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Cell cycle arrest and DNA endoreduplication following p21$^{Waf1/Cip1}$ expression

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p21$^{Waf1/Cip1}$ is a major transcriptional target of p53 and has been shown to be one of the principal mediators of the p53 induced G1 cell cycle arrest. We show that in addition to the G1 block, p21$^{Waf1/Cip1}$ can also contribute to a delay in G2 and expression of p21$^{Waf1/Cip1}$ gives rise to cell cycle profiles essentially indistinguishable from those obtained following p53 expression. Arrest of cells in G2 likely reflects an inability to induce cyclin B1/cdc2 kinase activity in the presence of p21$^{Waf1/Cip1}$, although the inefficient association of p21$^{Waf1/Cip1}$ and cyclin B1 suggests that the mechanism of inhibition is indirect. Cells released from an S-phase block were not retarded in their ability to progress through S-phase by the presence of p21$^{Waf1/Cip1}$, despite efficient inhibition of cyclin E, A and B1 dependent kinase activity, suggesting that p21$^{Waf1/Cip1}$ is inefficient at inhibiting replicative DNA synthesis *in vivo*. Interestingly, significant numbers of cells released from the p21$^{Waf1/Cip1}$ activated G2 block undergo endoreduplication, passing through another S-phase before undergoing mitosis. This supports a function of the mitotic kinases in both entry into mitosis, and also in preventing re-replication of DNA following S-phase and suggests a role for p21$^{Waf1/Cip1}$ in coupling DNA synthesis and mitosis. Unlike p53, which induces apoptosis in these cells, extended expression of p21$^{Waf1/Cip1}$ resulted in the expression of a senescent-like phenotype in these p53 null, pRB null tumor cells.

Keywords: p21$^{Waf1/Cip1}$; cell cycle arrest; cyclin dependent kinase; endoreduplication; senescence

Introduction

Cell cycle control is a tightly regulated process during which alternating rounds of DNA synthesis (S-phase) and mitosis (M-phase) are coordinated by a series of checkpoints. Progression through the cell cycle is driven by a family of cyclin-dependent kinases (cdks) whose activity is regulated through binding of cyclin regulatory molecules (Sherr, 1993; Hunter and Pines, 1994). Cyclin D/cdk4 and cyclin D/cdk6 complexes regulate early events in the G1 phase of the cell cycle, while cdk2 activity regulated by cyclins A and E appears to be critical for the initiation of DNA synthesis. Transit through mitosis is regulated by cdc2 (cdk1) in combination with cyclin B1, although cyclin A may also play some role. In addition to the positive regulation of cdk activity provided by cyclins, there are an increasing number of regulatory mechanisms that serve to inhibit cdk activity. Primary amongst these is the expression of cdk inhibitors (CDKIs) that can bind to and inhibit cyclin/cdk complexes (Sherr and Roberts, 1996). These fall into two classes, the INK family which bind to cdk4 and cdk6 and inhibit complex formation with D-cyclins, and the p21$^{Waf1/Cip1}$ family that bind to cyclin/cdk complexes and inhibit kinase activity.

The p21$^{Waf1/Cip1}$ family of CDKIs include p21$^{Waf1/Cip1}$, p27 and p57 and are broad range inhibitors of cyclin/cdk complexes (Gu *et al*., 1993; Harper *et al*., 1993; Xiong *et al*., 1993; Firpo *et al*., 1994; Polyak *et al*., 1994; Toyoshima and Hunter, 1994; Lee *et al*., 1995; Matsuoka *et al*., 1995). The crystal structure of p27/cyclin A/cdk2 indicated that this family of proteins make specific interactions with both cyclin and cdk (Russo *et al*., 1996), and this is reflected by primary sequence conservation within the p21$^{Waf1/Cip1}$ family members (Harper *et al*., 1993; Xiong *et al*., 1993; El-Deiry *et al*., 1994; Noda *et al*., 1994; Polyak *et al*., 1994; Toyoshima and Hunter, 1994; Lee *et al*., 1995; Matsuoka *et al*., 1995). This is supported by both *in vitro* and *in vivo* evidence for complex formation between p21$^{Waf1/Cip1}$ and cyclin A/cdk2, cyclin E/cdk2 and cyclin D/cdk4/6 complexes (Gu *et al*., 1993; Harper *et al*., 1993; Xiong *et al*., 1993; Hall *et al*., 1995). By contrast, the major mitotic cyclin B1/cdc2 complex associates less efficiently with p21$^{Waf1/Cip1}$, and the kinase activity associated with this complex remains relatively insensitive to even high levels of p21$^{Waf1/Cip1}$ *in vitro* (Harper *et al*., 1993; Xiong *et al*., 1993; Hall *et al*., 1995; Dulic *et al*., 1998). Interestingly, all of these inhibitors also serve as adapter proteins that allow assembly of the cyclins and cdks and target their nuclear localization. Therefore, at low levels p21$^{Waf1/Cip1}$ contributes to the formation of active cyclin/cdk complexes, which are then inhibited as p21$^{Waf1/Cip1}$ levels increase (Zhang *et al*., 1994; LaBaer *et al*., 1997).

A common theme in the cellular functioning of the CDKIs is their induced expression in response to a broad spectrum of cellular stresses, thus allowing the cell to regulate cell cycle progression in response to extracellular and intracellular cues (Sherr and Roberts, 1996). One of the most significant cellular stresses is the response to DNA damage, where p53-mediated p21$^{Waf1/Cip1}$ induction has been shown to play a critical role (Brugarolas *et al*., 1995; Deng *et al*., 1995; Waldman *et al*., 1995; Polyak *et al*., 1996). p53 levels are elevated in response to DNA damage, associated with an increase in the half-life of the protein, and through its sequence-specific DNA binding function, p53 is able to enhance the transcription of a number of genes, including p21$^{Waf1/Cip1}$ (Bates and Vousden, 1996).

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The p53-mediated response to genotoxic stress is characterized by the inhibition of cell cycle progression at both G1 and G2 checkpoints, and in some cells apoptotic cell death. The importance of p53's role in the DNA damage response is underlined by its mutation in many human tumours, allowing both the survival of abnormal cells and replication of damaged DNA, which in turn can lead to genomic instability and the acquisition of further oncogenic abnormalities.

The generation of mice homozygously null for p21$^{Waf1/Cip1}$ has shown that p21$^{Waf1/Cip1}$ is not essential for development and these animals fail to show the increased rate of tumorigenesis associated with p53 loss. Fibroblasts from these animals, however, are impaired in their ability to arrest in Gl following DNA damage (Brugarolas *et al*., 1995; Deng *et al*., 1995), and similar results have been reported for both human diploid fibroblasts and human tumour lines deleted for p21$^{Waf1/Cip1}$ (Waldman *et al*., 1995; Polyak *et al*., 1996; Brown *et al*., 1997). The latter cells have also suggested a role for p21$^{Waf1/Cip1}$ in both the p53-mediated G2 arrest response and the coupling of DNA synthesis and mitosis (Polyak *et al*., 1996; Waldman *et al*., 1996).

In addition to the ability to bind and inhibit cyclin/cdk complexes, p21$^{Waf1/Cip1}$ also has the ability to interact with PCNA (Xiong *et al*., 1992; Chen *et al*., 1995; Luo *et al*., 1995), to inhibit stress activated protein kinases (Shim *et al*., 1996) and interact with Gadd45 (Kearsey *et al*., 1995). *In vitro* studies showed that the interaction of p21$^{Waf1/Cip1}$ blocks the ability of PCNA to activate DNA polymerase δ, potentially by disrupting PCNA/Fen1 complexes (Chen *et al*., 1996; Warbrick *et al*., 1997). However, the subsequent inhibition of DNA synthesis was shown to be limited to replicative but not repair synthesis (Li *et al*., 1994; Shivji *et al*., 1994), leading to an attractive model in which DNA damage induced p21$^{Waf1/Cip1}$ expression, through p53, arrests DNA replication but allows repair of the damage to proceed. Indeed, functional p21$^{Waf1/Cip1}$ appears to be

