growth signals, such as serum and growth factors, and has been implicated in terminal differentiation and senescence [11–22]. Although the exact role(s) of p21 in these damage-independent situations remains to be elucidated, it may involve an interaction between p21 and the Cdks that are still present in non-growing cells. In support of this notion, p21 is known to interact with and regulate multiple Cdk–cyclins and, under certain situations, it can promote the assembly of the cyclin–Cdk complex [23,24]. Furthermore, in serum-stimulated cells, the p21-associated histone H1 kinase activity increases in parallel with increasing amounts of p21 [17].

In normal human cells, p21 exists in a quaternary complex with a Cdk, a cyclin and the proliferating cell nuclear antigen (PCNA) [24]. One unique feature of p21 that distinguishes it from the other Cdk inhibitors is its dual inhibition of the activities of Cdks and PCNA [25–27]. As an auxiliary factor for DNA polymerases δ and ϵ, PCNA facilitates the loading of the polymerases onto DNA

templates and increases the processivity of the polymerases [28]; PCNA is required for both DNA replication and DNA repair [29–31]. Although the total cellular level of PCNA is relatively constant through the cell cycle, it is tightly associated with the nucleus only in S-phase cells and in UV-irradiated non-S-phase cells [32–34]. Under such conditions, a subset of PCNA is resistant to detergent extraction from the nucleus.

Recent studies have revealed that different domains of p21 interact with Cdks and PCNA, and that both of these domains can independently inhibit DNA replication when present in cells [35–38]. This strengthens the notion that p21 can arrest cell-cycle progression by inhibiting both Cdks and PCNA. *In vitro*, p21 preferentially inhibits long-range DNA synthesis while allowing short-patch synthesis to proceed, such as that which occurs during nucleotide excision–repair [39,40].

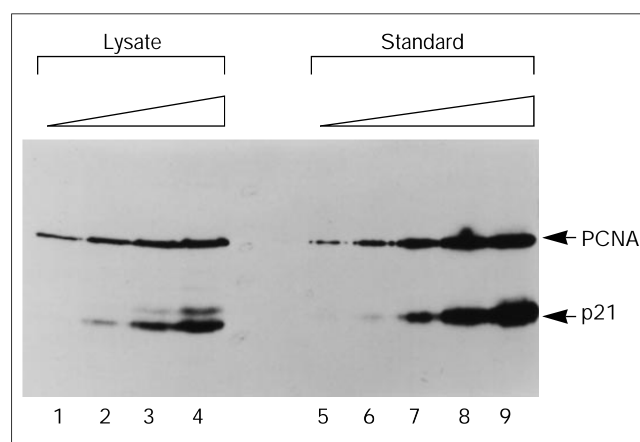
The ability of p21 to bind simultaneously to both Cdks and PCNA has led to the suggestion that it may mediate the coordination between DNA replication, DNA repair and cell-cycle progression [25,39]. To investigate this further, we used immunofluorescence staining to visualize the subcellular distribution of p21 and PCNA under different physiological conditions. The results show that DNA replication, but not DNA repair, correlates with low levels of p21. The kinetics of p21 induction and the extent of its association with the nuclear structure differed in response to DNA damage or serum stimulation. After DNA damage, we also observed a similar nuclear distribution of both p21 and PCNA that was dependent on the DNA-repair capacity of the cell. These results provide *in vivo* evidence for the involvement of p21 and PCNA in a damage-dependent cell-cycle checkpoint.

Results

DNA repair, but not DNA replication, is compatible with high levels of p21 in the nucleus

Previous biochemical studies have shown that a two-to-five-fold molar excess of p21 over the level of PCNA can completely inhibit the latter's function in DNA replication [25,27,40]. One issue that has arisen from this observation concerns the physiological relevance of the p21:PCNA ratios used in these *in vitro* assays. We have therefore examined the relative amounts of p21 and PCNA in an asynchronous population of human WI38 diploid fibroblasts by quantitative immunoblotting experiments. As shown in Figure 1, various amounts of WI38 cell lysate were compared with known amounts of purified PCNA and histidine-tagged p21 proteins. Based on multiple experiments, we estimated that the molar ratio of p21 to PCNA (monomer) was close to 1:1 *in vivo*, representing ~$2–5 \times 10^5$ molecules per cell. After UV irradiation, the level of p21, but not of PCNA, increased five-to-ten fold ([41] and our unpublished observations); these observations *in vivo* therefore mimic the conditions *in vitro*, where p21 inhibited PCNA's function in DNA replication.

