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The G_2 Checkpoint Is Maintained by Redundant Pathways

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While p53 activity is critical for a DNA damage-induced G_1 checkpoint, its role in the G_2 checkpoint has not been compelling because cells lacking p53 retain the ability to arrest in G_2 following DNA damage. Comparison between normal human foreskin fibroblasts (HFFs) and HFFs in which p53 was eliminated by transduction with human papillomavirus type 16 E6 showed that treatment with adriamycin initiated arrest in G_2 with active cyclin B/CDC2 kinase, regardless of p53 status. Both E6-transduced HFFs and control (LXSN)-transduced cells maintained a prolonged arrest in G_2; however cells with functional p53 extinguished cyclin B-associated kinase activity. Down regulation was mediated by p53-dependent transcriptional repression of the CDC2 and cyclin B promoters. In contrast, cells lacking p53 showed a prolonged G_2 arrest despite high levels of cyclin B/CDC2 kinase activity, at least some of which translocated into the nucleus. Furthermore, the G_2 checkpoint became attenuated as p53-deficient cells aged in culture. Thus, at late passage, E6-transduced HFFs entered mitosis following DNA damage, whereas the age-matched parental HFFs sustained a G_2 arrest. These results indicate that normal cells have p53-independent pathways to maintain DNA damage-induced G_2 arrest, which may be augmented by p53-dependent functions, and that cells lacking p53 are at greater risk of losing the pathway that protects against aneuploidy.

The inability to arrest or undergo apoptosis in response to negative signals is a hallmark of cancer cells. In some cell types, DNA damage leads to cell cycle arrest, presumably to allow time for repair so that cells do not replicate or segregate damaged DNA (27, 32) or to eliminate damaged cells from the proliferative pool (10). While normal cells are capable of arresting in G_1 and G_2 in response to a genotoxic stress, cells lacking the commonly mutated tumor suppressor gene, p53 (19, 24), arrest solely in G_2. DNA damage leads to stabilization of p53 and consequently transcriptional up regulation of the cyclin-dependent kinase (CDK) inhibitor, p21 (12, 21, 61), resulting in arrest in G_1.

Although the necessity for p53 in the DNA damage-induced G_2 checkpoint has been ruled out by the fact that cells without p53 function are capable of arresting in G_2, a role has been suggested in a variety of experimental systems. Overexpression of p53 in p53-null human fibroblasts led to both G_1 and G_2 block (1). Rat embryo fibroblasts transfected with human *ras* and the temperature-sensitive mutant *ts*p53^{Val135} arrest in G_1 and G_2 when shifted to the permissive temperature (40, 56). Expression of wild-type p53 in human ovarian cancer cell line by using *ts*p53^{Val135} led to arrest in G_2 but not G_1 (58). The role of p53 in the G_2 checkpoint, however, has yet to be demonstrated from DNA damage induction through to arrest, in one experimental system (reviewed in reference 60), or in primary cells. Furthermore, the general observation that p53-depleted cells are capable of a DNA damage-induced arrest in G_2 needs to be reconciled with any mechanism proposed for p53 in the G_2 checkpoint.

Studies on the G_2 transition and checkpoint have focused on CDC2 and its positive regulatory subunit, cyclin B. The kinase activity of this complex and levels of cyclin B oscillate with the cell cycle (14, 15). After binding with cyclin B, the kinase activity of CDC2 is dependent on the phosphorylation status of CDC2. CDC2 undergoes an activating phosphorylation on threonine 161 by CDC7/cyclin H and immediate inhibitory phosphorylation on tyrosine 15 by Wee1 kinase (39, 47) and threonine 14 by Myt1 kinase (36). Activation of cyclin B/CDC2 kinase activity, and subsequent progression into mitosis, is then dependent on the dephosphorylation of the inhibitory sites by CDC25C (15, 18). When faced with genotoxic stress (44), unreplicated DNA (54), or negative cellular signaling (3), cyclin B/CDC2 kinase activation is inhibited and cells arrest in G_2. An increase in tyrosine-phosphorylated forms of CDC2 has been associated with DNA damage (26, 45, 50, 59) and inactive kinase (33). Although DNA damage-induced activation of Wee1 kinase may be involved in the mechanism of this inhibitory phosphorylation of CDC2, much evidence in both fission yeast and human cells (17, 48, 51) points to inhibition or sequestration of CDC25C by the 14-3-3 σ protein. 14-3-3 σ is a member of a family of proteins that is expressed in response to a variety of signals, including epithelial differentiation and DNA damage (reviewed in references 34 and 49). The 14-3-3 proteins show sequence homology with the DNA damage-induced Rad24 and Rad25 proteins of fission yeast and have been demonstrated to bind and possibly sequester the activating CDC25C phosphatase for cyclin B/CDC2, thereby leading to a G_2 arrest. A possible mechanism for p53's role in the G_2 checkpoint has been reported to involve p53-mediated transcriptional activation of 14-3-3 σ (23), though that model does not explain how cells depleted of p53 are capable of a DNA damage-induced G_2 arrest.

Transcriptional regulation of the cyclin B, CDC2, and CDC25 genes have also been proposed as a means to modulate the G_2 checkpoint. Irradiation of HeLa cells resulted in an arrest in G_2, the maintenance of which correlated with down regulation of cyclin B mRNA and protein (41, 42). This decrease in mRNA levels was in part due to decreased stability of

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the cyclin B message (7, 38); CDC2 and CDC25 transcription was also decreased (7). p53 overexpression in p53 null EJ bladder cancer cells led to arrest in both G_1 and G_2/M, with decreased CDC2 and cyclin B transcript levels (57). The down regulation of CDC2 and cyclin B transcripts was attributed to the senescent phenotype (55) rather than a specific function of p53. Recent evidence shows cyclin B and CDC2 protein and mRNA down regulation in a p53- and possibly p21-dependent manner (2, 9), and p53's role may lie in the transcriptional repression of the cyclin B promoter (25).

Cells which have initiated a G_2/M checkpoint in response to DNA damage can succumb to a variety of fates, including apoptosis (reviewed in reference 13), prolonged permanent arrest (35), recovery after repair of DNA damage (reviewed in reference 43), or adaptation to the damage, allowing progression through the cell cycle with the DNA damage that initially evoked the arrest (52). Although roles for p53 in apoptosis and DNA repair have been described, p53's role in the G_2 checkpoint and adaptation remains to be elucidated. Experimental systems which utilize immortalized cells, tumor cell lines, and cells lacking functional p53 that have been grown in culture for multiple population doublings acquire uncharacterized genetic abnormalities, which can confound the interpretation of p53's role. By comparing colon carcinoma cell lines that differed in p53 status and utilizing extensively passaged human fibroblasts, Bunz et al. concluded that p53 induction of p21 is necessary to sustain G_2 arrest after DNA damage (5). We have used a model system in which p53 is depleted from primary human cells by transduction with the retrovirus LXSN, carrying the human papillomavirus type 16 (HPV 16) E6 oncogene (herein referred to as E6 cells). These cells were monitored throughout their proliferative life span and compared to the vector-transduced controls (herein called LXSN cells). We demonstrate that the initiation of the G_2 checkpoint is a p53-independent event and show that both LXSN and E6 cells sustain a prolonged G_2 arrest, although their mechanisms to maintain the arrest differ. Finally, as E6 but not LXSN cells undergo multiple population doublings, the ability to sustain G_2 arrest is lost, reminiscent of neoplastic progression.