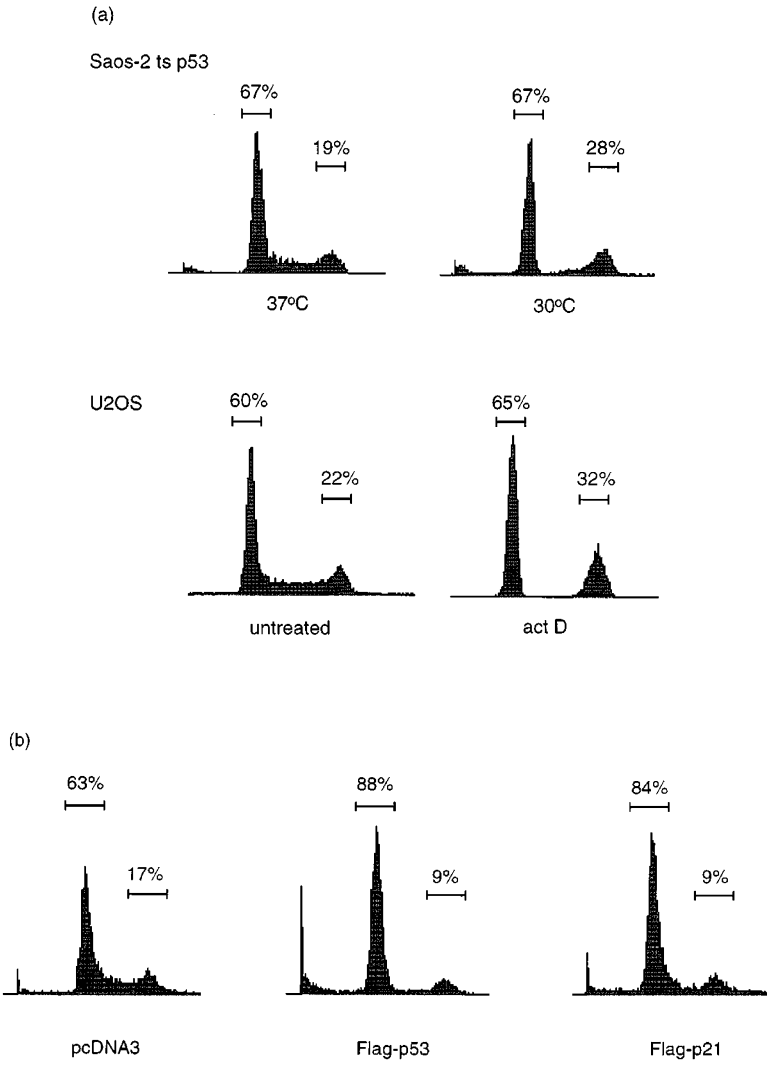


Figure 1 p53 induced cell cycle arrest. (**a**) Cell cycle analysis of Saos-2 cells stably expressing the human temperature sensitive p53143A (Saos-ts p53) mutant either at the restrictive temperature (37°C) or 24 h following shift to the permissive temperature (30°C) and U20S cells either untreated or 24 h following treatment with 5 nM actinomycin D. (**b**) CD20 co-transfection was utilised to analyse the cell cycle profiles of Saos-2 cells transiently transfected with 20 μg of the indicated plasmids and CD20 positive cells were identified using a FITC conjugated anti-CD20 antibody. Percentage of live cells (excluding cells with less that 2N DNA content) in G1 and G2/M phases are indicated

required for efficient repair of DNA damage (McDonald *et al*., 1996).

A role for both p53 and p21$^{Waf1/Cip1}$ has also been implicated in cellular aging (Harvey *et al*., 1993; Noda *et al*., 1994; Atadja *et al*., 1995; Bond *et al*., 1995). p53 levels accumulate as diploid fibroblasts age, with a commensurate increase in the levels of p21$^{Waf1/Cip1}$ and this coincides with a gradual slowing of cellular growth and inhibition of cyclin/cdk activity. Many studies have shown that fibroblasts homozygously deleted for either p53 or p21$^{Waf1/Cip1}$ escape from senescence, suggesting that these proteins play a critical role in the process (Harvey *et al*., 1993; Deng *et al*., 1995; Brown *et al*., 1997). In agreement with this concept, a recent study has shown that expression of exogenous p53 in the p53-null tumour line EJ results an increase in cell size, change in morphology and the induction of senescence associated β-Galactosidase activity, all characteristics of senescent cells (Sugrue *et al*., 1997). It seems clear, however, that many other factors are also important in the induction and maintenance of the senescent phenotype.

In this study we show that p21$^{Waf1/Cip1}$ can induce both a G1 arrest and G2 delay, but fails to significantly delay progress through S-phase in the p53/pRB null osteosarcoma cell line, Saos-2. p21$^{Waf1/Cip1}$ expression significantly inhibited all cyclin dependent kinase activity and reversal of the p21$^{Waf1/Cip1}$ expression resulted in unscheduled DNA replication in the G2 blocked cells. Sustained p21$^{Waf1/Cip1}$ expression resulted in dramatic morphological changes and the expression of senescence associated β-Galactosidase activity.