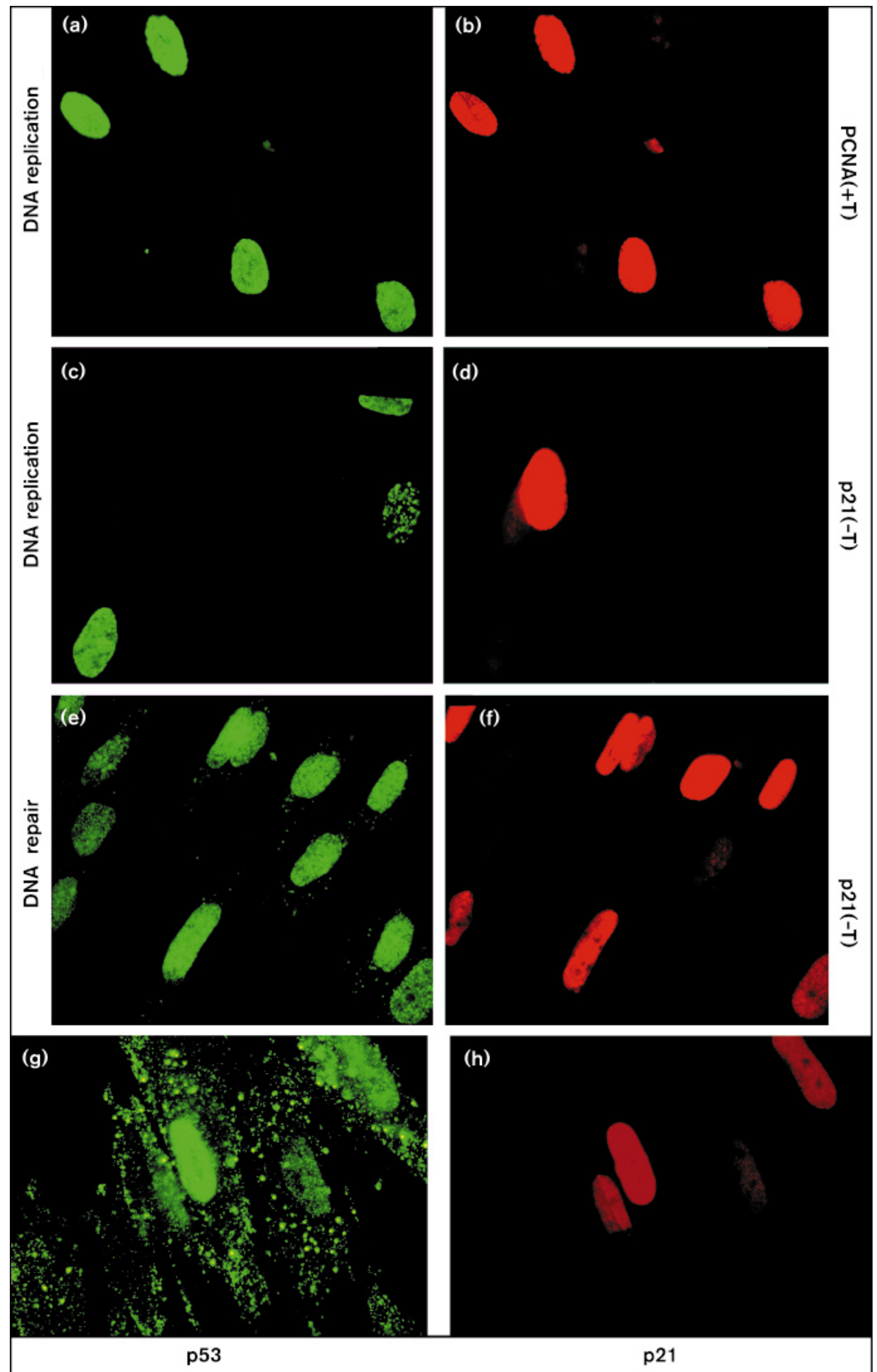
Figure 1



Quantitation of PCNA and p21 in WI38 human cells. Sub-confluent WI38 cells were harvested and lysed directly in Laemmli sample buffer. The amounts of lysate loaded in lanes 1–4 were equivalent to 1.5, 3, 6 and 12×10^4 cells, respectively. For quantitation purposes, 0.6, 1.2, 2.5, 5 and 10 ng of purified PCNA and p21 were loaded in lanes 5–9, respectively.

To further investigate the relationship between S-phase DNA synthesis and the level of p21, on a single-cell basis *in vivo*, asynchronous human diploid WI38 cells were pulse-labeled with bromodeoxyuracil (BrdU) and subsequently stained for incorporated BrdU and the endogenous p21 protein (Fig. 2c,d). After comparing the p21 and BrdU staining in many microscopic fields, we found that only a small proportion of the cells (10–20 %) contained high levels of p21 localized in the nucleus, and that none of these p21-positive cells incorporated BrdU. This is consistent with earlier reports demonstrating that p21 inhibits the functions of both Cdks and PCNA in DNA replication [25–27]. As a control, we also compared PCNA staining with the BrdU incorporation (Fig. 2a,b). As observed previously [32], only S-phase cells contained high levels of detergent-insoluble PCNA, whereas PCNA in non-S-phase cells was easily extractable with non-ionic detergent (data not shown; but see Fig. 4). Thus, in a proliferating population of normal human fibroblasts, cells with detergent-insoluble PCNA contained low levels of p21. Taken together, these data suggest that, unlike PCNA, p21 is not associated with active DNA replication in S-phase cells. From the data shown in panels of Fig. 2a–d, it is not possible to tell the exact cell-cycle stage of the p21-positive cells. However, as high levels of p21 have been found in senescent cells and p21 can be induced by serum, we speculate that the p21-positive cells in the asynchronous WI38 population may be in either the G0 or early G1 phase of the cell cycle.

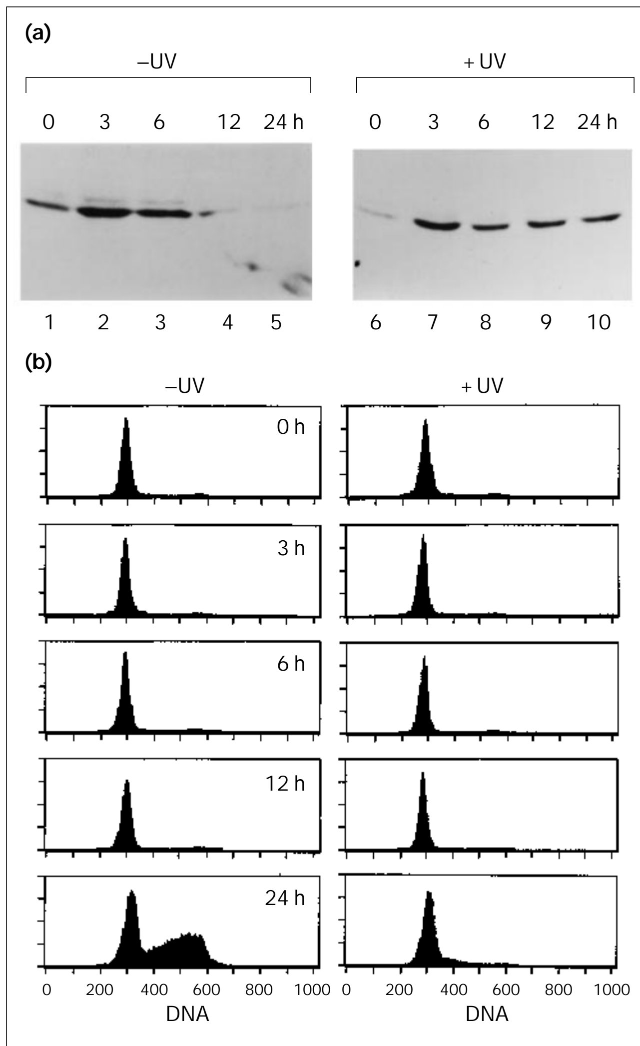
Figure 2



High levels of p21 are compatible with DNA repair but not DNA replication. **(a–d)** Asynchronous WI38 cells were labeled briefly with BrdU and stained with a flourescine isothiocyanate (FITC)-conjugated anti-BrdU antibody (a,c), together with either an anti-PCNA monoclonal antibody (b) or anti-p21 polyclonal serum (d), followed by a Texas Red-conjugated secondary antibody. Cells stained for PCNA were pretreated with Triton X-100 before fixation to reveal the detergent-insoluble PCNA present only in S-phase cells. Non-S-phase cells are not visible in (a,b). **(e,f)** Synchronized G0-phase cells were irradiated at 12 J m^{-2} and released into G1 phase by serum addition in the presence of BrdU. (e) The relatively weak repair signal was amplified using an anti-BrdU antibody, a biotinylated anti-mouse IgG antibody and FITC-conjugated streptavidin. (f) The polyclonal antiserum directed against p21 was used to detect p21 in the damaged cells. **(g,h)** Cells were treated as in (e,f), except without BrdU labelling. Samples were subsequently immunostained with (g) a monoclonal antibody directed against human p53 (FITC staining) and (h) the polyclonal antiserum directed against p21 (Texas Red staining).

Using different *in vitro* systems for DNA replication and nucleotide excision–repair, it was demonstrated previously that p21 has a differential effect on these two types of PCNA-dependent DNA synthesis [39,40]. To extend these observations *in vitro*, the levels of p21 were compared with the extent of DNA repair in cells undergoing active DNA repair following UV irradiation. Synchronized WI38 cells in G1 phase of the cell cycle were used to minimize the BrdU staining signal from S-phase DNA replication that would otherwise obscure the relatively weak signal generated by DNA repair. As shown in Figure 2e,f, all the irradiated cells underwent DNA repair and, concomitantly, the majority of cells (~75 %) contained abundant amounts of p21 protein in the nucleus. As

Figure 3



Serum and UV irradiation lead to different kinetics of p21 induction. Cells were synchronized by serum deprivation, treated with or without UV and then released from growth arrest by the addition of serum. Samples were harvested at different time points and analyzed **(a)** by immunoblotting using polyclonal anti-p21 serum or **(b)** by FACS analysis for cell-cycle distribution. In this and the following experiments, samples at time zero were harvested without further incubation in serum-containing media.

expected, cells deficient in nucleotide excision–repair did not generate the BrdU staining signal (data not shown). This result indicates that, unlike DNA replication, active DNA repair can occur in the presence of high levels of p21 (compare Fig. 2c,d with 2e,f). Interestingly, some variation in the levels of p21 was observed between cells within the synchronized population. This may result from different kinetics of p53 induction within individual cells. This is supported by the observation that the UV-irradiated cells containing higher levels of p21 also showed stronger nuclear staining for p53 (Fig. 2g,h). In conclusion, the immunofluorescence study is consistent with findings *in vitro* that p21 has different effects on the function of PCNA in DNA replication and DNA repair.