MATERIALS AND METHODS

Cell culture and media. Primary human fibroblasts derived from neonatal foreskin (HFFs) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (complete medium) at 37°C and 5% CO_2. Cells were transduced with LXSN vector or LXSN-16E6 by infection with amphotropic viruses containing each vector as previously described (20). Population doubling level (PDL) counts began with first plating after G418 selection. Cells were counted at each passage; population doublings were determined and added to the previous value. E6 and LXSN cells were stored in liquid nitrogen, in DMEM with 15% FBS and 10% dimethyl sulfoxide. Mouse embryo fibroblasts (MEFs) derived from p53 null ($p53^{-/-}$) and p21 null ($p21^{-/-}$) transgenic mice were a gift from Chris Kemp. MEFs were cultured in DMEM supplemented with 10% FBS.

Cell cycle synchronization. E6 and LXSN cells were grown to confluence and remained so for 24 h. They were released from density arrest by replating at 1×10^6 to 2×10^6 cells per 150-mm-diameter tissue culture plates, or 5×10^5 to 7.5×10^5 cells per 100-mm-diameter plate, in DMEM 10% FBS with aphidicolin, to allow cell cycle progression and synchronization to the G_1/S border. After remaining in aphidicolin (Sigma) at 3 μg/ml for 24 h, the cells were washed twice with phosphate-buffered saline (PBS) and refed DMEM–10% FBS. After 3 to 5 h (enough time for cells to proceed into the cell cycle), the treatment group of cells were pulsed with 2 mM adriamycin (ADR; stock solution in PBS) for 1 h at 37°C. They were washed twice in PBS and received complete medium. The cells were harvested for total cellular protein and total RNA and fixed for flow cytometry at several time points after release from synchronization.

Flow cytometry. Cells were fixed at variable time points after release from density-aphidicolin synchronization. For each time point, cells were trypsinized and fixed with 70% ethanol. The fixed cells were then stained with propidium iodide (50 μg/ml) with RNase (5 μg/ml). The stained cells were analyzed for DNA content by fluorescence-activated cell sorting (FACS) in a FACScan (Becton Dickinson Instruments). Cell cycle fractions were quantified with CellQuest (version 1.2; Becton Dickinson).

Western blotting. After trypsinization, cells were washed with cold PBS and lysed with WE 16th lysis buffer (Tris-HCl [50 mM, pH 7.5], NaCl [250 mM], EDTA [5 mM], Nonidet P-40 [1%], glycerol [20%], sodium orthovanadate [0.5 mM], β-glycerophosphate [80 mM], sodium fluoride [50 mM], phenylmethyl sulfonyl fluoride [1 mM], leupeptin [25 μg/ml], aprotinin [10 μg/ml], pepstatin [10 μg/ml]). Lysates were sonicated on ice, clarified by centrifugation at 14,000 rpm and stored at −70°C. Protein concentrations were determined by the DC protein assay (Bio-Rad). Nuclear and cytoplasmic extracts were prepared by hypotonic lysis with Dounce homogenization followed by high-salt extraction of the pelleted nuclei according to the basic protocol (1a); 20 μg of total cell lysates was loaded on sodium dodecyl sulfate (SDS)–10 or 12% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). Western blot analyses were performed with mouse monoclonal anti-human cyclin B1 (Pharmingen), anti-cyclin E (Pharmingen), anti-p53 (Oncogene Science Ab6), and anti-histone H1 (Upstate Biotechnology), rabbit polyclonal anti-CDC2 (Oncogene Science), and anti-Raf1 (Santa Cruz) antibodies. Secondary antibodies used were 1:35,000 anti-mouse–horseradish peroxidase (Jackson Immunoresearch Laboratories) and 1:20,000 goat anti-rabbit–peroxidase (Boehringer Mannheim) conjugates. Detection was by chemiluminescence (DuPont NEN Research Products) and exposure to X-Omat-Blue film (Kodak).

Kinase assays. Immunoprecipitations were performed by incubating 100 μg of whole lysate with a 1:50 (vol/vol) ratio of mouse monoclonal anti-cyclin B1 (PharMingen) on ice for 20 min. Protein G-Sepharose (Pharmacia Biotech), equilibrated 1:1 (vol/vol) with H1 wash buffer (Tris-HCl [25 mM, pH 7.5], NaCl [125 mM], $MnCl_2$ [10 mM], dithiothreitol [1.0 mM]), was added to each sample. The samples were rotated at 4°C for 1 h. Precipitated protein pellets were washed twice in lysis buffer, twice in H1 wash buffer, and once in kinase reaction buffer (Tris-HCl [50 mM], NaCl [70 mM], $MnCl_2$ [10 mM], dithiothreitol [1 mM]). Samples were pelleted and incubated for 30 min (a reaction time which has been determined to be in the linear range of the kinase reaction) at 37°C with 25 μl of reaction mix containing kinase reaction buffer with H1 histone (40 μg), unlabeled ATP (10 μM), and [γ-^{32}P]ATP (10 μCi; 10 μCi/μl). Kinase reactions were stopped with the addition of 25 μl of 4× running buffer (Tris-HCl [0.25 M, pH 6.8], SDS [8%], glycerol [40%], β-mercaptoethanol [20%], bromophenol blue [0.05%]) and boiling for 5 min. Of the resultant reaction mix, 15 μl was loaded onto an SDS–12% polyacrylamide gel, and the proteins were separated by electrophoresis. Gels were stained with Coomassie blue to verify equal loading of histone, dried, exposed to X-Omat film (Kodak), and developed.

Northern blotting. Total cellular RNA was prepared with a Qiagen RNeasy mini kit and quantified (Beckman DU-64, Nucleic Soft Pac module, Warburg program); 10 μg of RNA was run on 1% agarose–formaldehyde gels, transferred to Hybond-N membranes (Amersham), and hybridized to ^{32}P-labeled DNA probes. Probes for CDC2, cyclin B1, and 36B4 were made by digesting and gel purifying fragments from plasmids pSP73/CDC2 (gift of L. Bonin), pLXSN/cyclin B1 (gift of J. Pines), and pGEM5/36B4 (gift of J. Gudas). The 514-bp *Pvu*II fragment of cyclin B1 and the 445-bp *Acc*I/*Bgl*II fragment of CDC2 were labeled by PCR performed with [^{32}P]dCTP and a single antisense primer corresponding to the 3′ sequence of the fragment. The 36B4 fragment was radioactively labeled by a random hexamer priming kit (Boehringer Mannheim).

Cotransfection assays. Cytomegalovirus (CMV)-driven mammalian expression vector was used, with and without the p53 gene (30). Reporter constructs were created as follows. Sequences 927, 2,239, and 367 bp upstream of the cyclin B, CDC2, and c-*fos* ATG start sites, respectively, were amplified from human genomic DNA, using oligonucleotides constructed with a *Kpn*I 5′ and *Nco*I 3′ restriction enzyme recognition sequence overhangs. Each promoter fragment was ligated into pGEMT (Promega), expanded, and subjected to *Kpn*I-*Nco*I digestion. *Kpn*I/*Nco*I gel-purified fragments were ligated into pGL3 Basic vector (Promega), containing the luciferase gene in frame with the ATG of the 3′ *Nco*I site of the ligated promoter. Promoter-luciferase constructs containing the p53 response element (RE) (pCAST2Bluc and pCAST2Hluc [30]) were used as a control. Expression vector (4 μg), either with or without the p53 gene, was cotransfected with 4 μg of cyclin B-pGL3, CDC2-pGL3, cfos-pGL3, or p53 RE-luciferase, using Lipofectamine (Life Sciences), into $p53^{-/-}$ MEFs. $p21^{-/-}$ MEFs were cotransfected with 4 μg of expression vector, with or without p53 and 4 μg of cyclin B-pGL3 or CDC2-pGL3. The untransfected control cells underwent mock transfection with no plasmids added to the Lipofectamine mixture. Luciferase activities were assessed 48 h after transfection, using reagents from Promega, and relative light units (RLU) emitted from 20 μg of cell lysate was quantified for each sample (Monolight 2010 Luminometer; Analytical Luminescence Laboratory). Transfection efficiency was determined by dot blotting, probing for the luciferase gene, using DNA obtained from an aliquot of cells used for luciferase activities. RLU values were corrected for transfection efficiency and protein concentration. Data from three separate trials were arbitrarily normalized with the RLU/microgram of protein values from samples cotransfected with both pCMVp53 and cyclin B-luciferase from each trial.