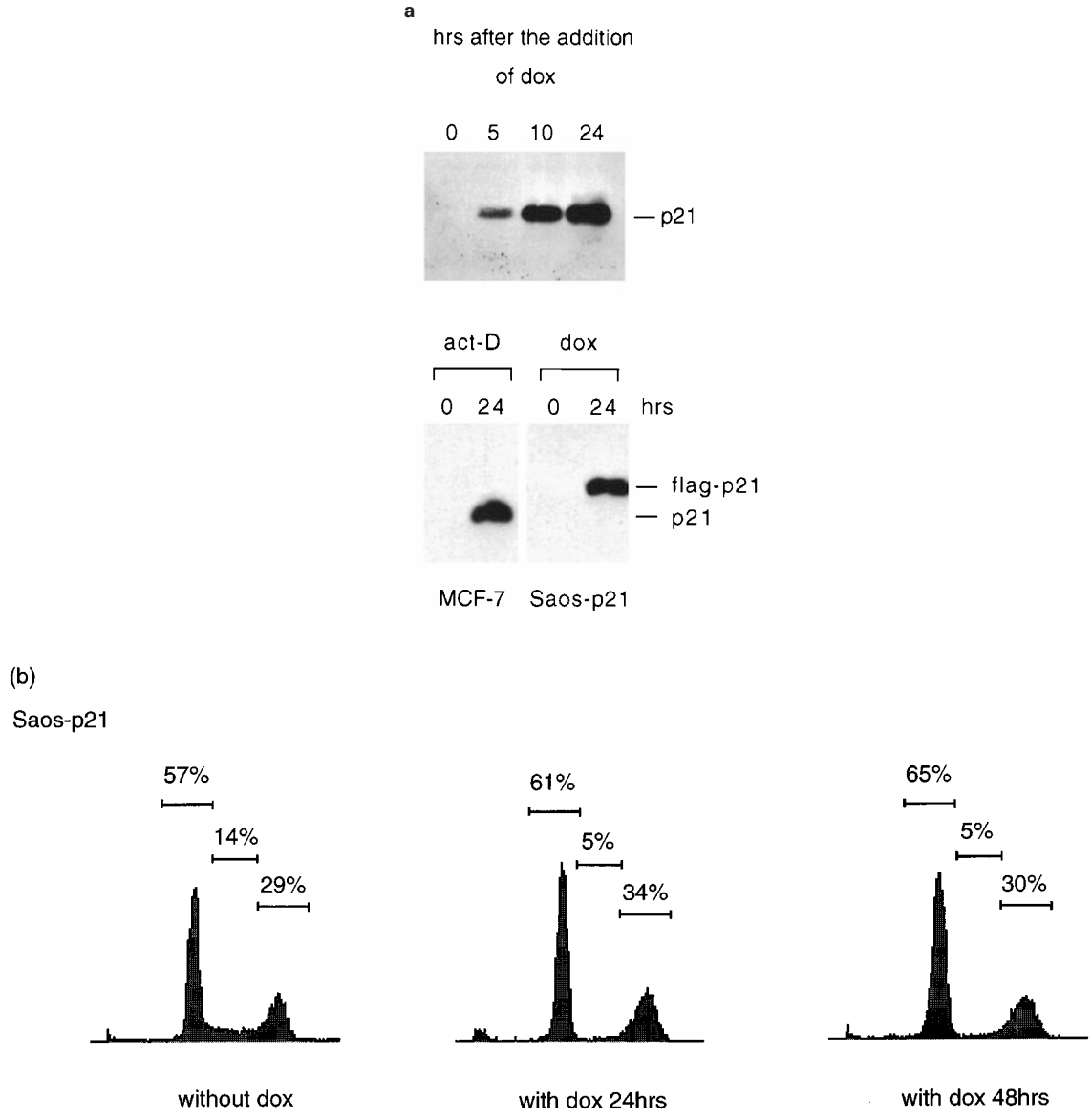


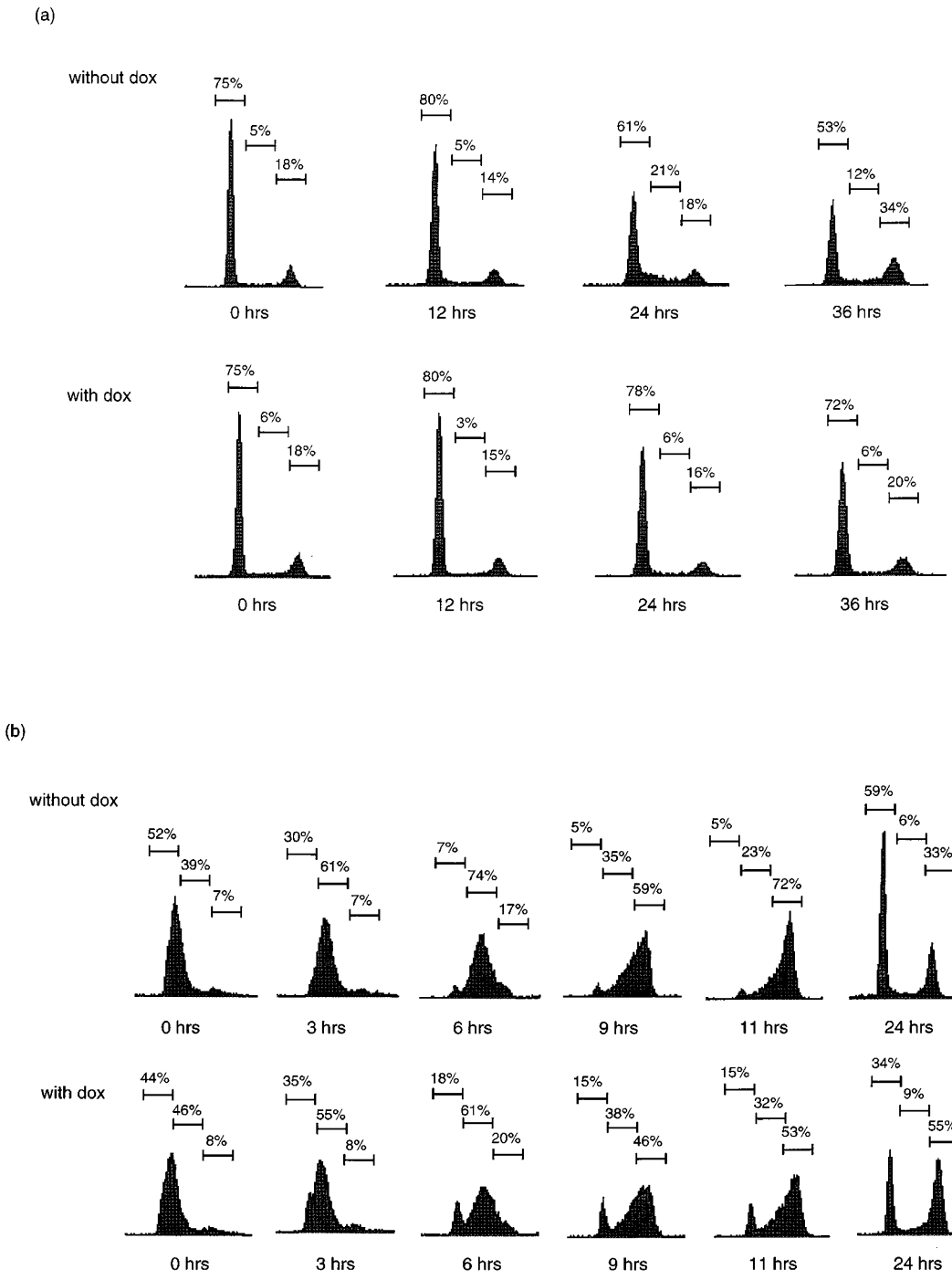
Figure 2 Characterization of p21$^{Waf1/Cip1}$ inducible cell line. (**a**) Saos-2 cells inducibly expressing Flag-p21$^{Waf1/Cip1}$ (Saos-p21) were treated with of 2 μg/ml of doxycycline and protein expression monitored by Western analysis, using an anti-Flag antibody, at the indicated times. For comparison, levels of p21$^{Waf1/Cip1}$ expression following activation of p53 by actinomycin-D treatment of a wild type p53 expressing cell (MCF-7) are shown. (**b**) Saos-p21 were either untreated or treated with 2 μg/ml doxycycline for 24 or 48 h (as indicated) and DNA profiles analysed by flow cytometry. The percentage of cells in the G1, S and G2/M phases of the cell cycle are indicated

Results

Inducible expression of p21Waf1/Cip1 in Saos-2 cells

Analysis of Saos-2 cells stably transfected with a human temperature sensitive p53 (ts p53) mutant showed that following shift to the permissive temperature, activation of p53 function resulted in both G1 and G2 blocks which were similar to those seen following DNA damage of the wild type p53 expressing human cell line U2OS (Figure 1a). However, similar transient transfections of p53 into the same cell line resulted in a predominantly G1 arrest (Figure 1b), suggesting that this was not a good assay to study the p53-mediated G2 arrest.

In order to gain further insight into the possible role of $p21^{Waf1/Cip1}$ in p53-mediated G1 and G2 arrests, a Flag-epitope tagged $p21^{Waf1/Cip1}$ construct was generated. Transient transfection of this construct into Saos-2 cells resulted in an efficient G1 arrest in a similar manner to p53 (Figure 1b) and in agreement with published data. Given the bias towards G1 arrest seen with p53 in transient assays, we sought to ask whether inducible $p21^{Waf1/Cip1}$ expression could also arrest cells in the G2 phase. To this end we generated a tetracycline inducible Saos-2 cell line for Flag-$p21^{Waf1/Cip1}$ (Saos-p21) and were able to demonstrate inducible expression of $p21^{Waf1/Cip1}$ protein after addition of the tetracycline analogue doxycycline (dox) (Figure 2a). The levels of $p21^{Waf1/Cip1}$ expression in this inducible system were



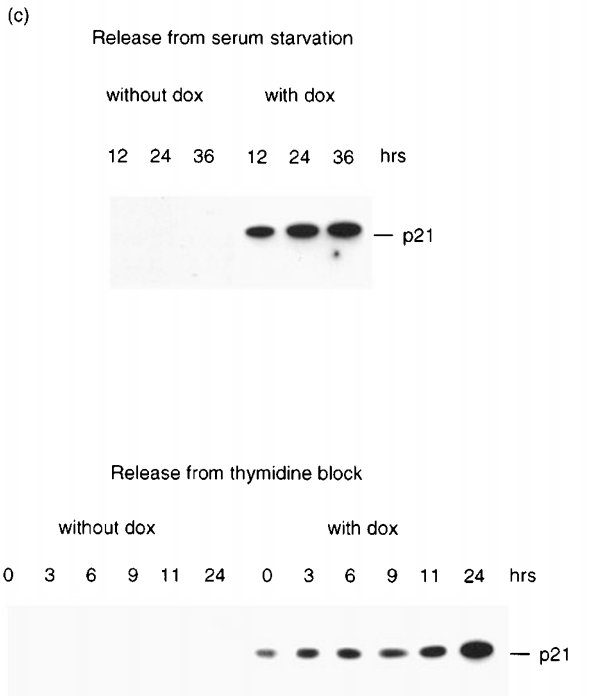
similar to the levels of endogenous p21Waf1/Cip1 seen following DNA damage of wild type p53 expressing MCF-7 cells. Analysis of DNA profiles by flow cytometry showed that induction of p21Waf1/Cip1 expression in logarithmically growing cells resulted in a loss of cells from S-phase and accumulation of cells in G1 and G2 in a manner similar to that seen for p53 (Figure 2b). Induction of p21Waf1/Cip1 for longer times showed a gradual loss of cells from G2 and accumulation of cells in G1, suggesting that the G2 block may be less complete than the G1 block. A similar redistribution of cells was noted in DNA profiles from actinomycin D treated U2OS cells and Saos-ts p53 at the permissive temperature (data not shown). Consistent with previous results, expression of p21Waf1/Cip1 did not contribute to an enhanced apoptotic rate.

p21Waf1/Cip1 expression contributes to G1 and G2 arrests but does not impede S-phase progression

The known functions of p21Waf1/Cip1 as an inhibitor of cyclin dependent kinases and PCNA suggest a potential role for this protein in the inhibition of normal progress through all stages of the cell cycle. To more closely examine the consequences of p21Waf1/Cip1 expression we analysed the effect of p21Waf1/Cip1 expression in cell populations synchronized at different stages of the cell cycle.