The induction of p21 by serum and DNA damage, and the effects on cell-cycle progression

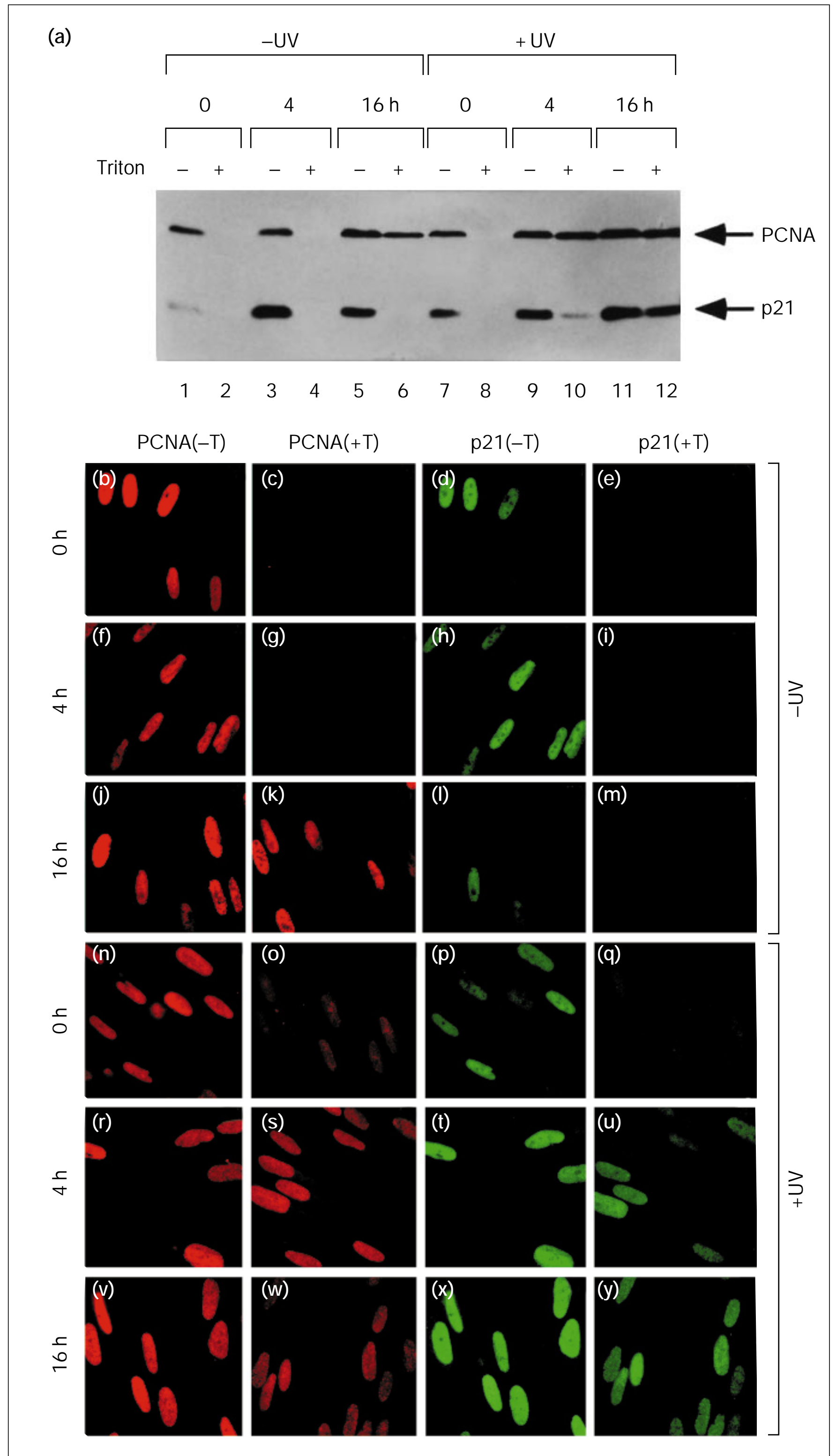
To compare the induction of p21 by serum and UV damage, we conducted a kinetic study on WI38 cells synchronized by serum starvation and then restimulated. At different time points after the addition of serum, the levels of p21 were determined by immunoblotting and the cell-cycle distribution assessed by an analysis of DNA content using fluorescence-activated cell sorting (FACS). As quiescent cells exited from G0 into G1 phase, the expression of p21 initially increased but then diminished as the cells progressed into late G1 and early S phase (Fig. 3a, lanes 1–5, and 3b). This result is consistent with previous kinetic studies of p21 mRNA levels following serum stimulation of quiescent fibroblasts [11,20]. It is also worth noting that a minor portion of the p21 protein seemed to migrate more slowly when analysed by SDS–PAGE. This may represent the phosphorylated form(s) of p21, as observed previously [23].

We then investigated the effects of DNA damage on the level of p21, by immediately irradiating synchronized cells with UV before serum release. As expected, the cell-cycle progression was prevented (compare the 24 hour time points in Fig. 3b); in fact, UV irradiation seemed to block the cell-cycle progression for a prolonged period up to 48 hours (data not shown). Based on the results shown in Figure 2e, these cells were undergoing active DNA repair. However, at the same time, the cellular levels of p21 were elevated and sustained over a long period of time (Fig. 3a, lanes 6–10). Serum and UV irradiation therefore differ in their ability to induce p21 and to maintain the elevated levels of p21.

PCNA is known to be tightly associated with the nuclear structure in UV-irradiated cells, presumably because of its involvement in post-damage events such as DNA repair [33,34]. Given the strong interaction between p21 and PCNA, it is possible that p21 in damaged cells might also be refractory to detergent extraction; it was reported recently that, after UV-irradiation of IMR90 cells, a considerable fraction of p21 became resistant to extraction with Triton [41]. We were interested to know whether p21 induced by either serum or DNA damage would behave differently in this regard. Synchronized cells were treated with or without UV irradiation, released from serum starvation and collected at different time points. The cell pellets were either lysed directly in protein sample buffer or subjected to extraction with Triton X-100 prior to resuspension in lysis buffer. The samples were then immunoblotted to determine the amount of total and Triton-resistant PCNA and p21. In the absence

