Defective G₂ Checkpoint Function in Cells from Individuals with Familial Cancer Syndromes


ABSTRACT

The early events in the G₂ checkpoint response to ionizing radiation (IR) were analyzed in diploid normal human fibroblasts (NHFś) and fibroblasts from patients with two heritable cancer syndromes. Exposure to γ-irradiation of asynchronously growing NHFs resulted in a rapid reduction in the number of cells in mitosis (G₂ delay) and was accompanied by a quantitatively similar reduction in the p34\textsuperscript{cdcf} /cyclin B in vitro histone H1 kinase activity as compared with sham-treated controls. This G₂ delay was strong by 1 h following exposure to IR, maximal by 2 h, and was accompanied by an accumulation of tyrosine-phosphorylated p34\textsuperscript{cdcf} molecules. In contrast, fibroblasts from individuals with ataxia telangiectasia displayed significantly less reduction of the mitotic index or histone H1 kinase activity after IR. Low passage fibroblasts from individuals with Li-Fraumeni syndrome having one wild-type and one mutated p53 allele were similar to NHFs in their immediate G₂ checkpoint response to IR, as were NHFs expressing the human papilloma virus type 16 E6 gene product (functionally inactivating p53) and low passage cells from p53-deficient mouse embryos. However, the p53-deficient fibroblasts were genomically unstable and became defective in their early G₂ checkpoint response to IR. Furthermore, immortal Li-Fraumeni syndrome fibroblasts lacking wild-type p53 displayed an attenuated G₂ checkpoint response. These results link the early events in G₂ checkpoint response to IR in NHFs with the immediate G₂ checkpoint response in immortal cells from patients with two heritable cancer syndromes. Exposure to IR, UV, DNA alkylating agents, or exposure to metabolic poisons by delaying the transitions from G₁ to S, or from G₂ to mitosis, or by reducing the rate of replication initiation in S phase (12–16). By delaying progression through the cycle, it is believed that cells gain additional time for repair processes to remove potentially mutagenic, clastogenic, or lethal lesions before the genome is altered irreversibly during DNA replication or mitotic segregation. Cells from patients with the familial cancer syndrome AT display defects in the induction of these cell cycle delays. In comparison to normal controls, AT cells display less G₁ delay, less inhibition of replication initiation, and less G₂ delay in their immediate response to IR (13, 17–20). One current hypothesis is that the predisposition to cancer of AT patients is a consequence of defects in cell cycle delay mechanisms (21). Recent studies with cells from patients with another heritable cancer syndrome, LFS, have shown that cells that are defective for the wild-type p53 tumor suppressor gene product have increased chromosomal instability (22), especially in the presence of metabolic poisons (10, 11). Additionally, human tissue culture cells that lack wild-type p53 are defective in the G₂ delay in response to IR (23–25).

Delays in proliferation in response to environmental stress appear to involve components normally involved in cell cycle control. The G₁ delay in response to ionizing radiation-induced DNA damage has been shown to be associated with increased levels of expression of p53 (24, 26), p21\textsuperscript{cip1/waf1} (27, 28), and GADD45 (29), and with decreased activities of members of the cdk family (30, 31). The transition from G₂ to mitosis is driven by a catalytic activity referred to as MPF, which is composed of the 34 kDa cdc2 serine/threonine protein kinase and cyclin B proteins (32) and is intricately regulated. (reviewed in Refs. 33 and 34). Specifically, as higher eukaryotic cells progress through S into G₂, p34\textsuperscript{cdcf} molecules associate with cyclin B proteins, which are increasing in abundance at this time. Concomitant with this association, p34\textsuperscript{cdcf} molecules become phosphorylated on threonine residue 161 (35–38), and on threonine residue 14 and tyrosine residue 15 (39–42) which are located in the putative ATP-binding domain of p34\textsuperscript{cdcf}. At the point when the cell commits to entry into division, residues Thr-14 and Tyr-15 are dephosphorylated (43–50) and the p34\textsuperscript{cdcf} /cyclin B kinase complex is activated as MPF, driving the cell into mitosis.