To examine the effect of p21Waf1/Cip1 on G1/S progression, Saos-p21 cells were serum starved, with or without doxycycline, and then released into serum containing medium (Figure 3a). In the absence of doxycycline induced p21Waf1/Cip1 expression cells were seen to re-enter DNA synthesis within 24 h following release and by 36 h were transiting between G2/M and a new G1 phase. Activation of p21Waf1/Cip1 prior to release from serum starvation essentially prevented S-phase transit, and these cells remained blocked at the G1/S phase boundary. Interestingly, the G1 peak became broader with time, suggesting that a proportion of the cells were attempting to initiate DNA synthesis, but the lack of cells progressing further into S-phase indicates that was an inefficient process. p21Waf1/Cip1 expression was confirmed by Western blotting (Figure 3c).

p21Waf1/Cip1 has been shown to interact with PCNA and efficiently inhibit DNA replication in *in vitro* systems. Nevertheless, activation of p53 (and thereby p21Waf1/Cip1) does not lead to the accumulation of cells in S-phase, suggesting either that p53 does not activate p21Waf1/Cip1 during S-phase or that p21Waf1/Cip1 expression does not necessarily inhibit DNA synthesis. In order to examine this more closely, Saos-p21 cells were serum starved, then released into thymidine to establish a block at the G1/S phase transition. Cells were then treated with doxycycline to activate p21Waf1/Cip1 expression and released from the thymidine block (Figure 3b). In contrast to the uninduced cells, a small proportion of the p21Waf1/Cip1 induced cells became arrested at a point in G1, the majority of the p21Waf1/Cip1 expressing cells, however,



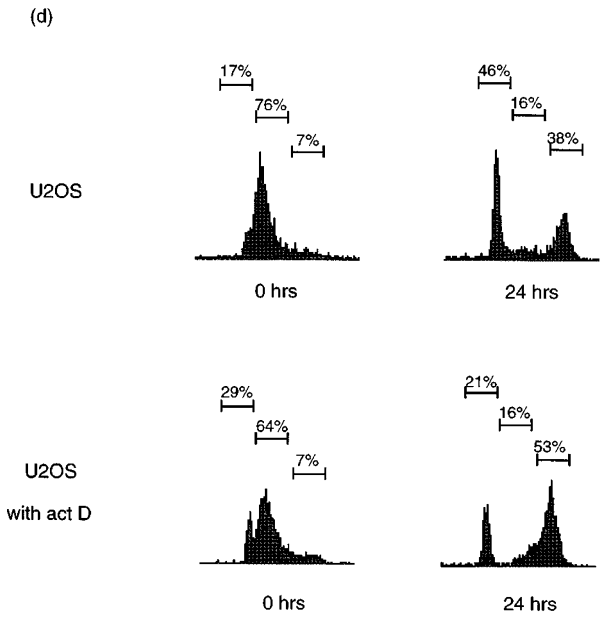
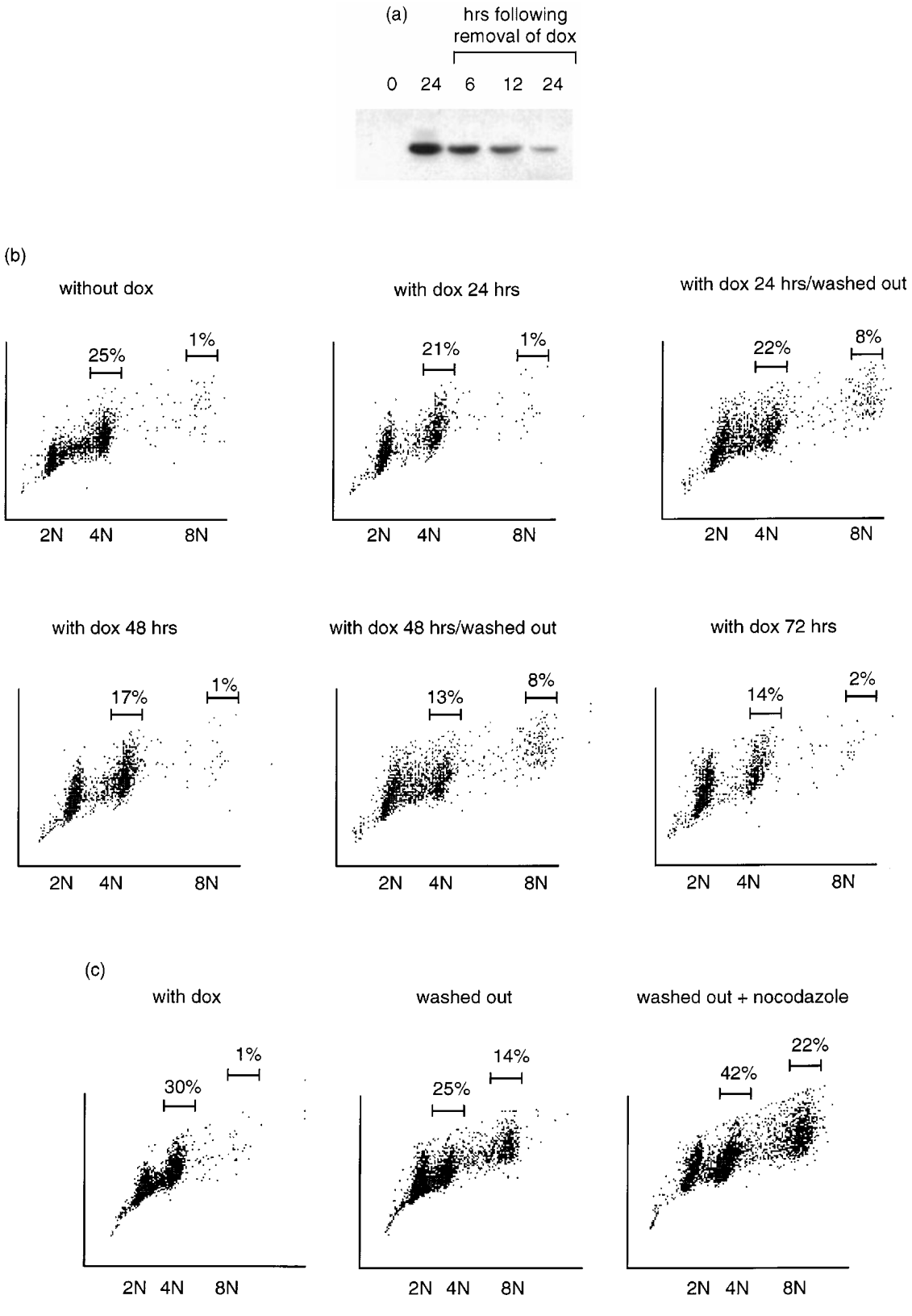


Figure 3 Analysis of p21Waf1/Cip1-mediated G1 and G2 arrests. (**a**) Saos-p21 cells were quiesced in serum free medium for 48 h, and stimulated to re-enter the cell cycle in the presence (with dox) or absence (without dox) of p21Waf1/Cip1 expression. At the indicated times following restimulation cells were collected for flow cytometry and Western blot analysis (**c**). (**b**) Saos-p21 cells were blocked at the G1/S boundary by incubation with 2 mM thymidine and p21Waf1/Cip1 induced (with dox) or not (without dox). The cells were released into fresh medium and collected for flow cytomtery and Western blot analysis (**c**) at the indicated times. (**c**) p21Waf1/Cip1 expression at the indicated times following release from quiescence (top) and a thymidine block (bottom) as monitored by anti-Flag Western blot anlaysis. (**d**) DNA profiles of U2OS cells arrested at the G1/S transition by thymidine block and 24 h following release with or without treatment with the DNA damaging drug actinomycin D. The percentage of cells in the G1, S and G2/M phases are indicated

moved through DNA synthesis at a rate very similar to that seen in uninduced cells. While p21$^{Waf1/Cip1}$ expressing cells released from the thymidine block failed to show any obvious defect in S-phase progression, they were clearly delayed in progress through G2/M compared to uninduced cells. p21$^{Waf1/Cip1}$ expression was confirmed by Western blotting (Figure 3c). Similar results were also seen, when thymidine blocked U2OS cells were treated with the DNA damaging drug actinomycin D (Figure 3d) which efficiently induces expression of p53 and p21$^{Waf1/Cip1}$. These results suggest that p21$^{Waf1/Cip1}$-mediated inhibition of DNA synthesis does not significantly block initiated replicative DNA synthesis *in vivo*.