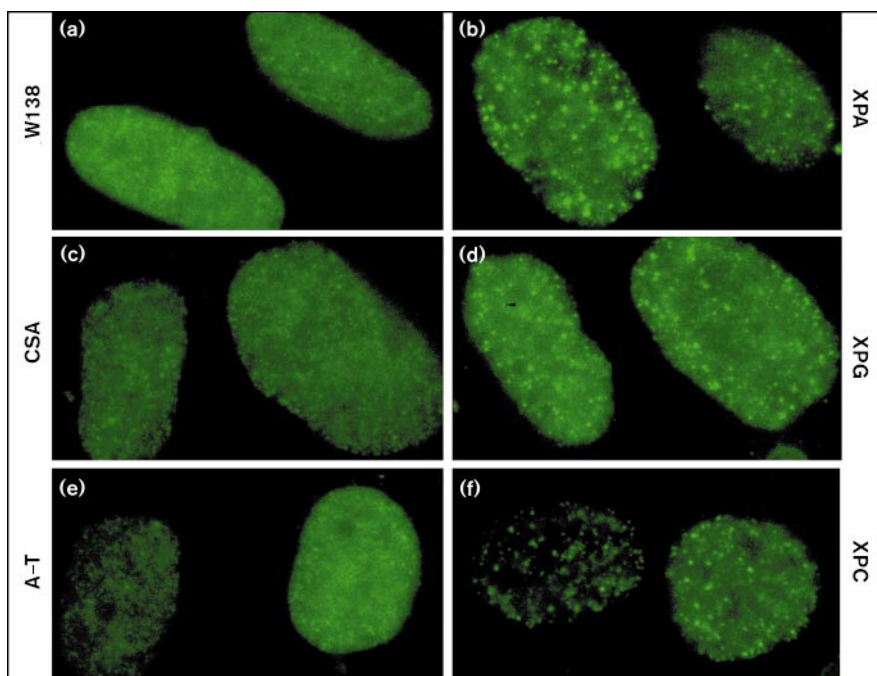
of UV damage, most of the p21 was Triton-soluble at all three time points (Fig. 4a, lanes 1–6). PCNA, however, became resistant to detergent extraction 16 hours after serum release (Fig. 4a, lanes 5,6), at which point the cells

Figure 4



Association of PCNA and p21 in the nucleus following UV damage. **(a)** WI38 cells were growth-arrested by serum starvation. Non-irradiated (lanes 1–6) and UV-irradiated (lanes 7–12) cells were then harvested 0, 4 and 16 hours after incubation in serum-containing media, and the cellular proteins with (+) or without (–) detergent extraction were analyzed by immunoblotting using an anti-PCNA antibody and anti-p21 polyclonal serum. **(b–m)** Non-irradiated and **(n–y)** UV-irradiated cells were collected at different time points following DNA damage and extracted with (+T) or without (–T) Triton X-100, as described in Materials and methods. Cells were stained for PCNA (FITC staining) and p21 (Texas Reds staining). For direct comparison, the photographs were taken using the same exposure time.

Figure 5



Distinct patterns of p21 and PCNA in repair-proficient and repair-deficient cells. WI38 and various mutant cells were synchronized, irradiated with UV and released into serum-containing media. See the text for a detailed description of the mutant cells. After 4 h incubation in serum-containing media, cells were extracted with detergent and stained with anti-p21 polyclonal serum. In this figure and in Fig. 6, the relative positions of the two cells from the same field were adjusted using the Adobe Photoshop software program in order to generate more compact pictures.

started to enter S phase (as determined by BrdU incorporation and FACS analysis; data not shown). This is consistent with the role of PCNA in DNA replication during S phase. In contrast, when cells were UV-irradiated at the time of serum release, cell-cycle progression was blocked (Fig. 3) and the damaged DNA was actively repaired (Fig. 2e,f). Accordingly, PCNA was resistant to Triton extraction at the 4 and 16 hour time points after DNA damage (Fig. 4a, lanes 9–12). Strikingly, a substantial amount of the p21 protein became resistant to Triton extraction 16 hours after UV irradiation (Fig. 4a, lanes 11 and 12), unlike the control without UV treatment (Fig. 4a, lanes 5,6). The resistance to detergent extraction was unlikely to be a result of non-specific UV cross-linking for two reasons. Firstly, the UV dosage used in this experiment was relatively low. Secondly, the amount of Triton-insoluble PCNA and p21 was negligible at the zero time point, even though the cells had been UV irradiated, suggesting a UV-induced time-dependent nuclear association of both proteins.

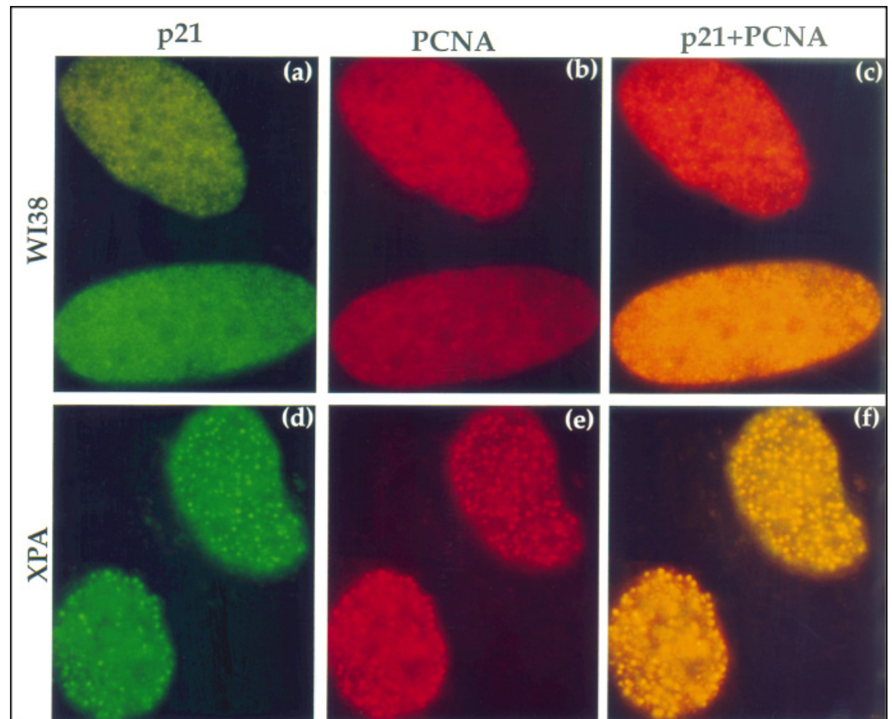
As both PCNA and p21 can interact with several other proteins involved in cell-cycle regulation, we stripped the blot shown in Figure 4 and reprobed it with polyclonal antisera directed against Cdc2, Cdk2, cyclins A, D and E, and the replication proteins RPA and RF-C. Unlike PCNA and p21, none of these proteins displayed UV-dependent resistance to Triton extraction (data not shown). A similar observation was made recently by Pagano *et al.* [41]. The association of p21 with the nuclear structure is therefore likely to result from its direct interaction with PCNA.

To confirm the results obtained by the immunoblotting analysis, we directly examined the nuclear distribution of PCNA and p21 by immunofluorescence staining in non-irradiated or irradiated cells, with or without detergent extraction prior to fixation. Under similar conditions to those used in the previous experiment, detergent-insoluble PCNA appeared in both non-irradiated cells in S-phase (Fig. 4j,k) and UV-irradiated cells undergoing DNA repair (Fig. 4n,o,r,s,v,w). In non-irradiated cells, p21 was not detected in the Triton insoluble form in the nucleus (Fig. 4d,e,h,i,l,m). In contrast, a significant amount of detergent-insoluble p21 was detected in the UV-irradiated cells, but only after a time delay (Fig. 4p,q,t,u,x,y). Together with the earlier kinetic studies, these data reveal distinct properties of p21 when induced by damage-dependent and independent pathways.