MI. Asynchronous cells growing on slides were either treated continuously with 100 nM ADR in complete medium or untreated (placed in complete medium). At 24 h, cells were fixed with 100% ice-cold methanol. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) and visualized by fluorescence microscopy, and mitotic figures were counted, with oil immersion optics.

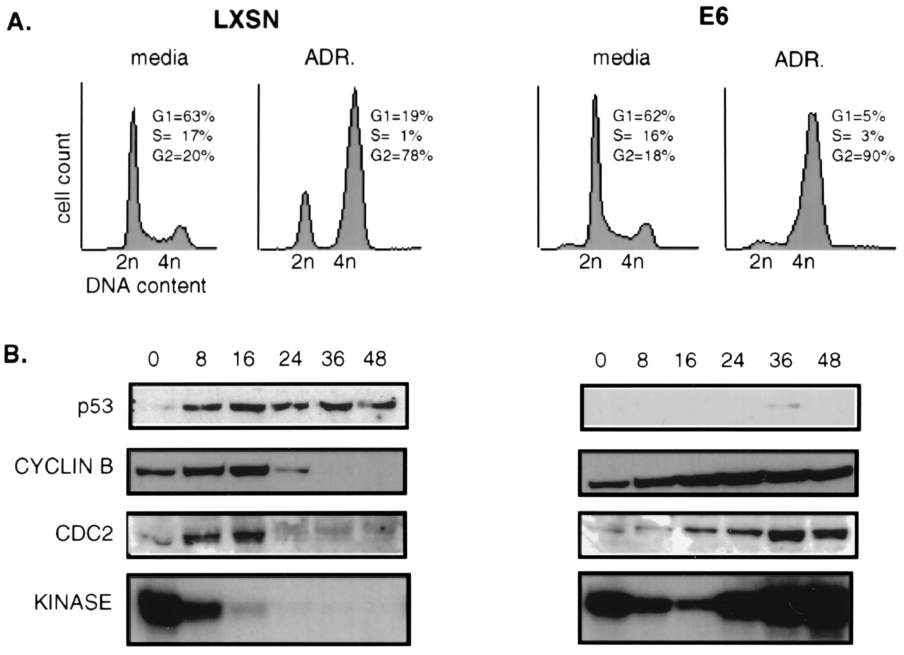


FIG. 1. DNA damage-induced G_2 arrest occurs independently of p53 status. Asynchronous cells were continuously treated with complete medium containing 100 nM ADR. (A) Cell cycle profiles show the LXSN cells to arrest in both G_1 and G_2, while the E6 cells solely arrest in G_2, by 24 h after ADR exposure. (B) Total protein was harvested at the indicated hour. p53, cyclin B, and CDC2 protein levels were determined by Western blotting analysis. The same protein extracts were used for cyclin B-associated kinase activities performed on histone H1. CDC2 protein is represented as two bands; the slower-migrating band represents the phosphorylated inactive form of CDC2, and the faster-migrating band represents the unphosphorylated active form of CDC2.

Over 2,000 cells were counted for each cell type and population doubling level (PDL). Mitotic index (MI) was defined as the percentage of cells in mitosis. Data for adriamycin-treated cells are presented as MI of ADR-treated cells/MI of untreated cells.

RESULTS

The DNA damage-induced arrest in G_2 occurs independently of p53 status. The DNA damage-induced checkpoints, in relation to p53 status, were examined by comparing early-passage HFFs transduced with the LXSN vector alone (LXSN-HFFs) or carrying the HPV 16 E6 oncogene (E6-HFFs). Asynchronously growing cells were treated with the DNA-damaging drug ADR. As anticipated, p53 protein levels rose rapidly in LXSN cells (Fig. 1B), and FACS analysis 24 h postexposure showed arrest in G_1 and G_2 (Fig. 1A). In contrast, E6 cells were depleted of a G_1 population and had a marked increase in the G_2 population without any detectable p53. Although both cell types showed an accumulation of cells in G_2, examination of cyclin B/CDC2-associated kinase activity showed clear differences between the LXSN and E6 cells. Cyclin B-associated kinase activity decreased by 8 h postexposure in LXSN-HFFs and was extinguished by 16 h, though cyclin B and CDC2 protein levels were unchanged during this time. By 24 h there was a dramatic down regulation of cyclin B and CDC2 protein that persisted over 48 h. E6-HFFs showed a similar pattern of reduced cyclin B-associated kinase activity for the first 16 h postexposure; however, cyclin B-associated kinase activity then increased over time; cyclin B and CDC2 protein levels were stable throughout.

The active CDC2 kinase in the E6 cells was not associated with apoptosis, as judged by the lack of a sub-G_1 population (Fig. 1A) and by lack of the characteristic morphological changes of disrupted nuclear membranes or of nuclear condensation, segmentation, and fragmentation (data not shown). Immunofluorescence with DAPI staining of nuclei and tubulin staining of cytoskeleton confirmed an interphase morphology (data not shown). Therefore, it appeared that the p53-depleted E6 cells remain arrested with 4n DNA content and no evidence of early mitosis or apoptosis, yet with active cyclin B-associated kinase activity.

DNA damage-induced decrease in cyclin B and CDC2 is dependent on p53. The E6 protein has been shown to have other activities in addition to its ability to target p53 for degradation. To attribute the differences between the LXSN and E6 cells to their p53 status, E6 mutants that vary in the ability to degrade p53 were each transduced into HFFs (Fig. 2A). Three mutants were used; 16E6-8S9A10T fails to bind or degrade p53 (reference 16 and Fig. 2C), 16E6-Δ151 fails to bind the tumor suppressor hDLG (human homologue of the *Drosophila* discs large tumor suppressor protein) (29) but retains p53 binding and degradation, and the double mutant 16E6-8S9A10T/Δ151 fails to bind either tumor suppressor. Asynchronously growing HFFs expressing wild-type or mutant E6 protein were exposed to ADR and analyzed for cell cycle position, p53 and cyclin B protein levels, and cyclin B/CDC2 kinase activity. Cells expressing the E6 proteins that eliminated p53, i.e., wild-type E6 and E6-Δ151 cells, arrested with a 4n DNA content and substantial levels of cyclin B and B-associated kinase activity (Fig. 2B and C). In contrast LXSN, 16E6-8S9A10T, and 16E6-8S9A10T/Δ151 cells, which are incapable of degrading p53, arrested in G_1 and G_2 with increased p53 and greatly reduced cyclin B and associated kinase activity. These results confirm that G_2 arrest can occur independently of p53 status whereas decreased cyclin B protein and kinase activity is correlated with loss of p53. The persistence of cyclin B was not unique to p53 depleted human fibroblasts, as the same result was obtained in mammary epithelial cells transduced with E6 (data not shown). Examination of the response to other DNA-