Reversal of p21$^{Waf1/Cip1}$ induced G2 arrest results in endoreduplication

Given that p21$^{Waf1/Cip1}$ expression is dependent on the presence of doxycycline, removal of the drug should inhibit p21$^{Waf1/Cip1}$ protein expression and reverse its cellular effects. Indeed, this is the case, and removal of doxycycline resulted in rapid reduction of p21$^{Waf1/Cip1}$ protein levels (Figure 4a) and the resumption of essentially normal cell cycle progression. However, we did note that on release of cells from p21$^{Waf1/Cip1}$-mediated cell cycle arrest a small proportion of the cells (approximately 5%) appeared to attain an 8N DNA content (Figure 4b). We reasoned that this endoreduplication must represent cells blocked in the G2 phase that entered DNA synthesis as opposed to the more appropriate mitosis. Consistent with this idea, increasing the proportion of the cells blocked in G2 by prior thymidine block caused a commensurate increase in the proportion of cells attaining an 8N DNA content (approximately 15%). Further treatment with nocodazole, a spindle inhibitor which arrests cells in G2/M, indicated that up to 40% of the cells acquired a 8N DNA content (Figure 4c). Analysis of cells which had incorporated BrdU during this experiment showed that whilst DNA synthesis was limited to 2N–4N cells in populations released from a thymidine block but not activated for p21$^{Waf1/Cip1}$, BrdU incorporating cells released from the p21$^{Waf1/Cip1}$ induced G2 block showed both 2N–4N and 4N–8N DNA contents (Figure 4d).

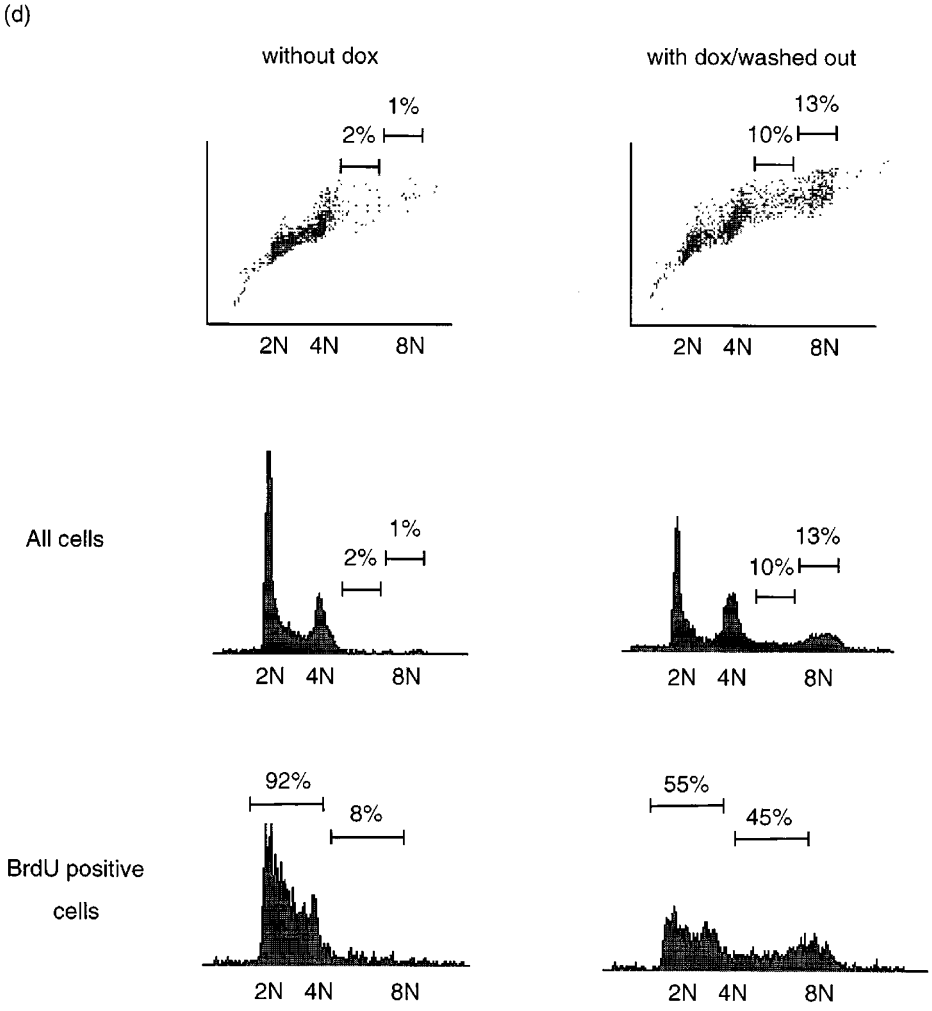


Figure 4 Release from p21$^{Waf1/Cip1}$-mediated G2 arrest can result in endoreduplication. (**a**) Time course showing decrease in p21$^{Waf1/Cip1}$ expression levels following removal of doxycyline. (**b**) DNA profiles of logarithmically growing Saos-p21 cells treated with doxycycline for the indicated times. Where indicated p21$^{Waf1/Cip1}$-mediated arrest was reversed by replacement of the medium (washed out). Doublet discriminator plots are shown (x-axis propidium iodide (PI) peak area, y-axis PI peak width) as the endoreduplicated cells are more clearly identifiable than in the DNA histograms (see **c**). (**c**) Saos-p21 cells were synchronised at the G1/S boundary by thymidine block and released in the presence of doxycycline for 24 h to establish a p21$^{Waf1/Cip1}$-mediated G2 arrest (with dox). The p21$^{Waf1/Cip1}$ arrest was subsequently reversed by washing out the doxycycline and incubating in fresh medium for 24 h. Cells were collected for flow cytometry either immediately (washed out) or following overnight incubation with the mitotic spindle inhibitor nocodazole (washed out + nocodazole). The position of 2N, 4N and 8N DNA contents and the percentage of cells with 4N and 8N DNA contents are indicated. (**d**) Saos-p21 cells were blocked with thymidine and a p21$^{Waf1/Cip1}$-mediated G2-arrest was established by release of the cells into doxycycline; cells released in the absence of doxycycline served as a control. Twenty-four hours after the doxycycline was washed out, cells were pulsed for 1 h with 10 μM BrdU and collected for cell cycle analysis. Flow cytometry profiles are shown as doublet discriminator plots (top panel) and DNA histograms of all the cells (middle panel) and only those cells which have incorporated BrdU (lower panel). The position of 2N, 4N and 8N DNA contents and the percentage of cells with between 4–8N and 8N DNA contents are indicated.

These results suggest that a proportion of cells undergoing a p21$^{Waf1/Cip1}$ G2 arrest lose the ability to follow a normal cell cycle progression after release from the block, but rather enter a second round of DNA synthesis.

Cyclin dependent kinase activity

Cyclin dependent kinase activity is essential for both G1/S and G2/M progression, although different kinase complexes are important at different stages of the cell cycle. The D-cyclins appear to play a major role in allowing entry into the cell cycle and progress through the restriction point, but the absence of pRB and the overexpression of p16 in Saos-2 cells means that the D-cyclins are unlikely to have a major role in regulating cell cycle transitions in these cells. We therefore, analysed the consequences of p21$^{Waf1/Cip1}$ induction on the activity of cyclin A, cyclin E and cyclin B1 complexes. Induction of p21$^{Waf1/Cip1}$ in logarithmically growing cells resulted in an efficient inhibition of cyclin A, cyclin E (Figure 5) and cdk2 (data not shown) activity, consistent with previous reports. Surprisingly, cyclin B1 associated kinase activity was also impaired to a similar extent to cyclin E. Given that cyclin B1 has a primary role in the regulation of the G2/M transition, any loss of cyclin B1 kinase activity would be expected to greatly impair entry into mitosis.