The molecular mechanisms involved in G₂ delay following ionizing radiation exposure in NHFs and fibroblasts from patients with familial cancer syndromes have not been elucidated. G₂ delay following DNA damage has been investigated in several immortal rodent and human tumor cell lines, and has been reported to be associated with an inhibition of cdk activity and an accumulation of the tyrosine-phosphorylated form of p34\textsuperscript{cdcf} proteins in these immortal cell lines (16, 30, 51–53). Reduced levels of cyclin B expression have also been reported following exposure to high levels of IR (54). However, induced by IR, UV, DNA alkylating agents, or exposure to metabolic poisons by delaying the transitions from G₁ to S, or from G₂ to mitosis, or by reducing the rate of replication initiation in S phase (12–16). By delaying progression through the cycle, it is believed that cells gain additional time for repair processes to remove potentially mutagenic, clastogenic, or lethal lesions before the genome is altered irreversibly during DNA replication or mitotic segregation. Cells from patients with the familial cancer syndrome AT display defects in the induction of these cell cycle delays. In comparison to normal controls, AT cells display less G₁ delay, less inhibition of replication initiation, and less G₂ delay in their immediate response to IR (13, 17–20). One current hypothesis is that the predisposition to cancer of AT patients is a consequence of defects in cell cycle delay mechanisms (21). Recent studies with cells from patients with another heritable cancer syndrome, LFS, have shown that cells that are defective for the wild-type p53 tumor suppressor gene product have increased chromosomal instability (22), especially in the presence of metabolic poisons (10, 11). Additionally, human tissue culture cells that lack wild-type p53 are defective in the G₂ delay in response to IR (23–25).

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immortal cells have already lost some elements of normal control of cellular proliferation in that they have acquired the capacity to proliferate indefinitely in tissue culture. In addition, it has been suggested that there are significant differences in cell cycle control mechanisms between rodent and human cell lines (55, 56). We felt that it was critical that the molecular events involved in the G2 checkpoint response be investigated in normal diploid human cells and then contrasted with cells from individuals predisposed to cancer development, particularly since it has been suggested that defects in cell cycle checkpoints can contribute to the process of neoplastic transformation by, at least in part, driving the genomic instability observed in cancer cells (10, 11, 22-25, 57, 58). The results of our studies suggest that defective G2 checkpoint control may also contribute to human cancer development as one of the consequences of genomic instability.

MATERIALS AND METHODS

Human Cells and Culture Conditions. NHF1 and NHF3 are human fibroblast cultures derived from foreskins of apparently healthy neonates (59). Secondary cultures of these cells were used at passage levels 8-14 (NHF1) and 5-10 (NHF3). AT fibroblasts were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository [Camden, NJ (strain designations GM08391 and GM06647)], the National Institute on Aging, Aging Cell Repository [Camden, NJ (strain AG03088)], and from Malcolm Paterson [Cross Cancer Institute, Edmonton, Alberta, Canada (strain CRL1343)]. These cells were used at passage levels 13-22. Low passage LFS fibroblasts designated MDAH041 were determined to have one wild-type p53 allele and one allele with a frame shift mutation at codon 184 (11). A derivative population [MDAH041(high); passage >120] underwent spontaneous immortalization in culture and was found to be heterozygous for the mutation at codon 184. Similarly, another LFS strain (MDAH087, used at passage 15) was found to be heterozygous for a point mutation at codon 248 of p53 but homozygous for this mutation after spontaneous immortalization [MDAH087(high); passage >160] (11). Low passage LFS fibroblasts designated MDAH174 were determined to have one wild-type p53 allele and one codon 175 mutant allele.4 Again, a derivative population [MDAH174(high)] underwent spontaneous immortalization in culture and was found to have again lost the wild-type p53 allele.4 NHF1 cells expressing the HPV16 E6 oncoprotein, designated NHF1 E6 (used between either passage 4 and 10 or passage 14 and 20 from infection), or the retroviral expression vector alone, designated NHF1 neo (used at passage 13 from infection), were used in our experiments. Normal human neonatal foreskin fibroblast cultures expressing the HPV16 E6 gene (NHF4 E6) or vector alone (NHF4 neo) were obtained with the use of the same retroviral expression vectors as above (61) and were used between passages 6 and 10 from establishment of the cultures and between passages 3 and 7 from infection. Human fibroblasts were grown at 37°C in a humidified atmosphere of 5% CO2 in MEM supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. Mouse cells were obtained from embryos of wild-type or p53-deficient mice (62, 63). Embryos were digested with trypsin to release single cells. Cells were grown in DMEM with 10% fetal bovine serum. Exponentially growing cell cultures were subjected to the same movements in and out of incubators as were the normal fibroblasts.