Cells deficient in global repair display distinct detergent-insoluble patterns of p21 and PCNA

DNA repair is one of the major post-damage events occurring in irradiated cells. We therefore hypothesized that the differential distribution patterns of p21 and PCNA in the presence or absence of irradiation might be related to the response of the cellular repair machinery to DNA damage. To further assess the relationship between DNA repair and the subcellular localization of p21, we compared the nuclear staining patterns of p21 in normal and several

Figure 6



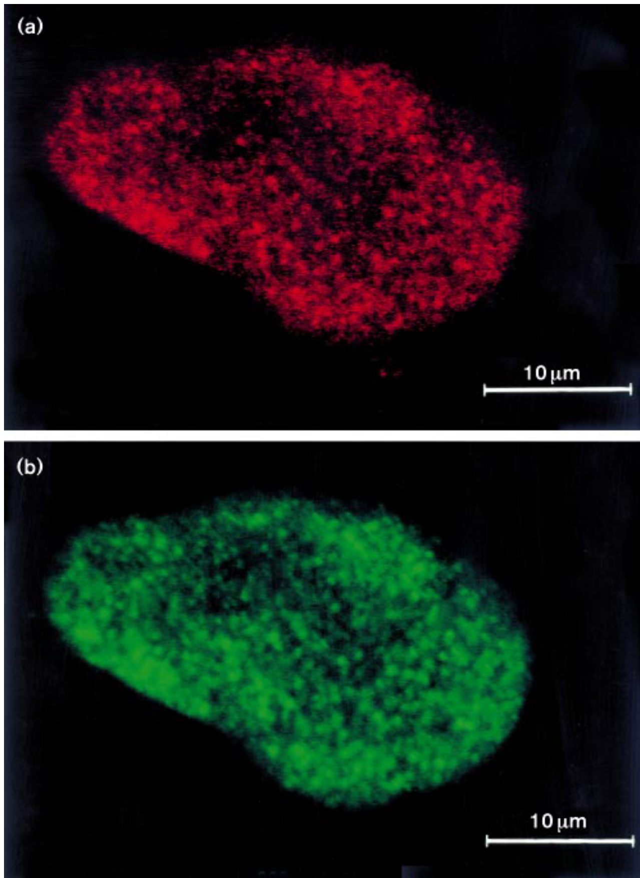
(a–c) WI38 and **(d–f)** XPA cells were treated as described in Figure 5. UV-irradiated cells were stained with anti-PCNA and anti-p21 monoclonal antibodies. To superimpose the p21 and PCNA patterns, photographs with double exposure are also shown (c,f).

mutant cell lines that are defective in various DNA-repair pathways. Xeroderma pigmentosum (XP) is a human genetic disease characterized by an extreme sensitivity to UV irradiation [42]. The different complementation groups of the XP syndrome represent defects in multiple gene products that are involved in nucleotide excision–repair [43–45]. For example, the XPA group lacks the activity of a protein that recognizes damaged DNA, whereas the XPG group has a defect in the enzyme which is normally responsible for making an incision in the DNA on one side of the lesion. As a result, the overall rate of nucleotide excision–repair is greatly reduced in both of these mutant cells. Cockayne's syndrome (CS) is caused by defects in transcription-coupled DNA repair, and cells from patients with CS exhibit reduced repair only on the transcribed strand of transcriptionally active genes; in contrast, global genome repair is normal in these cells. Another XP group, XPC, is proficient in transcription-coupled repair, yet is deficient in global repair of the remaining genome. Finally, ataxia–telangiectasia (A–T) is a human genetic disease characterized by an extreme sensitivity to ionizing irradiation [42].

The detergent-insoluble p21 staining patterns in all cell types could be divided into two categories — those with small and numerous speckles (Fig. 5a,c,e), and those with much larger but fewer dots (Fig. 5b,d,f). Interestingly, this difference seems to correlate with the ability of the cells to execute global DNA repair. Cells capable of global DNA repair (WI38, CSA and A–T) exhibited the more homogeneous nuclear staining pattern of Triton-insoluble p21, even though some cells (CSA) were defective for transcription-coupled repair. In contrast, cells that were deficient in global repair (XPA, XPG and XPC) contained p21 that displayed the larger punctate staining pattern.

Miura *et al.* [46] reported the detection of two types of Triton-insoluble PCNA in UV-irradiated normal and XPA cells. The Triton-insoluble PCNA staining pattern in normal cells appeared earlier than that in XPA cells, and the fluorescent speckles in XPA cells were larger in size but smaller in number. This was somewhat surprising as PCNA is thought to be involved in a late step in the excision–repair pathway [29,31], presumably after the recognition of lesion by the XPA protein [47,48]. The p21 staining patterns in the two categories described in Figure 5 are similar to the PCNA staining patterns described by Miura *et al* [46]. To determine whether the two types of p21 staining patterns correlated with PCNA localization, W138 and XPA cells were extracted with Triton-containing buffer and immunostained using using both anti-p21 and anti-PCNA antibodies. The distribution of p21 and PCNA was essentially coincident in both cell types, with the result being more obvious in XPA cells (Fig. 6d–f) than in normal W138 cells (Fig. 6a–c). To confirm the co-localization of p21 and PCNA in normal W138 cells, confocal microscopy was employed to compare localization of the two proteins within a defined optical section of the nucleus. The pattern of p21 and PCNA staining was very similar and in most

Figure 7



Triton-insoluble patterns of **(a)** PCNA and **(b)** p21. WI38 cells, treated as in Figure 6, were examined using a confocal laser scanning microscope. A 0.5 μm optical section of both staining patterns is shown.

cases overlapped in the optical section obtained (0.5 μm; Fig. 7). This demonstrates that in UV-irradiated wild-type cells, the p21 and PCNA staining patterns co-localize, concomitant with their tight nuclear association.

Discussion

The p21 levels in the nucleus can be induced either by mitogenic stimuli or DNA damage. Our results indicate that serum stimulation causes a temporary induction of p21 protein in early G1 phase, and a gradual decline when cells progress into S phase. The induction of p21 protein by serum addition seems to parallel that of p21 mRNA [11]. The low levels of p21 in S phase cells are consistent with the ability of p21 to inhibit DNA replication [25–27]. UV irradiation, on the other hand, leads to a sustained induced level of the p21 protein. A similar pattern of p21 induction by ionizing irradiation has been reported recently [49]. The current work also suggests an intimate association between p21 and PCNA following UV irradiation *in vivo*. Both p21 and PCNA become Triton-insoluble and co-localize in the nucleus of irradiated cells, supporting the notion that the interaction between p21 and PCNA plays an important role in the cellular response to DNA damage. The elevated levels of p21 following DNA damage correlate with increased levels of nuclear p53. Recent genetic evidence in both human and mouse cells demonstrates that p21 is a major downstream effector of p53 in causing cell-cycle arrest following DNA damage [50–52].