To analyse this effect more closely Saos-p21 cells were synchronized at the G1/S phase boundary and released in the presence or absence of doxycycline (Figure 6a). Immunoprecipitation of cyclin B1 complexes as the cells progressed through the remainder of the cycle clearly showed that cyclin B1 associated kinase activity was activated as the uninduced cells entered mitosis, and this was greatly impaired by the presence of p21$^{Waf1/Cip1}$ (Figure 6b). Analysis of protein expression in the induced cells, showed that cyclin Bl was still being expressed, and while cyclin A efficiently associated with p21$^{Waf1/Cip1}$, cyclin B1 was not present in p21$^{Waf1/Cip1}$ complexes at significant levels (Figure 6c). These data are consistent with previous reports showing that cyclin B1 complexes are inefficiently bound and inhibited by p21$^{Waf1/Cip1}$, indicating that cyclin B1 associated kinase activity may be indirectly inhibited by p21$^{Waf1/Cip1}$.

Activation of markers of senescence

While short term induction of p21$^{Waf1/Cip1}$ had little obvious effect on Saos-2 cell shape, we noted that induction of p21$^{Waf1/Cip1}$ for 72 h or longer caused the cells to undergo a striking change in morphology, becoming much flatter and larger. Along with the morphological changes, some apoptotic cell death was also noted and occasionally very large cells were seen that showed a striated appearance; a similar phenotype has previously been reported by others (Sheikh *et al*., 1995) (Figure 7). These gross morphological changes could be explained by either initiation of a process of differentiation or by the induction of senescence. Analysis of expression patterns for three osteoblast differentiation markers (alkaline phosphatase, osteopontin and osteocalcin (Aubin *et al*., 1995)) failed to show an indication of differentiation (data not shown). However, senescence associated β-Galactosidase activity was clearly detectable following 5 days of p21$^{Waf1/Cip1}$ induction, indicating that these cells have acquired at least some characteristics of a senescent phenotype (Figure 7).

Discussion

Cell cycle inhibition by p21$^{Waf1/Cip1}$

We have used a tetracycline inducible system to examine the effect of p21$^{Waf1/Cip1}$ expression at different stages of the cell cycle in a human osteosarcoma cell line Saos-2. Inducible expression of p21$^{Waf1/Cip1}$ resulted in inhibition of cell cycle progression at both the G1/S and the G2/M transitions. Expression of p21$^{Waf1/Cip1}$ efficiently inactivated cyclin E and cyclin A dependent kinase activity, and was able to block the entry of cells into DNA synthesis after release from serum starvation. These data are also consistent with the ability of transiently transfected p21$^{Waf1/Cip1}$ to induce a G1 arrest and shows the p21$^{Waf1/Cip1}$-mediated G1 arrest function can be active despite the absence of functional pRB, although a recent study has shown that the G1 block is

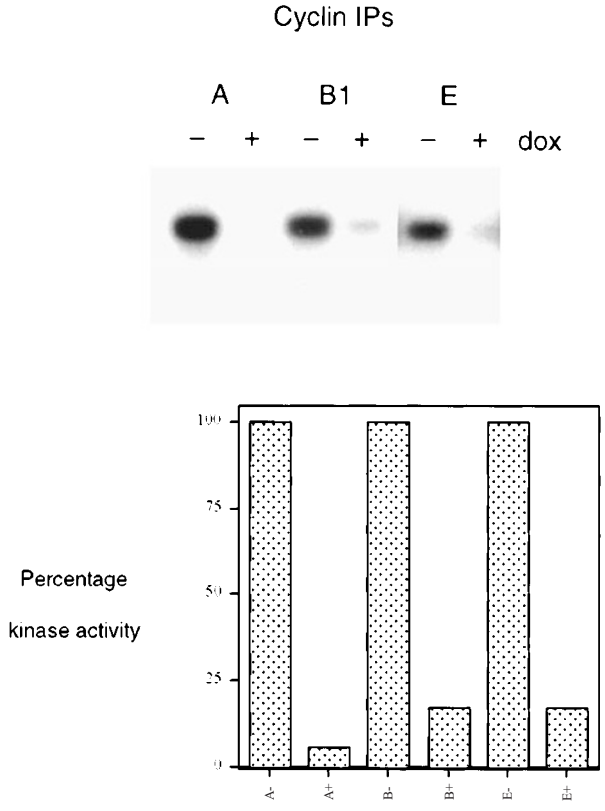


Figure 5 Inhibition of cyclin-associated kinase activity by p21$^{Waf1/Cip1}$. Logarithmically growing Saos-p21 were either untreated (−) or treated with doxycyline for 24 h (+), cyclin A, B1, and E complexes inimunoprecipitated and kinase activity towards histone H1 monitored. The cyclin E associated kinase activity was less intense that cyclin A and B1 and consequently a longer exposure is shown. These data were quantified using a phosphorimager and plotted as a percentage of the starting activity