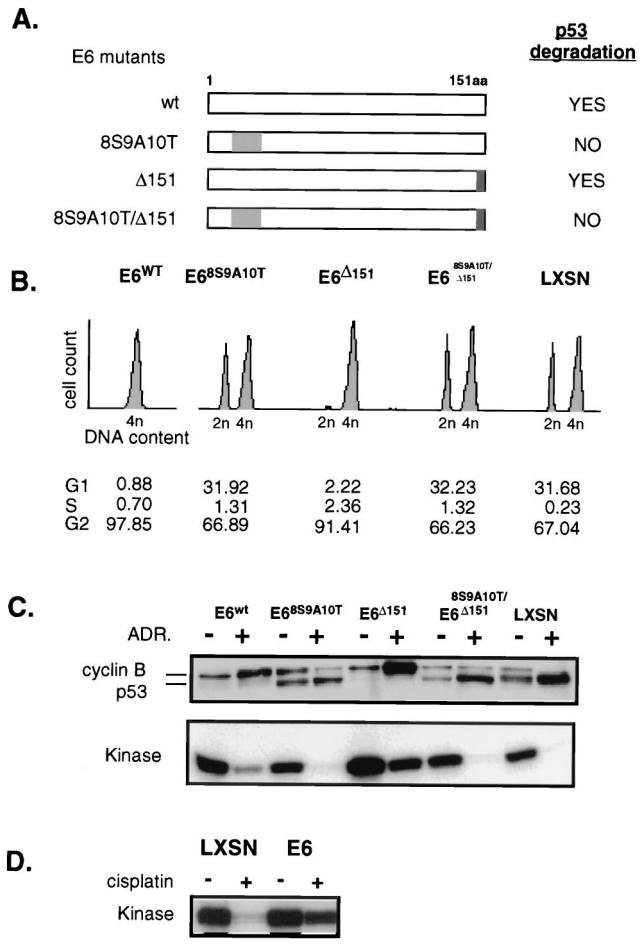


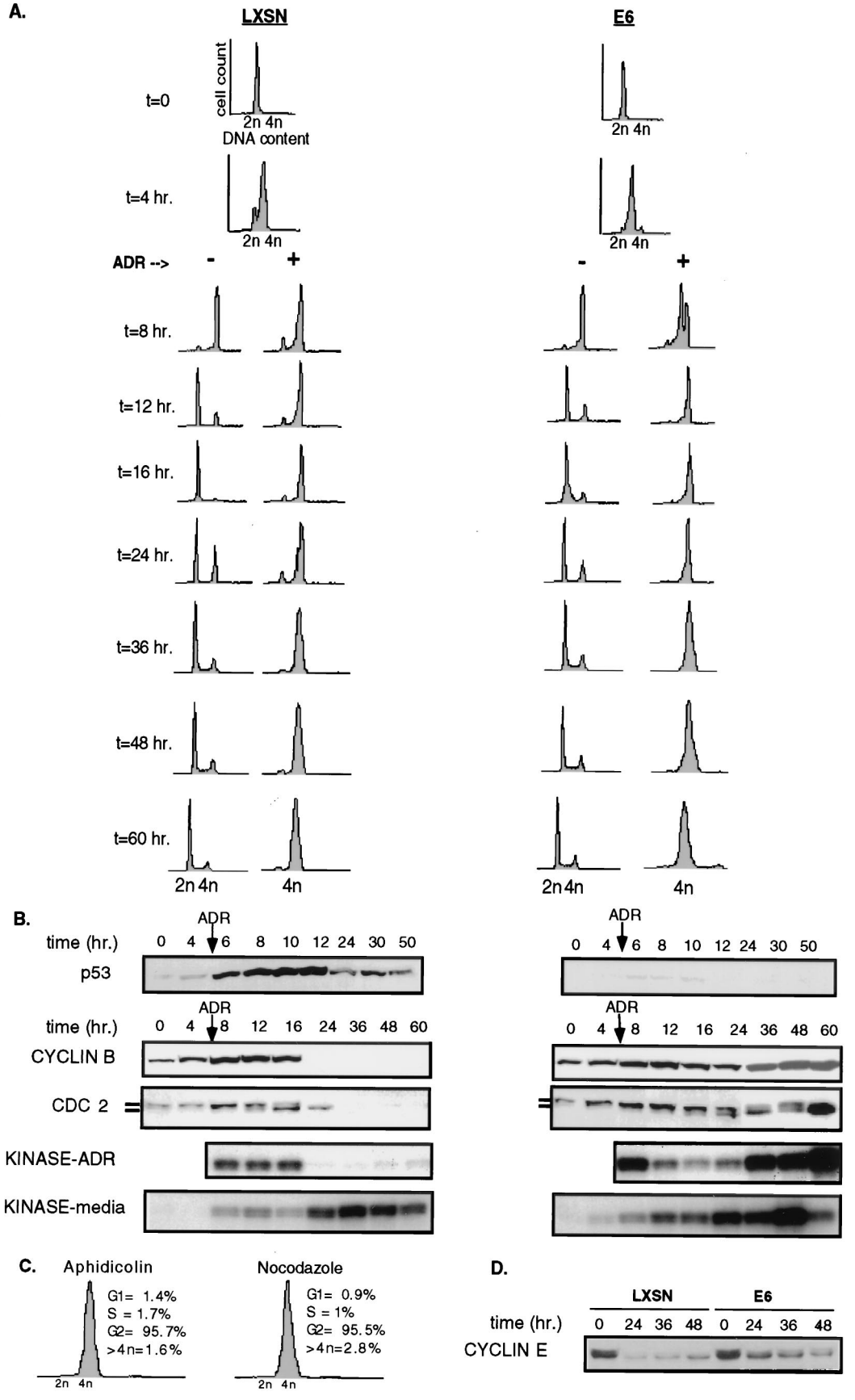
FIG. 2. Down regulation of cyclin B, CDC2, and kinase activity is a function of p53, rather than E6 or agent of DNA damage used. (A) E6 mutants vary in the ability to degrade p53. E6 is a 151-amino-acid protein, depicted in a linear diagram. Amino acids 8 to 10 are responsible for binding to p53, leading to its degradation. The gray shading of this region indicates E6 mutants with replacement of these amino acids such that p53 binding is disrupted and p53 degradation does not occur. The darker shading indicates deletion of the C-terminal amino acid, 151, which would disrupt E6 binding to hDLG, as described in the text. (B) Asynchronous HFFs transduced with the various mutants were treated with complete medium containing 100 nM ADR for 24 h. Cell cycle profiles were analyzed by flow cytometry. (C) Western blotting and kinase assays were performed with proteins harvested from same cells with and without continuous exposure to ADR for 24 h as for panel B. Cyclin B and kinase down regulation occurs only in the cells capable of DNA damage induced up regulation of p53. (D) The p53-dependent down regulation of kinase activity is not a unique finding in cells DNA damaged with ADR. E6-HFFs and LXSN-HFFs were treated with cisplatin (1 μg/ml) continuously for 24 h. Cyclin B-associated kinase activity assays performed with protein lysates obtained from cells treated or untreated, as indicated, show the LXSN cells with extinguished kinase activity, while the E6 cells maintain high kinase activity.

damaging agents including low-dose actinomycin D (data not shown) extended the generalizability of these findings. Cisplatin exposure results in the addition of adducts to the DNA and leads to induction of p53 in LXSN cells and to G_2 arrest in both LXSN and E6 cells (reference 22 and data not shown). Despite the different DNA-damaging mechanism, cyclin B-associated kinase activity was suppressed only in the LXSN cells (Fig. 2D). Thus, the down regulation of cyclin B that occurred with maintenance of prolonged G_2 arrest was not dependent on the type of DNA damage or on the cell type but rather on the presence of p53.