One question that arises from these experiments concerns the exact mechanism that leads to the different kinetics of p21 expression. Given that the kinetics of induction of the p21 protein and mRNA are quite similar (this study and [11]), transcriptional regulation is likely to play a major role in p21 induction. For example, following serum stimulation, certain serum-responsive transcription factors may bind to the promoter region of the p21 gene and activate gene expression. On the other hand, UV is known to induce an elevated level of p53 over a relatively long period [53], and this could account for the sustained high level of the p21 protein observed in this study. It is tempting to speculate that the p21 induced by these two mechanisms might be involved in different processes. As p21 is capable of promoting the formation of the Cdk–cyclin complex under certain conditions [23], it is conceivable that the transitory increase in the level of p21 stimulated by serum may be required to increase the Cdk activity in early G1 phase to facilitate the progression from the G0 to the G1 phase of the cell cycle. In the presence of DNA damage, however, a longer period may be needed to completely repair lesions throughout the genome before the cell-cycle machinery can resume normal progression.

In *S. cerevisiae*, the protein encoded by the *SIC1* gene (Sic1p) is an inhibitor of the B-type cyclin-dependent kinases that control entry into S phase [54,55]. Sic1p is degraded by a ubiquitin-dependent mechanism at the transition from G1 to S phase, thereby releasing the Cdk to activate the initiation of DNA replication. Because p21 seems to be removed from the nucleus as cells enter S phase, it is possible that p21 controls entry into S phase by inhibiting specific cyclin–Cdks in a similar manner to the way that Sic1p inhibits the Cdks that activate S phase in yeast. It would therefore be of interest to determine whether similar cell-cycle regulated proteolysis of p21 occurs in mammalian cells.

Previous studies have shown that there are two forms of PCNA, with regard to its association with the nuclear structure — detergent-insoluble PCNA in S-phase or UV-irradiated cells, and detergent-soluble PCNA in normal non-S-phase cells [32–34]. Given the known biochemical function of PCNA in DNA synthesis [29–31], the tight association of PCNA with the nucleus in S-phase and irradiated cells is consistent with its involvement in DNA replication and nucleotide excision–repair. However,

several lines of evidence suggest that the exact role(s) of PCNA in DNA replication and repair may not be identical. During DNA replication, PCNA serves as an auxiliary factor for DNA polymerase δ and, together with RF-C, facilitates the loading and processivity of the polymerase. Although PCNA is essential for nucleotide excision–repair, the nature of DNA templates and the length of newly synthesized DNA is quite different from those in DNA replication, and some studies have indicated that polymerase ε, rather than polymerase δ, may play the major role in DNA repair [48,56,57]. Furthermore, PCNA has been shown to facilitate the catalytic turnover of repair-specific enzymes involved in the incision–excision step of nucleotide excision–repair [31], presumably a result of its interaction with repair-specific proteins. Genetic studies have also led to the discovery of yeast PCNA mutants that are specifically impaired in DNA repair but not DNA replication [58]. In addition, the fact that p21 inhibits the function of PCNA in SV40 DNA replication *in vitro*, but does not interfere with its function in nucleotide excision–repair, suggests that PCNA acts differently in these two processes [39,40]. Finally, immunofluorescence studies have demonstrated that PCNA becomes tightly associated with the nucleus immediately after DNA damage, even at low temperature and in the absence of an energy source [34], and that PCNA can be Triton-insoluble even in repair-deficient cells following DNA damage [46].

It is intriguing that the Triton-insoluble p21 and PCNA in repair-deficient cells give rise to distinct patterns of nuclear distribution. The precise nature of the bright speckles in XP cells remains unclear. One simple explanation is that they represent some low-level nucleotide excision–repair activity in UV-irradiated XPA cells. However, this hypothesis is not supported by the observation that the granular pattern of PCNA in XPA cells occurs even when no DNA repair is detected [46]. An alternative explanation is that these fluorescent sites represent other DNA-repair pathways, which may be less readily detectable by thymidine incorporation. For example, base excision–repair involves PCNA and is thought to be required for the removal of pyrimidine hydrates, a minor lesion generated by UV irradiation [42]. A third hypothesis is that the tight association of p21 and PCNA with the nuclear structure is involved in other aspects of the cellular damage response.

As discussed above, the known biochemical function of PCNA in DNA repair is to facilitate polymerase δ/ε-dependent repair synthesis, and it is believed that the Triton-insoluble PCNA in UV-irradiated cells is involved in repair synthesis [29,31]. Current models based on *in vitro* data suggest that PCNA only functions after the execution of the incision stage of nucleotide excision–repair [47,48]. However, the observation that repair-deficient cells have a distinct staining pattern for Triton-insoluble p21 and PCNA raises the intriguing possibility that PCNA, together with p21, may play a role in nucleotide excision–repair prior to the actual incision event catalyzed by the XPA and XPG proteins. For example, upon DNA damage, p21 may bind to PCNA and this may contribute to initial sensing of the damage by the cellular machinery. If the repair machinery fails to repair the lesions promptly, p21 and PCNA may accumulate at those damaged sites, causing the fluorescent speckles in the repair-deficient cells. This, in turn, would suggest a role for p21 and PCNA in a step of nucleotide excision–repair that is independent of the activity of XPA or XPG proteins, which function during the early stages of damage recognition and incision. Although the exact molecular basis for this phenomenon awaits further study, our data strongly suggest that the nuclear localization of p21 and PCNA following UV damage is dependent upon the competence of the cellular repair machinery. The tight association of p21 with PCNA and the nucleus following DNA damage supports the notion that p21, together with PCNA, serves as an important link between DNA replication, DNA repair and cell-cycle progression.

Conclusions

The results in the current study support the notion that p21 plays a pivotal role in cellular response to DNA damage by inhibiting the onset of S-phase DNA replication while allowing active DNA repair. We have shown that the presence of p21 in damaged cells not only is compatible with active DNA repair, but a substantial fraction of it is also tightly associated with the nuclear structure in a manner that is similar to that of PCNA. Moreover, the detergent-insoluble nuclear staining patterns of p21 and PCNA are dependent on the capacity of cells to repair damaged regions of the genome. Based on these data, we propose that the interaction between p21 and PCNA is significant in coordinating the multiple post-DNA damage events, including checkpoint control, inhibition of DNA replication and efficient DNA repair. Our results also support the notion that p21 induced by the p53-independent pathways, such as serum stimulation, may play a distinct role in cell-cycle progression.

Materials and methods

Antibodies, cell lines and tissue culture

The purified anti-PCNA antibody from an autoimmune patient was kindly provided by M. Mathews. The polyclonal antiserum directed against p21 was generated by G.H. and D.B. The monoclonal antibodies directed against p21 (Ab-1) and against p53 (Ab-2) were purchased from Oncogene Science. The two anti-p21 antibodies gave rise to essentially the same results and were used interchangeably in the experiments. WI38 cells were purchased from ATCC and grown in DMEM plus 10 % fetal bovine serum. The following human genetic mutant cell lines were purchased from Coriell Cell Repositories and grown in DMEM containing 20 % serum: XPA (GM00710B); XPC (GM03176); XPG (GM03021A); CSA (GM01856B); A–T (GM05823B). For the synchronization experiments, cells were starved in the serum-free media for 2–3 d and subsequently transferred to serum-containing media. For UV irradiation, synchronized cells were washed with PBS and irradiated at

12 J m^{-2} using a 254 nm UV lamp calibrated by a UV radiometer. The cells were then incubated in fresh media for various periods of time. The human PCNA and histidine-tagged p21 used for the quantitative immunoblotting experiment were purified as published previously [25].