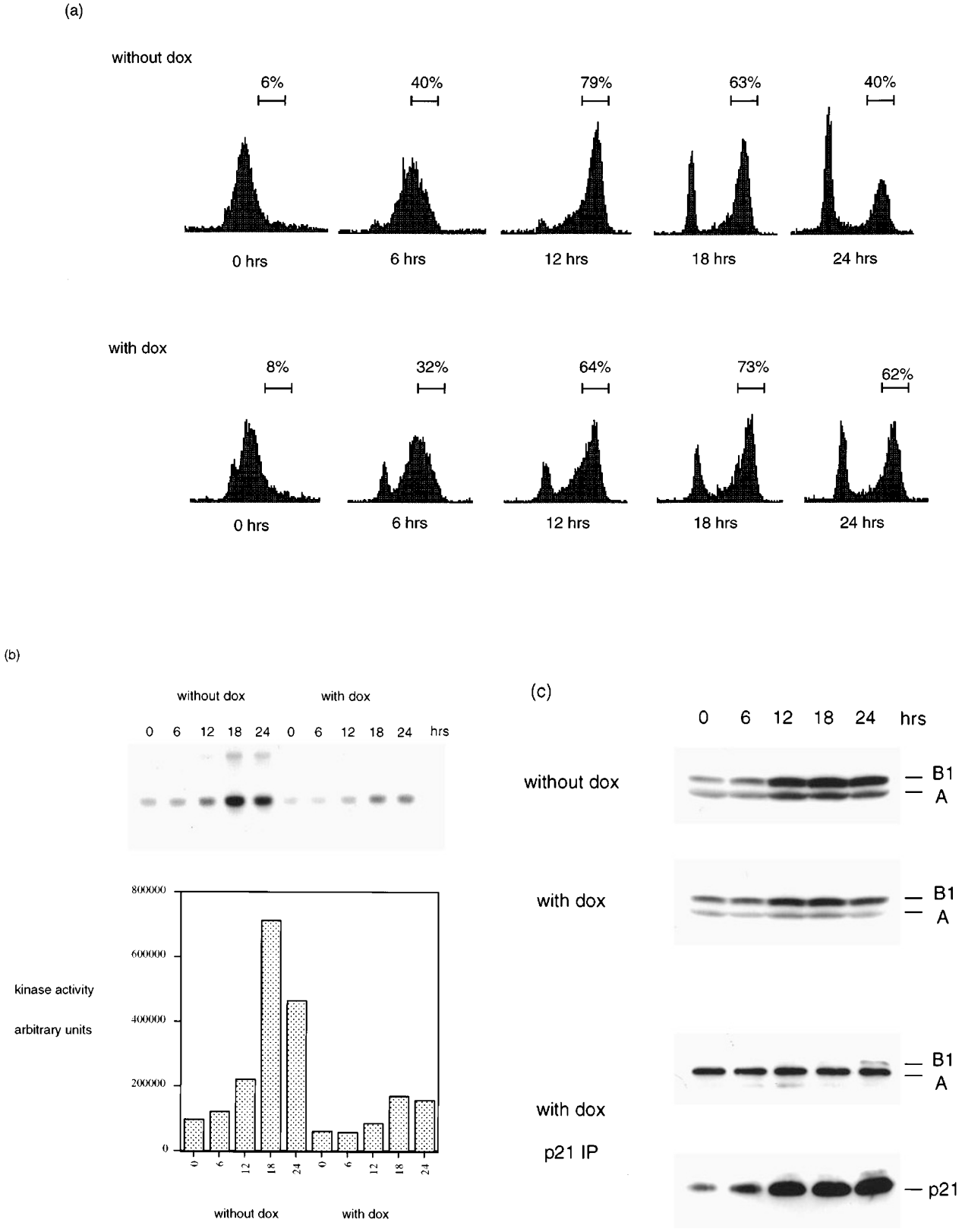


Figure 6 $p21^{Waf1/Cip1}$-mediated inhibition of cyclin B1 is indirect. (**a**) DNA profiles of Saos-p21 cells released from a thymidine block in the presence (with dox) or absence (without dox) of $p21^{Waf1/Cip1}$ expression. The percentage of cycling cells (excluding those blocked in G1 by $p21^{Waf1/Cip1}$ induction) in the G2/M phase of the cell cycle is indicated. (**b**) Cyclin B1 associated kinase activity towards histone H1 in cells released from a thymidine block. Radioactive incorporation into histone H1 was monitored by autoradiogram (top) and data quantified using a phophorimager (bottom); kinase activity is indicated in arbitrary units. (**c**) Western analysis of cyclin A and cyclin B1 levels in whole cell lysates of cells released from a G1/S block in the presence (with dox) or absence (without dox) of $p21^{Waf1/Cip1}$ expression. Cells released from the G1/S block in the presence of $p21^{Waf1/Cip1}$ were also subjected to immunoprecipitation with an anti-Flag antibody, and the precipitation of $p21^{Waf1/Cip1}$, cyclin A and cyclin B1 monitored by Western blotting

stronger in cells with functional pRB (Niculescu *et al*., 1998). Previous studies have shown that, while D-cyclin activity is dispensable for cell cycle progression in pRB-null cells, cyclin E activity is essential (Lukas *et al*., 1994) and, therefore, the ability of p21$^{Waf1/Cip1}$ to inhibit G1 progression is probably due to its ability to inhibit cdk2 kinase activity.

Our study also demonstrates a role for p21$^{Waf1/Cip1}$ in the activation of a G2 arrest, a contention supported by other recent reports (Dulic *et al*., 1998; Medema *et al*., 1998; Niculescu *et al*., 1998). This is likely to reflect the inability of cells with induced p21$^{Waf1/Cip1}$ to activate cyclin B1 associated kinase activity at the G2/M transition. *In vitro* results have shown that cyclin Bl/cdc2 complexes are inefficiently bound by p21$^{Waf1/Cip1}$, and this is reflected by the relative insensitivity of cyclin B1 associated kinase activity to p21$^{Waf1/Cip1}$ (Harper *et al*., 1993; Xiong *et al*., 1993; Hall *et al*., 1995). This point of view is supported by the relatively small amount of cyclin B1 that co-immunoprecipitates with p21$^{Waf1/Cip1}$ *in vivo* both in our system and others (Harper *et al*., 1993; Xiong *et al*., 1993; Dulic *et al*., 1998). While we can not rule out that this small amount of cyclin B1 associated with p21$^{Waf1/Cip1}$ can account for the inhibition of cyclin B1 kinase activity, it seems more likely that cyclin B1 kinase activity is inhibited by an indirect mechanism. Interestingly in this regard, previous work in Xenopus has shown that activation of cyclin B/cdc2 kinase depends on the prior activation of cdk2 kinase activity (Guadagno and Newport, 1996), and given the extremely efficient inhibition of cdk2 activity in our cells following p21$^{Waf1/Cip1}$ activation (approximately 40-fold), this could account for inability to activate the cyclin B1 kinase. Alternatively, the p21$^{Waf1/Cip1}$-mediated arrest could block cell cycle progression at a point in early G2 or late S-phase prior to cyclin B1/cdc2 activation, and indeed a role for cyclin A/cdk2 in S-phase exit has been seen in other systems (Krek *et al*., 1995).

A role for p21$^{Waf1/Cip1}$ in inhibiting DNA synthesis has been suggested by several studies and although this is most likely to be mediated through the interaction with PCNA (Li *et al*., 1994; Shivji *et al*., 1994; Waga *et al*., 1994), there is also some evidence for a contribution of inhibition of the cyclin dependence kinases (Ogryzko *et al*., 1997). Despite these functions of p21$^{Waf1/Cip1}$, we noted here that expression of p21$^{Waf1/Cip1}$ in cells at the beginning of S-phase was not sufficient to substantially impede their progress through DNA synthesis, as also shown by another recent report (Medema *et al*., 1998). This is consistent with many studies in both normal and tumor cells which show that activation of p21$^{Waf1/Cip1}$ through p53 results in loss of cells from S-phase (Kuerbitz *et al*., 1992) (Figure 1a). These results are also consistent with studies in human fibroblasts (Di Leonardo *et al*., 1994; Linke *et al*., 1997), where DNA damage induced activation of p53 during late G1 failed to impede progress though S-phase, despite accumulation of hypophosphorylated pRB, but lead to the accumulation of cells in G2/M.

The cell cycle consequences of p21$^{Waf1/Cip1}$ induction mirror the consequence of activation of p53 following either temperature shift of Saos-ts p53 or DNA damage of wild-type p53 expressing cells. This coupled with the observation that some p21$^{Waf1/Cip1}$ deficient human cells fail to arrest in G2 in response to p53 (Polyak *et al*., 1996) suggests that p21$^{Waf1/Cip1}$ may, in addition to its well established role in p53-mediated G1 arrest, contribute to p53-mediated G2 arrest. In this regard it should be noted, however, that p21$^{Waf1/Cip1}$ independent mechanisms of G2 arrest have been reported (Levedakou *et al*., 1995) and other p53 target genes have also been implicated (Hermeking *et al*., 1997). Despite this, it seems likely that p21$^{Waf1/Cip1}$ plays some role in p53-mediated G2 arrest and these data further enhance the merits of p21$^{Waf1/Cip1}$ as the major cell cycle inhibitory target of p53.

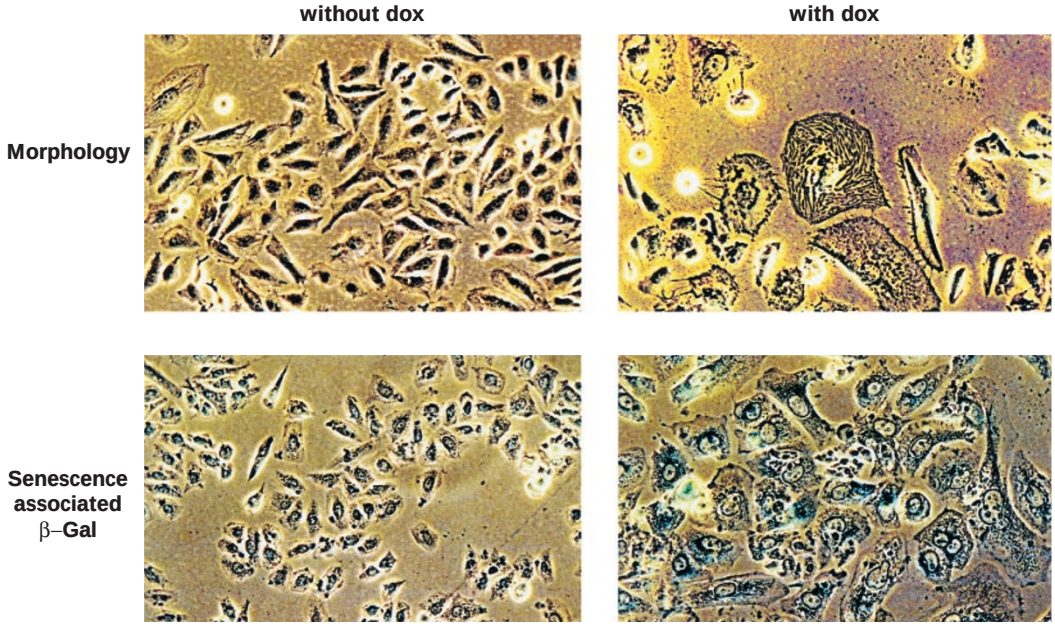


Figure 7 Senescence induced by prolonged expression of p21$^{Waf1/Cip1}$ in Saos-2 cells. Changes in morphology (upper panel) and senescence associated β-Galactosidase activity (lower panel) in Saos-p21 cells treated with doxycycline for 5 days