Cyclin B-associated kinase activity during maintenance of the G_2 checkpoint varied with p53 status. Comparison of the G_2 checkpoint between asynchronously growing LXSN and E6 cells is complicated by G_1 arrest in cells with functional p53, resulting in a smaller proportion of LXSN cells arrested in G_2. The possibility that the differences observed between the LXSN and E6 cells were due to the different proportion of cells in G_2 was addressed. Both cell types were synchronized at G_1/S by density arrest followed by passage to lower density into medium containing the reversible DNA polymerase inhibitor aphidicolin. Reentry into the cell cycle was achieved with washing and replacing with aphidicolin-free medium. DNA damage pulse treatment with ADR caused both LXSN and E6 cells to arrest predominantly in G_2 as the first functional DNA damage-induced checkpoint. Synchronization also allowed an analysis of the kinetics of initiation and maintenance of G_2 arrest. Figure 3A shows that both populations were arrested with a 2n content by aphidicolin; untreated cells proceeded through S phase into G_2 by 8 h, and the majority of the cells completed mitosis and were in the G_1 phase of the cell cycle by 12 h postsynchronization. Most of the ADR-treated LXSN cells also reached G_2 by 8 h and remained arrested in G_2 for at least 60 h; a small subpopulation remained in G_1 due to a greater propensity for the LXSN cells to arrest in G_0 with synchronization. The E6 cells reached G_2 with generally the same kinetics and also remained arrested in G_2. The initiation of the G_2 checkpoint can be considered to take place 3 to 5 h after the pulse of ADR, or between 8 and 12 h after release from aphidicolin as shown in Fig. 3A, at the time when cells without DNA damage enter mitosis. Maintenance of the G_2 arrest under these conditions of DNA damage is prolonged, extending beyond 60 h.

p53 accumulated rapidly after exposure to ADR in the LXSN cells (Fig. 3B). Interestingly, cyclin B and CDC2 protein levels and their associated kinase activity increased as the cells accumulated in G_2 and at the initiation of the G_2 checkpoint (8 to 12 h); cyclin B and CDC2 protein levels fell dramatically between 16 and 24 h (Fig. 3B). Predictably, E6 cells did not show induction or accumulation of p53. As in the LXSN cells, cyclin B and CDC2 levels increased as E6 cells entered G_2 and initiated the G_2 checkpoint. However, in contrast to the extinction of cyclin B-associated kinase seen in the cells with functional p53, maintenance of G_2 arrest in cells lacking p53 occurred with persistent cyclin B and CDC2 and high levels of cyclin B-associated kinase activity. Generally, high levels of cyclin B-associated kinase activity herald entry into mitosis, but no morphologic signs of mitosis were observed, and the cell cycle profile indicated that the cells remained with a 4n DNA content. The high level of kinase activity found in the ADR-treated E6 cells represents a large population of E6 cells containing kinase activity, as the kinase reactions are carried out with excess substrate and for a reaction time within the linear range of the kinase activity (data not shown).

Although there was no evidence of a subpopulation of cycling E6 cells after the G_2 checkpoint had been initiated, either by FACS or by microscopic evidence of mitosis (see also Fig. 6), this possibility was formally addressed given that such a cycling population could contribute to cyclin B/CDC2 kinase activity. Synchronized ADR-treated E6 cells that arrested in G_2 were reexposed to aphidicolin for an additional 24 h, such that any cells passing the G_2/M checkpoint and completing mitosis would be arrested at the subsequent G_1/S restriction point with a 2n DNA content. Only 1.4% of E6 cells had a 2n DNA content, and 1.7% had a DNA content between 2n and 4n (Fig. 3C). As a control, synchronized ADR-treated E6 cells were blocked in mitosis with nocodazole; 0.9% of the cells


A.
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0 4 6 8 10 12 24 30 50
p53
0 4 8 12 16 24 36 48 60
CYCLIN B
CDC 2
KINASE-ADR
KINASE-media
C.
Aphidicolin
G1= 1.4%
S = 1.7%
G2= 95.7%
>4n=1.6%
Nocodazole
G1= 0.9%
S = 1%
G2= 95.5%
>4n=2.8%
D.
LXSN
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0 24 36 48 0 24 36 48
CYCLIN E

were found in G_1, and 1.0% were found in S (Fig. 3C). This indicated that <1% of cells could have passed the DNA damage-induced G_2 block and completed mitosis.

Another possibility that could account for the reactivation cyclin B/CDC2 kinase activity is that E6 cells progressed through mitosis without cytokinesis. To identify cells with a 4n content that had entered G_1, cyclin E levels were assayed (Fig. 3D). Neither the LXSN nor E6 cells showed a dramatic increase in cyclin E protein levels. Furthermore, if E6 cells were to have adapted and entered G_1 without cytokinesis, these cells would enter S phase and reduplicate their DNA; however, cells with >4n DNA content never exceeded 3% of the E6-HFF population (Fig. 1, 3A, and 3C).

Taken together, these results indicate that the initiation of a G_2 arrest in response to DNA damage was not dependent on p53 function. Inhibition of cyclin B/CDC2 kinase, which has been implicated in DNA damage-induced G_2 arrest, did not occur with initiation of the G_2 checkpoint but rather occurred with maintenance of G_2 arrest in cells with functional p53. Otherwise normal cells, in which p53 has been eliminated, also respond to DNA damage with a sustained G_2 arrest, with no more than 3% of cells exiting G_2. E6 cells contrast from the parental cells by remaining in G_2 despite active cyclin B/CDC2 kinases.

Subcellular localization of cyclin B and CDC2 in ADR-treated E6 cells. One explanation for the active cyclin B/CDC2 kinase activity in the G_2-arrested E6 cells could be that the cyclin B/CDC complexes are excluded from the nucleus, as suggested from the observation that p21 promotes nuclear localization of mitotic cyclin/CDKs (11). To test this hypothesis, nuclear and cytoplasmic extracts were harvested 36 h after treatment with ADR, during maintenance of G_2 arrest. Cyclin B and CDC2 were distributed in both the nuclei and cytoplasm of E6 cells, and cyclin B-associated kinase activity was isolated from both cytoplasmic and nuclear fractions (Fig. 4). Thus, at least some active cyclin B/CDC2 complexes translocated into the nuclei of ADR-treated E6 cells. LXSN cells treated with ADR showed no cyclin B, a small amount of cytoplasmic CDC2, and no detectable kinase activity in either fraction. Western blotting to cytoplasmic and nuclear controls (Raf1 and histone H1 proteins, respectively) showed that separation of the cytoplasmic and nuclear components was achieved, as the cytoplasmic fractions did not show histone bands and the nuclear component showed only trace contamination with Raf1 (Fig. 4). These results rule out the possibility that E6 cells maintain their G_2 arrest, by excluding active cyclin B/CDC2 kinase from the nucleus.