BrdU labeling and immunofluorescence staining

For detecting DNA replication, asynchronous cells were labeled with 10 μM BrdU (Amersham) for 10–15 min. To detect the relatively weak signal from DNA repair, UV-irradiated cells were incubated in media containing 100 μM BrdU for 3–4 h. The immunofluorescence procedure was performed essentially as described [59]. Upon harvesting, cells were fixed in 2 % formaldehyde for 15 min at room temperature and then permeabilized in 0.2 % Triton X-100 for 5 min on ice. Cells were incubated with the primary antibodies and the secondary antibodies (FITC- or Texas Red-conjugated) for 1 h each at room temperature. For detergent extraction, cells before fixation were pretreated with a hypotonic buffer containing 0.1 % Triton, as described [41].

For BrdU staining, cells after staining of the first antigen were fixed again with formaldehyde, denatured with 4 M HCl for 10 min at room temperature, and neutralized by incubation with 0.1 M sodium borate (pH 8.5) and PBS. The DNA replication signal was detected by a FITC-conjugated anti-BrdU antibody (Boehringer). DNA-repair signal was detected by a procedure described previously [41], using an anti-BrdU monoclonal antibody (Amersham), in conjunction with a biotinylated anti-mouse IgG antibody and FITC-conjugated streptavidin (Vector Laboratories). Under these conditions, UV-irradiated cells, but not control G1 phase cells, gave rise to BrdU staining.

Samples were examined and photographed on a Nikon Microphot-FXA microscope using 60 x and 100 x plan-apochromat lenses. Unless otherwise specified, pictures were taken on Kodak Ektachrome 400 x film using the automatic exposure feature of the microscope. The final composite figures were made by using a Polaroid SprintScan 35 slide scanner and Adobe Photoshop software program, and printed on a Codonics NP-1600 Photographic Network Printer. For the confocal pictures, 0.5 μm optical sections were obtained using a Zeiss LSM10 confocal laser scanning microscope and a 63 x/1.3 N.A. objective with a HeNe (λ: 543 nm) and Argon Ion (λ: 488 nm) lasers.

Protein analysis

For analyzing the total amount of p21 and PCNA, cells were harvested and directly resuspended in Laemmli sample buffer. To extract cells with detergent, the protocol by Pagano *et al.* [41] was followed with some minor modifications. Essentially, cell pellets were resuspended in extraction buffer (50 mM Tris-HCl pH 7.4, 0.25 M NaCl, 0.1 % Triton-X100 1 mM EDTA, 50 mM NaF, 1 mM DTT, 0.1 mM Na_3VO_4) containing protease inhibitors. The sample was incubated on ice for 30 min and centrifuged at 14 000 cpm for 10 min. The resulting pellet was extracted again with the same buffer and resuspended in the same volume of Laemmli buffer as the one used for total protein. Proteins were resolved by SDS–PAGE on a 17 % gel, transferred to a nitrocellulose membrane and detected by a ECL immunoblotting detection kit (Amersham).

Fluorescence activated cell sorting (FACS) analysis

Samples were collected by trypsinization and resuspended in 100 μl PBS plus 1 % serum. The cells were fixed by adding 900 μl 100 % ethanol dropwise and incubating for 1 h at 4 °C. The fixed cells were washed once with PBS plus serum and resuspended in the same buffer containing 10 μg ml^{-1} DNase-free RNase and 25 μg ml^{-1} propidium iodide. After incubation at 37 °C for 30 min, the samples were analyzed on a EPICS Elite ESP flow cytometer (Coulter).

Acknowledgements

We thank M. Mathews for providing the anti-PCNA antibody; D. Spector for generating the confocal images; R. Derby for technical assistance in the immunofluorescence study; P. Burfiend for FACS analysis; S. Waga for the purified his-tagged p21 protein; G. Cullman for help in generating the color figures; and V. Ellison and A. Stenlund for critical reading of the manuscript. R.L. is a special fellow of the Leukemia Society of America. D.B. is an Investigator of the Howard Hughes Medical Institute. This research was supported by a grant to B.S. from the National Institutes of Health (CA 13106).

References

1. Hartwell LH, Kastan MB: **Cell cycle control and cancer.** *Science* 1994, **266**:1821–1828.
2. Nigg EA: **Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle.** *BioEssays* 1995, **17**:471.
3. Nurse P: **Ordering S. phase and M phase in the cell cycle.** *Cell* 1994, **79**:547–550.
4. Sherr CJ, M RJ: **Inhibitors of mammalian G_1 cyclin-dependent kinases.** *Genes Dev* 1995, **9**:1149–1163.
5. Peter M, Herskowitz I: **Joining the complex: cyclin-dependent kinase inhibitory proteins and the cell cycle.** *Cell* 1994, **79**:181–184.
6. El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, *et al.*: ***WAF1*, a potential mediator of p53 tumor suppression.** *Cell* 1993, **75**:817–825.
7. Gu Y, Turck CW, Morgan DO: **Inhibition of CDK2 activity *in vivo* by an associated 20K regulatory subunit.** *Nature* 1993, **366**:707–710.
8. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ: **The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases.** *Cell* 1993, **75**:805–816.
9. Dulic V, Kaufmann WK, Wilson SJ, Tlsty TD, Lees E, Harper JW, *et al.*: **p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G_1 arrest.** *Cell* 1994, **76**:1013–1023.
10. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D: **p21 is a universal inhibitor of cyclin kinases.** *Nature* 1993, **366**:701–704.
11. Li Y, Jenkins CW, Nichols MA, Xiong Y: **Cell-cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21.** *Oncogene* 1994, **9**:2261–2268.
12. Noda A, Ning Y, Venable SF, Pereira-Smith OM, Smith JR: **Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen.** *Exp Cell Res* 1994, **211**:90–98.
13. Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, *et al.*: **Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD.** *Science* 1995, **267**:1018–1021.
14. Parker SB, Eichele G, Zhang P, Rawls A, Sands AT, Bradley A, *et al.*: **p53-Independent expression of $p21^{cip1}$ in muscle and other terminally differentiating cells.** *Science* 1995, **267**:1024–1027.
15. Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D: **Induction of WAF1/CIP1 by a p53-independent pathway.** *Cancer Res* 1994, **54**:3391–3395.
16. Johnson M, Dimitrov D, Vojta PJ, Barrett JC, Noda A, Pereira-Smith OM, *et al.*: **Evidence for a p53-independent pathway for upregulation of SDI1/CIP1/WAF1/p21 RNA in human cells.** *Mol Carcinog* 1994, **11**:59–64.
17. Macleod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, *et al.*: **p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage.** *Genes Dev* 1995, **9**:935–944.
18. Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang X: **Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism.** *Proc Natl Acad Sci USA* 1995, **92**:5545–5549.
19. Zhang W, Grasso L, McClain CD, Gambel AM, Cha Y, Travali S, *et al.*: **p53-Independent induction of WAF1/CIP1 in human leukemia cells is correlated with growth arrest accompanying monocyte/macrophage differentiation.** *Cancer Res* 1995, **55**:668–674.
20. Sheikh MS, Li XS, Chen JC, Shao ZM, Ordonez JV, Fontana JA: **Mechanisms of regulation of *WAF1/Cip1* gene expression in human breast carcinoma: role of p53-dependent and independent signal transduction pathways.** *Oncogene* 1994, **9**:3407–3415.
21. Steinman RA, Hoffman B, Iro A, Guillouf C, Lieberman DA, el-Houseini ME: **Induction of p21 (WAF1/CIP1) during differentiation.** *Oncogene* 1994, **9**:3389–3396.
22. Jiang H, Lin J, Su ZZ, Collart FR, Huberman E, Fisher PB: **Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53.** *Oncogene* 1994, **9**:3397–3406.