Quantification of Radiation-induced Mitotic Delay. Fibroblasts were fixed on dishes by incubation with trypsin, which was inactivated by addition of serum-containing medium, and cells were sedimented by centrifugation. Alternatively, cells were scrapped off of dishes in cold PBS. The cell pellet was washed once with ice-cold HBSS or PBS, and then after resuspension in a small volume the cells were sedimented in 1.5-ml plastic tubes. The supernatant was removed and the pellets of cells were quickly frozen in an ethanol-dry ice bath. Pellets were stored at −70°C until further use. Thawed cell pellets were solubilized on ice in kinase lysis buffer with inhibitors [10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 5 mM β-glycerophosphate, 2 mM DTT, 5 mM sodium fluoride, 2.5 mM phenylmethylsulfonyl fluoride, 120 kmoberg IU (KIU)/ml aprotinin, 10 μg/ml leupeptin, and 1 mM sodium vanadate]. Lysates were clarified by centrifugation at 100,000 × g for 1 h at 4°C, and protein concentrations were determined with the use of the protein assay reagent procedure (Pierce, Rockford, IL) with BSA as the standard. For p34CDK2 protein kinase activity determinations, 25 μl of p10scl-agarose beads (5 mg/ml; Oncogene Science) were added to extracts containing 50 to −1000 μg of protein and tumbled for either 4 h or overnight at 4°C. Alternatively, immunoprecipitations were performed by incubation overnight at 4°C with either 5 μg of IgG of rabbit polyclonal anti-CDK2 28 amino acid-terminal specific antibody (Upstate Biotechnology, Inc., Lake Placid, NY), 2.5 μl of rabbit polyclonal anti-cyclin A 28 amino acid antibody (obtained from Giulio Draetta, Mitotix), or 2.5 μl of mouse monoclonal anti-cyclin B1 28 amino acid antibody (Upstate Biotechnology, Inc.), followed by incubation for 1 h with 25 μl of protein G-agarose beads (GBFBC-BRL). While the concentrations of protein in lysates varied from one experiment to another, the amount of protein in precipitation reactions was the same for irradiated cells and their sham-treated controls. Precipitated protein pellets were washed 3 times with ice-cold kinase lysis buffer and then resuspended in 20 μl of ice-cold histone h1 kinase buffer [20 mM HEPES (pH 7.3), 80 mM β-glycerophosphate, 20 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 50 mM MgCl2, 5 mM MnCl2, 1 mM DTT, 2 mM phenylmethylsulfonyl fluoride, 60 KIU/ml aprotinin, 10 μg/ml leupeptin, 10 mM cyclic AMP-dependent protein kinase-inhibitory peptide]. Twelve-μl reaction mix containing 10 μCi [γ-32P]ATP (~3000 Ci/mmol; Amersham), 1 mM unlabeled ATP, and 32 μg histone H1 protein (Boehringer Mannheim) were then added to each sample and incubated at 30°C for 15 min. Kinase reactions were stopped by the addition of an equal volume of 2x SDS sample buffer [4% SDS, 150 mM Tris·HCl (pH 6.8), 20% glycerol, 0.02% bromophenol blue, 2 mM sodium vanadate] and by boiling for 5 min. Proteins were separated by electrophoresis in 10% SDS-PAGE. Gels were stained with Coomassie blue to verify equal loading of histone protein, dried, and subjected to autoradiography with Hyperfilm MP (Amersham). The region of gels that contained histone H1 was excised, soaked in 30% hydrogen peroxide overnight, dried, and exposed to X-ray film. Autoradiography was quantitated on a Molecular Dynamics PhosphorImager and ImageQuant software.