Transcriptional regulation of cyclin B and CDC2. The mechanism involved in down regulation of cyclin B and CDC2 protein levels in the LXSN cells was explored further; Northern blotting was performed as an initial screen to determine whether p53 influenced cyclin B or CDC2 RNA levels (Fig. 5A). ADR-treated LXSN cells showed a decrease in cyclin B and CDC2 transcripts to undetectable levels by 19 h postexposure (24 h after aphidicolin release). E6 cells, however, showed stable levels of cyclin B and CDC2 mRNA. To determine whether the down regulation of RNA was mediated by p53-dependent repression of the cyclin B and CDC2 promoters, transient cotransfection assays were performed in $p53^{-/-}$ MEFs, using cyclin B or CDC2 promoters driving a luciferase reporter, with or without p53. p53 protein expression in the $p53^{-/-}$ MEFs was documented by Western blotting (data not shown). Reproducibly, p53 repressed the CDC2 promoter 5-fold and the cyclin B promoter 10-fold (Fig. 5B). In control transfections, the previously characterized p53-repressible c-*fos* promoter (31) showed twofold repression by p53 (Fig. 5B). The p53-inducible promoters of the beta interferon and human T-lymphocytic leukemia virus type 1 genes gave 12- and 5-fold-increased luciferase expression, respectively, when cotransfected with the p53 expression vector (data not shown).

Overexpression of p53 can lead to p21 induction, with its downstream effects on transcription via E2F, and arrest in G_1. To address the possibility that G_1 arrest or p21-mediated transcriptional repression can account for the cyclin B and CDC2 promoter down regulation seen with p53 overexpression, transient cotransfection assays were performed with p21-expressing MEFs (Fig. 5C). In these cells, p53 overexpression would not lead to p21-mediated transcriptional regulation; furthermore, $p21^{-/-}$ MEFs have been shown to be significantly deficient in the ability to undergo a G_1 arrest in response to DNA damage (4, 8); therefore, G_1 arrest alone could be excluded as an explanation of inactive cyclin B and CDC2 promoters. Expression of human p53 in CMV-p53 plasmid-transfected $p21^{-/-}$ MEFs was confirmed by Western blotting (data not shown). The cotransfection assays showed that the cyclin B

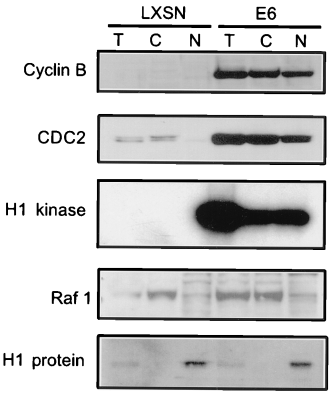

FIG. 4. Cyclin B and CDC2 translocate into the nucleus of ADR-treated E6 cells. Asynchronously growing E6-HFFs and LXSN-HFFs were treated continuously with 100 nM ADR for 36 h. Total (T), cytoplasmic (C), and nuclear (N) proteins were harvested 36 h after exposure as described in Methods and Materials; 20 μg of total, cytoplasmic, and nuclear extracts were loaded onto an SDS–12% polyacrylamide gel and transferred. Western blot analyses for cyclin B, CDC2, the cytoplasmic control (Raf1), and the nuclear control (histone H1) were performed. The ADR-treated LXSN cells show no cyclin B and diminished CDC2 protein levels, while the E6 cells show both cyclin B and CDC2 levels, in the cytoplasm and nucleus. Cyclin B-associated H1 kinase assays were performed on 100 μg of total, cytoplasmic, and nuclear extracts.

FIG. 3. G_2 checkpoint is initiated independent of p53 status and cyclin B/CDC2 kinase and maintained without adaptation. A time course experiment was performed on G_1/S-synchronized cells as described in Materials and Methods. Time zero ($t = 0$) indicates the time of release from synchronization. At 5 h after release ($t = 5$), half of the plates were pulsed with 2 μM ADR in complete medium for 1 h, followed by replacement with complete medium. The other half remained in complete medium and monitored like the untreated control. Flow cytometry (A) and western blotting and kinase assays (B) were performed on cells harvested at the times indicated. (C) To assess the presence of a cycling subpopulation, synchronized ADR-treated E6 cells, treated identically to the cells used for panel A, were reexposed at $t = 24$ to aphidicolin (3 μg/ml) or nocodazole (0.05 μg/ml) in complete medium for an additional 24 h. Cells were then fixed, stained, and subjected to flow cytometry. (D) E6-HFFs and LXSN-HFFs do not adapt to a DNA damaged-induced G_2 arrest. Asynchronous E6 and LXSN cells were pulsed with ADR (2 μM) for 1 h; protein was harvested over a time course and subjected to Western blotting with anti-cyclin E.