23. Zhang H, Hannon GJ, Beach D: **p21-containing cyclin kinases exist in both active and inactive states.** *Genes Dev* 1994, **8**:1750–1758.
24. Xiong Y, Zhang H, Beach D: **Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation.** *Genes Dev* 1993, **7**:1572–1583.
25. Waga S, Hannon GJ, Beach D, Stillman B: **The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA.** *Nature* 1994, **369**:574–578.
26. Strausfeld UP, Howell M, Rempel R, Maller JL, Hunt T, Blow JJ: **Cip1 blocks the initiation of DNA replication in *Xenopus* extracts by inhibition of cyclin-dependent kinases.** *Curr Biol* 1994, **4**:876–883.
27. Flores-Rozas H, Kelman Z, Dean FB, Pan Z-Q, Harper JW, Elledge SJ, *et al.*: **Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase δ holoenzyme.** *Proc Natl Acad Sci USA* 1994, **91**:655–8659.
28. Stillman B: **Initiation of chromosomal DNA replication in eukaryotes.** *J Biol Chem* 1994, **269**:7047–7050.
29. Shivji MKK, Kenny MK, Wood RD: **Proliferating cell nuclear antigen is required for DNA excision repair.** *Cell* 1992, **69**:367–374.
30. Prelich G, Tan CK, Kostura M, Mathews MB, So AG, Downey KM, *et al.*: **Functional identity of proliferating cell nuclear antigen and a DNA polymerase δ auxiliary protein.** *Nature* 1987, **326**:517–520.
31. Nichols AF, Sancar A: **Purification of PCNA as a nucleotide excision repair protein.** *Nucleic Acids Res* 1992, **20**:2441–2446.
32. Bravo R, MacDonald-Bravo H: **Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites.** *J Cell Biol* 1987, **105**:1549–1554.
33. Celis J, Madsen P: **Increased nuclear cyclin/PCNA antigen staining of non S-phase transformed human amnion cells engaged in nucleotide excision DNA repair.** *FEBS Lett* 1986, **209**:277–283.
34. Toschi L, Bravo R: **Changes in cyclin/proliferating cell nuclear antigen distribution during DNA repair synthesis.** *J Cell Biol* 1988, **107**:1623–1628.
35. Chen J, Jackson PK, Kirschner MW, Dutta A: **Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA.** *Nature* 1995, **374**:386–388.
36. Luo Y, Hurwitz J, Massague J: **Cell-cycle inhibition by independent CDK and PCNA binding domains in p21^{Cip1}.** *Nature* 1995, **375**:159–161.
37. Warbrick E, Lane DP, Glover DM, Cox LS: **A small peptide inhibitor of DNA replication defines the site of interaction between the cyclin-dependent kinase inhibitor p21^{WAF1} and proliferating cell nuclear antigen.** *Curr Biol* 1995, **5**:275–282.
38. Goubin F, Ducommun B: **Identification of binding domains on the p21^{Cip1} cyclin-dependent kinase inhibitor.** *Oncogene* 1995, **10**:2281–2287.
39. Li R, Waga S, Hanon G, Beach D, Stillman B: **Differential effects by the p21 CDK inhibitor on PCNA dependent DNA replication and DNA repair.** *Nature* 1994, **371**:534–537.
40. Shivji MKK, Grey SJ, Strausfeld UP, Wood RD, Blow JJ: **Cip1 inhibits DNA replication but not PCNA-dependent nucleotide excision–repair.** *Curr Biol* 1994, **4**:1062–1068.
41. Pagano M, Theodoras AM, Tam SW, Draetta GF: **Cyclin D1-mediated inhibition of repair and replicative DNA synthesis in human fibroblasts.** *Genes Dev* 1994, **8**:1627–1639.
42. Friedberg EC, Walker GC, Siede W: *DNA Repair and Mutagenesis.* Washington DC: American Society of Microbiology; 1995.
43. Hoeijmakers JHJ: **Nucleotide excision repair II: from yeast to mammals.** *Trends Genet* 1993, **9**:211–217.
44. Hanawalt P: **Transcription-coupled repair and human disease.** *Science* 1994, **266**:1957–1958.
45. Sancar A: **Mechanisms of DNA excision repair.** *Science* 1994, **266**:1954–1955.
46. Miura M, Domon M, Sasaki T, Kondo S, Takasaki Y: **Two types of proliferating cell nuclear antigen (PCNA) complex formation in quiescent normal and xeroderma pigmentosum group A fibroblasts following ultraviolet light (UV) irradiation.** *Exp Cell Res* 1992, **201**:541–544.
47. Mu D, Park C-H, Matsunaga T, Hsu DS, Reardon JT, Sancar A: **Reconstitution of human DNA repair excision nuclease in a highly defined system.** *J Biol Chem* 1995, **270**:2415–2418.
48. Aboussekhra A, Biggerstaff M, Shivji MKK, Vilpo JA, Moncollin V, Podust VN, *et al.*: **Mammalian DNA nucleotide excision repair reconstituted with purified protein components.** *Cell* 1995, **80**:859–868.
49. Di Leonardo A, Linke SP, Clarkin K, Wahl GM: **DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts.** *Genes Dev* 1994, **8**:2540–2551.
50. Deng C, Zhang P, Harper JW, Elledge SJ, Leder P: **Mice lacking p21*CIP1/WAF1* undergo normal development, but are defective in G_1 checkpoint control.** *Cell* 1995, **82**:675–684.
51. Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T, Hannon GJ: **Radiation-induced cell cycle arrest compromised by p21 deficiency.** *Nature* 1995, **377**:552–557.
52. Waldman T, Kinzler KW, Vogelstein B: **p21 is necessary for the p53-mediated G_1 arrest in human cancer cells.** *Cancer Res* 1995, **55**:5187–5190.
53. Lu X, Lane DP: **Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes?** *Cell* 1993, **75**:765–778.
54. Dahmann C, Diffley JFX, Nasmyth KA; **S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state.** *Curr Biol* 1995, **5**:1257–1269.
55. Schwob E, Bohm T, Mendenhall MD, Nasmyth K; **The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S. cerevisiae.*** *Cell* 1994, **79**:233–244.
56. Nishida C, Reinhard P, Linn S: **DNA repair synthesis in human fibroblasts requires DNA polymerase ε.** *J Biol Chem* 1988, **263**:501–510.
57. Wang Z-G, Wu X-H, Friedberg EC: **DNA repair synthesis during base excision repair *in vitro* is catalyzed by DNA polymerase ε and is influenced by DNA polymerase α and δ in *Saccharomyces cerevisiae.*** *Mol Cell Biol* 1993, **13**:1051–1058.
58. Ayyagari R, Impellizzeri KJ, Yoder BL, Gary SL, Burgers PMJ: **A mutational analysis of the yeast proliferating cell nuclear antigen indicates distinct roles in DNA replication and DNA repair.** *Mol Cell Biol* 1995, **15**:4420–4429.
59. Spector DL, Smith HC: **Redistribution of U-snRNPs during mitosis.** *Exp Cell Res* 1986, **163**:87–94.