Immunoblot Analyses. Protein extracts prepared as described above were analyzed by Western blotting for p34CDK2, cyclin B1, or tyrosine-phosphorylated proteins. Total protein in cell lysates or precipitated proteins were separated on 10% SDS-PAGE and then transferred to 0.2-μm nitrocellulose paper. For p34CDK2 analysis, blots were blocked with 5% Blotto:TBST, then incubated at 4°C overnight with affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to the p34CDK2 protein kinase carboxy terminus (GIBCO-BRL or Upstate Biotechnology, Inc., as indicated in figure legends) diluted in 1% Blotto:TBST. Blots were then incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG (Boehringer Mannheim) diluted in 1% Blotto:TBST; immunoreactive proteins were visualized by ECL (Amersham) and by exposure of blots to Hyperfilm (Amersham). For analysis of human cyclin B1 proteins, blots were blocked overnight at 4°C with 5% Blotto:TBST, followed by a 3-h incubation at room temperature with mAb to human cyclin B1 (Upstate Biotechnology, Inc.) in 1% Blotto:TBST. After removal of the primary antibody, blots were washed first with 1% Blotto:TBST containing 0.1% NP40 (64), then with excess Blotto:TBST, followed by a 1-h incubation at room temperature of anti-mouse IgG peroxidase-conjugated antibody (Boehringer Mannheim). Proteins were visualized by ECL as above.
For phosphotyrosine analysis, blots were blocked for 1 h with 2% BSA in TBS, followed by overnight incubation at 4°C with anti-phosphotyrosine monoclonal hybridoma supernatant (obtained from Deborah K. Morrison, ABL-Basic Research Program, Frederick, MD) diluted 1:1 in TBS with 0.2% Tween 20. Blots were then incubated with peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim) diluted 1:20,000 in TBS with 0.2% Tween 20 and then visualized with ECL. Relative abundances of the various forms of p34<sup>CD<sub>2</sub>C</sup> kinase were determined by scanning films of various exposure times with use of an LKB Ultrascan XL Enhanced Laser densitometer with Gel Scan XL 2.1 software (Pharmacia LKB Biotechnology, Uppsala, Sweden). The abundance of the slower migrating phosphorylated forms was normalized to the fastest migrating form.

RESULTS

Delay of Entry of Normal Diploid Human Fibroblasts into Mitosis following IR Treatment Corresponds with Inhibition of p34<sup>CD<sub>2</sub>C</sup>/Cyclin B Complexes. Exposure of diploid normal human fibroblasts (NHF1) in logarithmic growth phase to either 3 Gy γ-radiation (a dose that results in a reduction in colony-forming efficiency to 10% of unirradiated control levels) or 6 Gy γ-radiation produced a rapid reduction in the fraction of mitotic cells in the culture populations (Fig. 1A). By 1 h after exposure to either 3- or 6-Gy γ-radiation, treated cultures had only 22 or 13%, respectively, of the number of mitotic figures seen in the unirradiated, sham-treated control cell cultures. By 2 h, the reduction of mitotic figures was near maximal (4–6% of sham-treated controls following either 3- or 6-Gy γ-irradiation) and they persisted at approximately the same levels (3–4% of sham-treated controls) up to 4 h following irradiation.