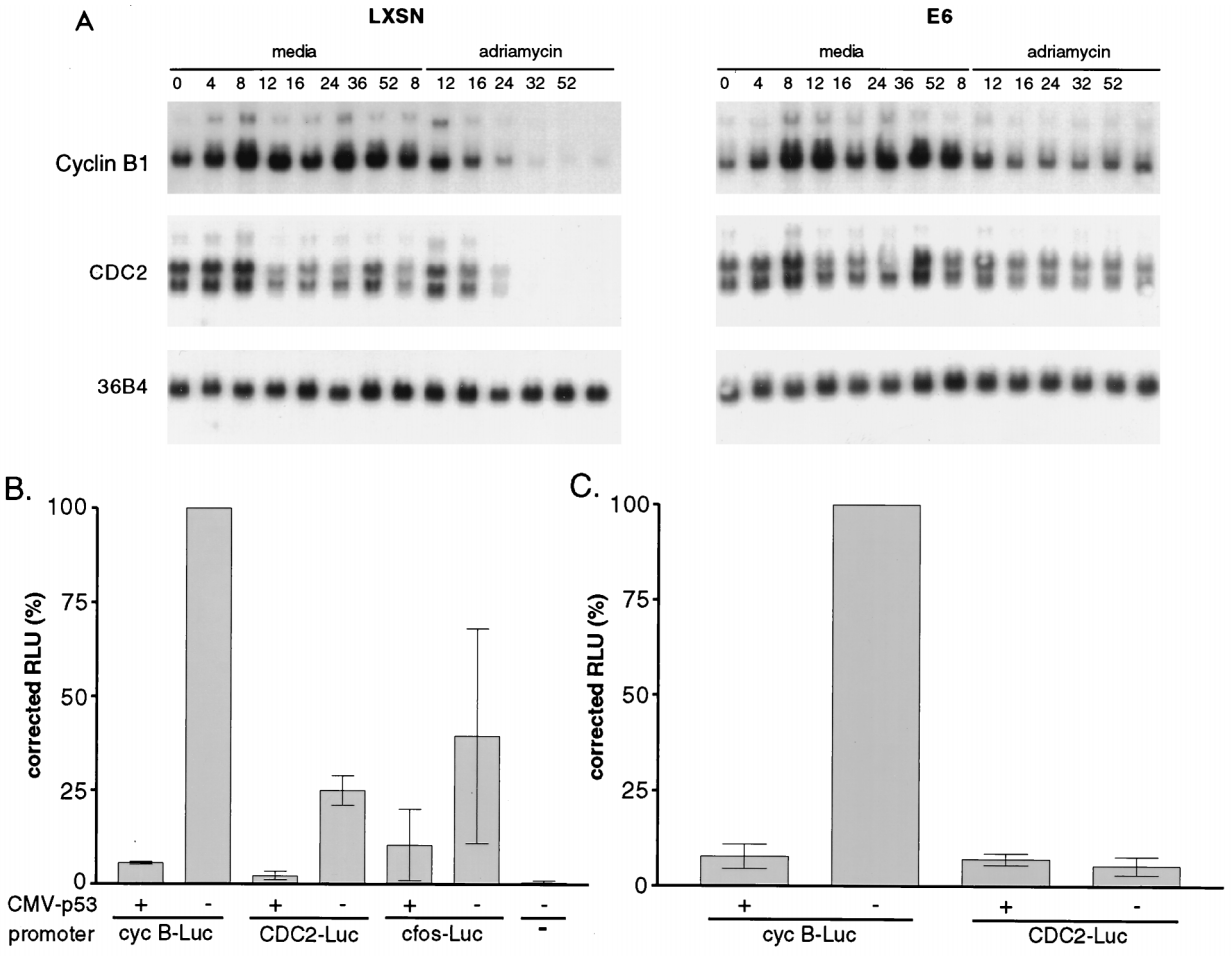

FIG. 5. p53's role in the G_2 checkpoint appears to be mediated by the transcriptional down regulation of cyclin B and CDC2. (A) E6-HFFs and LXSN-HFFs were synchronized and pulsed with 2 μM ADR for 1 h at 5 to 6 h after aphidicolin release. Cells were harvested for RNA at the indicated hour. Northern blotting was performed, and blots were probed with radiolabeled cyclin B, CDC2, and the loading control, 36B4. Lanes 1 to 8 indicate the hour of release from synchronization into complete medium; lanes 12 to 14 indicate the hours after aphidicolin release in the cells pulsed with ADR. (B) Cotransfection experiments show p53 to be a transcriptional repressor of cyclin B and CDC2 promoters. $p53^{-/-}$ MEFs were cotransfected with a CMV-driven expression vector (p53) or without p53 (−) and the various promoter-reporter constructs shown and described in Materials and Methods. Luciferase assays were performed on the protein lysates, and RLU values are normalized for transfection efficiency represented per microgram of protein. Error bars represent standard errors of the means of triplicate experiments. The untransfected control represents $p53^{-/-}$ MEFs that have been mock transfected. (C) Identical cotransfections were performed in $p21^{-/-}$ MEFs.

promoter was repressed by p53 in the $p21^{-/-}$ MEFs to a similar degree as in the $p53^{-/-}$ MEFs. The low basal activity of the CDC2 promoter in $p21^{-/-}$ MEFs made it difficult to analyze p53 repression.

The DNA damage-induced G_2 checkpoint becomes attenuated in p53-depleted cells at later population doubling levels. Previous studies have shown that gamma-irradiated E6 cells showed an attenuation of the G_2 checkpoint after multiple PDL, whereas PDL-matched LXSN cells did not (28). To test whether attenuation occurred in chemotherapy-treated E6 cells, the MI was determined 24 h after ADR treatment and compared to the MI of untreated cells (Fig. 6). For most of their life span in culture, both E6-HFFs and LXSN-HFFs displayed an intact G_2 checkpoint. By PDL 67, a subpopulation of E6-HFFs entered mitosis after DNA damage, and by PDL 70 to 76, 25 to 30% of the $p53^{-/-}$ cells had lost their G_2 checkpoint, whereas the LXSN cells arrested in G_2 checkpoint at all PDLs tested. Between PDL 80 and 90, both LXSN and E6 populations showed increased doubling time, crisis, or replicative senescence in culture, and checkpoint function could no longer be measured.

To further test if late-passage E6 cells have lost the ability to sustain a G_2 arrest, aphidicolin-synchronized late passage, presenescent E6 cells, which were pulsed with ADR, were then reexposed to aphidicolin to trap cells that had escaped G_2 in at the subsequent G_1/S phase. Figure 6B shows 11.3% of the E6 cells in G_1 and 11.9% in S phase, the latter indicating the percentage of cells cycling through S phase prior to the inhibition of DNA polymerase by aphidicolin. In contrast, only 4.2% of the cells could be found in G_1 after nocodazole block, and 4.7% were in S phase. Notably, no increase in the tetraploid population was seen with the late-passage E6 cells that were kept in nocodazole for 24 h. These data with late-passage E6 cells are markedly different from the results with early-passage E6 cells (Fig. 3C), indicating that p53 loss does not directly result in the inability to sustain G_2 arrest, but more likely the ensuing genetic instability resulting from p53 loss predisposes cells to lose the pathway that maintains G_2 arrest.

A role for p21 in the regulation of the G_2 checkpoint has recently received much attention (2, 5, 6, 9). Given that p21 may be induced in a p53-independent manner, it was possible that the sustained G_2 arrest seen in early-passage E6 cells was

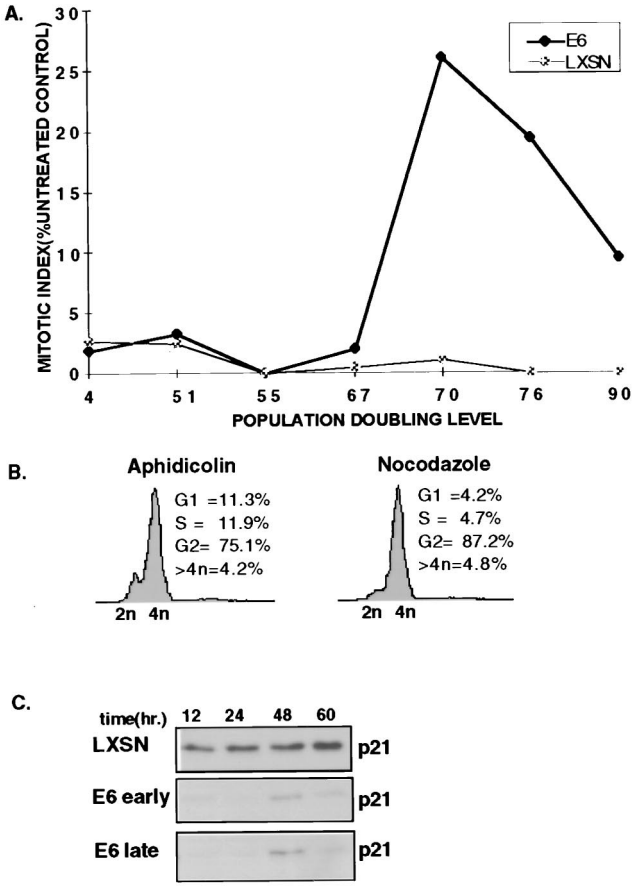

FIG. 6. Cells lacking p53 attenuate the DNA damage-induced G_2 checkpoint after multiple population doublings. (A) Asynchronously growing E6 and LXSN cells varying in PDL were continuously exposed to complete medium containing 100 nM ADR or medium alone for 24 h, fixed, and stained with DAPI, and the MI was determined. (B) Late-passage cells were synchronized by density arrest followed by aphidicolin synchronization. At 4 h after release, they were pulsed with 2 μM ADR for 1 h. At 24 h after release from synchronization, when G_2 maintenance would be expected, the medium was replaced with complete medium containing aphidicolin (3 μg/ml) or nocodazole (0.05 μg/ml) for an additional 24 h. Cells were then fixed, stained, and examined by flow cytometry. (C) p21 loss is not involved in the attenuation of the G_2 checkpoint in late-passage E6 cells. Synchronized, ADR-pulsed early-passage E6 and late-passage LXSN and E6 cells were harvested at the indicated time points. p21 levels were evaluated by Western blotting.

due to p53-independent induction of p21 and that p21 induction was compromised in late-passage E6 cells. As expected, LXSN cells showed high levels of p21 in response to DNA damage throughout their proliferative life span (late-passage LXSN cells are shown in Fig. 6C). Early- and late-passage E6 cells showed equivalent minimal induction of p21 in response to ADR treatment. Therefore, p53-independent induction of p21 did not play a role in sustaining G_2 arrest in early-passage E6 cells.