A role for p21Waf1/Cip1 in coupling DNA synthesis and mitosis

Of particular interest was the observation that release from a p21Waf1/Cip1 induced G2 arrest resulted in DNA endoreduplication in a significant proportion of the cells, suggesting that cells blocked in G2 by p21Waf1/Cip1 are somehow impaired in their ability to sense their position in the cell cycle following release. Although these cells appear to complete the second round of DNA synthesis, and accumulate with an 8N DNA content, stable lines of tetraploid cells could not be derived following release from the p21Waf1/Cip1 block, suggesting that the endoreduplicated cells were not stable or viable. Endoreduplication has previously been reported in both p21Waf1/Cip1 null cells, following a DNA damage induced G2 delay (Waldman *et al*., 1996), and in p21Waf1/Cip1 expressing cells lacking pRB (Niculescu *et al*., 1998). We would like to suggest that expression of p21Waf1/Cip1 in the G2 phase of the cell cycle can interfere with the normal checkpoints which prevent reinitiation of DNA synthesis in the absence of an intervening mitosis. This could occur through inhibition of the G2/M cyclin dependent kinases, as has been noted in yeast and human cells (Broek *et al*., 1991; Stem and Nurse, 1996; Itzhaki *et al*., 1997), or by altering complexes that are characteristic of later stages in the cell, such as the association between cyclin A and p107 (Zhu *et al*., 1995; Shiyanov *et al*., 1996). At present, however, we do not know which, if any, of these mechanisms contributes to endoreduplication in our cells.

p21Waf1/Cip1 can induce markers of senescence

Finally, the observation that prolonged expression of p21Waf1/Cip1 in these cells gives rise to expression of some senescent phenotypes is consistent with several studies showing a role for p21Waf1/Cip1 in cellular aging (Noda *et al*., 1994; Deng *et al*., 1995; Brown *et al*., 1997). While p53, and consequently p21Waf1/Cip1, levels accumulate in senescent cells, and the induction of p53 can result in senescence (Sugrue *et al*., 1997), the loss of p21Waf1/Cip1 can increase the proliferative capacity of diploid fibroblasts. These data, therefore, support a role for p53-mediated p21Waf1/Cip1 induction in the activation of senescence, although it seems clear that other proteins also play a role, with evidence that p21Waf1/Cip1 is not sufficient for normal replicative senescence (Bond *et al*., 1995). It is of interest that in our study this phenotype was seen in p53 and pRB deficient tumor cells, and given the apparent irreversibility of senescence presents a interesting possible therapeutic option.

Materials and methods

Cell culture and transfections

Saos-2, U2OS and Saos-p21 cells were maintained in DMEM supplemented with 10% FCS at 37°C. Saos-2 143A were generated by stably transfecting Saos-2 cells with pCMVp53 143A and clones were screened for p53 expression by Western blotting. Saos-2 p53 143A were maintained the same as the parental cells and the temperature sensitive p53 was activated by moving the cells to 30°C. To induce p53, U2OS or MCF-7 cells were treated with 5 nM actinomycin D as previously described (Hickman *et al*., 1997).

For transient transfection 8×10^5 Saos-2 cells were plated in 10 cm^3 dishes and transfected the following day using calcium phosphate co-precipitation. 20 μg of plasmid DNA was transfected, and an additional 7 μg of pCMVCD20 was included for CD20 co-transfections.

Plasmids

PCR was employed to remove 5′ and 3′ UTR sequences from the p53 and p21Waf1/Cip1 cDNAs and the same Kozak sequence (CGCACCATG) was placed in front of each coding domain. The Flag-epitope tag was introduced at the N-terminus of each protein to facilitate detection. For transient expression the constructs were inserted into the vector pcDNA3 (Invitrogen) and expression was driven by the CMV promoter.

Cell synchronization

To induce quiescence, cells were incubated in serum-free DMEM for 48 h, p21Waf1/Cip1 being induced by the addition of 2 μg/ml doxycycline for the final 24 h (as appropriate). To re-initiate cell cycle progression the medium was replaced with DMEM containing 10% FCS and 2 μg/ml doxycycline (as appropriate).

To synchronize cells at the G1/S phase transition, quiescent cells were released into DMEM containing 10% FCS and 2 mM thymidine. Following overnight incubation doxycycline was added (as appropriate), and the next day the cells were released into thymidine-free DMEM containing doxycycline (as appropriate). To reverse the p21Waf1/Cip1 arrest, cells were incubated in fresh DMEM containing 10% FCS overnight.

Inducible cell lines

Saos-2 cells were transfected with the Tet-on plasmid (Clontech) encoding the rTet repressor protein and neomycin resistant cells were selected in G418 for 2 weeks. Inducible expression of the rTet repressor protein was analysed in clones by transient transfection with a luciferase reporter gene under the control of a Tet responsive element (pTRE Clontech) and luciferase activity monitored in the presence or absence of the tetracycline analogue doxycycline. Clones were selected that had low background levels of expression in the absence of doxycycline but showed a large induction following the addition of 2 μg/ml doxycycline. This primary cell line was subsequently transfected with 10 μg of pTRE vector into which Flag-p21Waf1/Cip1 was inserted while co-transfection with 2 μg of pSV2Hyg allowed the selection of transfected cells in 100 μg/ml hygromycin over a 2 week period. The two highest expressing clones were selected for further analysis.

Immunoprecipitation and Western blotting

Flag-tagged p21Waf1/Cip1 was detected using the anti-Flag monoclonal antibodies M2 and M5 (Kodak IBI), cyclin A antisera were kindly provided by Julian Gannon and Tim Hunt, while cyclin E, B1 and cdk2 antibodies were purchased from Pharmingen.

For immunoprecipitations, purified immunoglobulin was covalently linked to protein A or protein G sepharose (as appropriate) using 5 mg/ml dimethylpimilidate in 200 mM sodium borate pH 9.2. Cells were lysed in NP40M (150 mM NaCl, 50 mM Tris pH 8.0, 1% Nonidet P-40, 1 mM PMSF, 0.1 mM NaF, 0. 1 NaV_3O_4) buffer, cleared by centrifugation and rotated with 5 μg of coupled antiserum for 2 h at 4°C. The immune complexes were collected by centrifugation,

washed in NP40M and either eluted using sample buffer, or used directly in kinase reactions.

Whole cell lysates were prepared by directly lysing cells in sample buffer as described previously (Bates *et al*., 1994). Proteins were separated on 10–15% SDS-polyacrylamide gels, as appropriate, and transferred to Hybond P (Amersham). Immune complexes were visualized using ECL (Amersham).

Flow cytometry

Cells were detached from the dish using trypsin, pooled with floating cells from the medium, washed in PBS and fixed in methanol at 4°C. After overnight incubation the methanol was removed and the cells washed in PBS before treating with 40 μg/ml RNase A and 20 μg/ml propidium iodide (P1) for 30 min. DNA profiles were analysed using a FACS Calibur flow cytometer. When CD20 co-transfection was employed, cells were processed as previously described (Zhu *et al*., 1993). For BrdU profiles, cells were pulsed with 10 μM BrdU for 1 h prior to fixation in methanol. The cells were subsequently stained with FITC-conjugated anti BrdU antibody (Pharmingen) and PI as previously described (Hickman *et al*., 1997).

Kinase assays

Kinase reactions were performed as described previously (Hickman *et al*., 1997). Briefly, cyclins were inununoprecipitated and immune complexes incubated in kinase buffer (50 mM Tris, 10 mM $MgCl_2$, 250 μM cold ATP and 5 μg Histone HI) and 5 μCi [γ^{32}P]ATP (Amersham) was added per reaction. Tubes were incubated at 37°C for 20 min, stopped by the addition of sample buffer and the proteins separated by SDS–PAGE. Results were visualized by autoradiography and quantified using a phosphorimager.

Senescence associated β-Galactosidase (SA-β-Gal) activity

Saos-p21 cells were either untreated or treated with doxycycline for 5 days, fixed in 3% formaldehyde and incubated overnight at 37°C in SA-β-Gal buffer (1 mg/ml X-Gal, 40 mM citric acid/sodium phosphate pH 6.0, 5 mM potassium ferrocyanide, 5 mm potassium ferricyanide, 150 mM NaCl and 2 mM $MgCl_2$) (Dimri *et al*., 1995). The next day cells were rinsed in PBS and photographed.

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