DISCUSSION

Our results demonstrated that initiation of the G_2 checkpoint in response to DNA damage was independent of p53 status, as both E6 and LXSN cells arrested in G_2 with similar kinetics. Interestingly, initiation of the G_2 checkpoint was also independent of inhibition of cyclin B/CDC2 kinase activity. Active cyclin B/CDC2 was present in both E6 and LXSN cells up to 16 h postexposure, the time by which initiation of the G_2 arrest had occurred in cells exposed to ADR. In this system, the outcome of DNA damage was a sustained arrest in G_2. In HFFs, the complete inhibition of cyclin B/CDC2 kinase activity occurred as a later event related to down regulation of the CDK and cyclin genes and was associated with the presence of functional p53. Cells depleted of p53 were equally capable of maintaining a G_2 arrest despite high cyclin B/CDC2 kinase activity that translocates to the nucleus.

In previous models of DNA damage-induced G_2 arrest, the common denominator on which all pathways converged was the kinase activity of cyclin B/CDC2. It has been assumed that active cyclin B/CDC2 kinase inevitably results in entry into mitosis and, if inactive, results in arrest at the G_2/M border. However, there has been evidence that has shown that although important, cyclin B/CDC2 is not the sole engine driving the cell cycle through G_2 and into mitosis. HeLa cells overexpressing a permanently active CDC2 mutant, CDC2AF, showed high levels of cyclin B protein and cyclin B/CDC2 kinase activity yet were still capable of a DNA damaged-induced G_2 delay (26). Work with *Aspergillus nidulans* has demonstrated parallel pathways controlling entry into mitosis. NIMA kinase (a mitotic kinase) as well as cyclin B/CDC2 must be active for *Aspergillus* to proceed into mitosis (46). NIMA kinase activity is not dependent on active cyclin B/CDC2 kinase, suggesting the existence of an independent and parallel pathway leading to mitosis. There is evidence for a NIMA-like mitotic pathway in vertebrate cells (37), and the closest human NIMA kinase homologue, Nek2 kinase (NIMA-related kinase), has been described (53). Nek2, or a kinase similar to it, may catalyze a G_2 transition pathway that is regulated in a p53-independent manner in response to DNA damage.

There is considerable evidence implicating cyclin B/CDC2 in the transition from G_2 to mitosis, and clearly, p53 targets cyclin B and possibly CDC2 for transcriptional repression during sustained G_2 arrest. However, cyclin B/CDC2 kinase activity either does not contribute to G_2 arrest or is not the only pathway mediating G_2 arrest, since cells lacking p53 remain arrested in G_2 without disrupting cyclin B/CDC2 activity. There are two possible scenarios to explain the sustained G_2 arrest: (i) there is a single pathway maintaining G_2 arrest which does not involve p53, p21, or cyclin B/CDC2, and the down regulation of cyclin B/CDC2 is unrelated to G_2 arrest; or (ii) there are redundant pathways, such that $p53^+$ cells have two mechanisms and $p53^-$ cells have one mechanism, and either is sufficient to sustain G_2 arrest. This model assumes that one of the pathways in the $p53^+$ cells involves cyclin B/CDC2. A variation of the second scenario is that $p53^+$ and $p53^-$ cells use distinct pathways to regulate G_2 arrest, either a p53-dependent down regulation of cyclin B/CDC2 or a p53-, CDC2-independent pathway, and that the latter serves as a default pathway if cells lose p53 function. While we have no data to rule out the first scenario, the extensive data linking cyclin B/CDC2 to the G_2/M transition make the possibility of redundant pathways an attractive hypothesis.

An important interpretation of our data, which has been experimentally addressed, is that the E6-expressing cells might have adapted and drifted out of G_2 and gone back into S phase, and the cell cycle status rather than lack of p53-mediated repression of cyclin B/CDC2 transcription could account for the maintenance of cyclin B and CDC2 activity. This possibility has been ruled out by the following data. First, if the ADR-treated E6 cells were to have proceeded into G_1, then an increase in cyclin E levels would be expected. This was not observed (Fig. 3D). Second, if the ADR-treated E6 cells adapted, they would reenter S phase for another round of DNA synthesis, as these cells do not have a G_1 checkpoint,

leading to an 8n DNA content. A population of cells with >4n DNA was not demonstrated by flow cytometry (Fig. 1 and 3A) up to 60 h or by a comparison of aphidicolin and nocodazole trapping of ADR-treated E6 cells after initiation and maintenance of the G_2 checkpoint (Fig. 3C). Third, if there were adaptation and progression through the cell cycle, we would also expect cyclical changes in cyclin B protein and mRNA levels, as we do for the untreated controls that progress through G_2/M. We show by Western blot (Fig. 3B) and Northern blot (Fig. 5A) analyses that this clearly does not occur for the ADR-treated E6 cells. This is in contrast to the fluctuation of cyclin B mRNA in the synchronous cycling untreated E6 cells (and untreated LXSN cells) (Fig. 5A). Finally, the promoter-reporter analyses presented in Fig. 5B and C were performed with $p53^{-/-}$ and $p21^{-/-}$ MEFs, to rule out the possibility that the effects are due to a specific phase of the cell cycle.

Attenuation of the DNA damage-induced G_2 checkpoint or the ability to sustain G_2 arrest occurred during the in vitro life span of HFFs expressing E6, but not HFFs transduced with control vector (see also reference 28). Indeed many established cell lines lack a functional G_2 checkpoint. Recently, it was shown that $p53^-$ colon carcinoma cell lines and p53 knockout human fetal fibroblasts (a method that requires extensive population doublings) could not sustain DNA damage induced G_2 arrest, whereas $p53^+$ lines could (5). Although that finding was used to conclude that p21 inhibition of CDC2 has a central role in sustaining a G_2 arrest, our data indicate that loss of G_2 arrest is a late event in $p53^-$ cells and that more likely an as yet uncharacterized mechanism that sustains G_2 arrest in $p53^-$ cells is lost due to the genetic instability accompanying prolonged proliferation without a G_1 checkpoint.

Unfortunately, knowing that loss of the ability to maintain a sustained G_2 arrest is only a secondary event related to p53 inactivation does not clarify which of the above two scenarios for G_2 control is correct. In the first model, the G_2 arrest mechanism, though not caused by p53, may be prone to loss in cells lacking a G_1 checkpoint or other p53-dependent functions. The second model would predict that there is an equal chance of losing either of the two G_2 arrest pathways; however, the chance of the $p53^+$ cells encountering inactivating mutations in both pathways is high. This would be the equivalent of familial cancer syndromes; when an inherited allele (or in this case, pathway) is nonfunctional, loss of the second allele results in tumors at an early age, in comparison with tumors in which both alleles need to be inactivated.

The details of the mechanism(s) sustaining G_2 arrest await discovery of the genes involved in the p53-independent pathway. Importantly, numerical and structural chromosomal abnormalities developed in the $p53^-$ cells only after loss of their G_2 checkpoint; loss of G_1 alone did not appear to be sufficient for the development of aneuploidy (28). This has important implications for the process of neoplastic progression, as the tolerance of aneuploidy is a feature of cancer cells. Attenuation of the G_2 checkpoint response appears to be involved in this tolerance of aneuploidy. This finding underscores the importance of understanding control of the G_2 checkpoint, as loss of this checkpoint frees the barrier to genomic instability.

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