To determine whether this delay of entry into mitosis in normal human fibroblasts was associated with inhibition of the p34<sup>CD<sub>2</sub>C</sup> protein kinase, cells were harvested 1, 2, and 4 h after exposure to either 3 or 6 Gy of γ-radiation, and in vitro histone H1 kinase activity was assayed from protein extracts (Fig. 1B). A rapid reduction in the histone H1 kinase activity was observed in the irradiated populations when compared with unirradiated, sham-treated controls. Inhibition of kinase activity reached near maximal levels by 1 h following exposure to γ-radiation, with maximal inhibition being reached by 2 h following irradiation with either 3- or 6-Gy doses (13 and 15% of sham-treated controls, respectively). We saw no significant difference in the response of NHFs to exposure to 3- or 6-Gy γ-radiation with respect to either delay of entry into mitosis or inhibition of histone H1 kinase activity (Fig. 1, A and B).

While there was an excellent correlation between the strong inhibition of entry into mitosis and the inhibition of activation of histone H1 kinase activity 2 h after exposure of NHFs to either 3- or 6-Gy γ-radiation (Figs. 1A, 2A, and 3), we repeatedly observed less reduction of the in vitro histone H1 kinase activity associated with p13<sup>SUCl</sup> relative to the reduction of the mitotic indexes. p13<sup>SUCl</sup> can bind other members of the cdk family in addition to p34<sup>CD<sub>2</sub>C</sup>, and these cdkS can contribute to the in vitro histone H1 kinase activity assayed from extracts of γ-irradiated populations. However, the majority of the in vitro histone H1 kinase activity associated with p13<sup>SUCl</sup> precipitates is likely to be attributable to the contribution of p34<sup>CD<sub>2</sub>C</sup>/cyclin B complexes (65–66). Previous work by O’Connor et al. (53) demonstrated that the G<sub>2</sub> delay induced by treatment of human CA46 lymphoma cells with nitrogen mustard was accompanied by a decrease in p34<sup>CD<sub>2</sub>C</sup>/cyclin B and p34<sup>CD<sub>2</sub>C</sup>/cyclin A protein kinase activity but not by a significant decrease in the p33<sup>CDK2</sup>/cyclin A protein kinase activity, which was the major cyclin A-associated kinase activity of their cells in S and G<sub>2</sub> (53). The strong inhibition of histone H1 kinase activity associated with p13<sup>SUCl</sup> precipitates that we observed following exposure of diploid human fibroblasts to IR appears to be due primarily to the inhibition of p34<sup>CD<sub>2</sub>C</sup>/cyclin B complexes. For example, in one experiment with NHF1 cultures where the mitotic index was reduced to 4% of controls by 2 h following exposure to 3-Gy γ-radiation, p13<sup>SUCl</sup>-associated kinase activity was 16% of sham-treated controls, but the kinase activity associated with p34<sup>CD<sub>2</sub>C</sup> carboxyl-terminal specific antibody immunoprecipitates was reduced to 8% of controls. Again, in another experiment with a low passage culture of diploid fibroblasts from another individual where the mitotic index was inhibited to 6% of sham-treated controls, but the kinase activity associated with cyclin B1 immunoprecipitates was reduced to 6% of controls. In that same experiment, the in vitro histone H1 kinase activity associated with cyclin A immunocomplexes was only reduced to 72% of sham-treated control levels following IR, probably reflecting some inhibition of p34<sup>CD<sub>2</sub>C</sup>/cyclin A protein kinase complex activity but little reduction in the p33<sup>CDK2</sup>/cyclin A protein kinase complex activity (53). Thus, it appears that the majority of the inhibition of in vitro histone H1 kinase activity we

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Fig. 1. Kinetics of delay into mitosis of normal human fibroblasts (NHF1) following exposure to 3- or 6-Gy γ-radiation. A, the mitotic index of radiation-treated logarithmically growing populations of NHF1 cells as a percentage of the mitotic index of sham-treated cells at various times after treatment. In all cases over 1000 cells were counted on each of at least 2 dishes. The results from treatment with 3-Gy γ-radiation, in both A and B, were from a single experiment, while those from treatment with 6-Gy γ-radiation were from the average of two independent experiments. B, the in vitro histone H1 kinase activity associated with p13<sup>SUCl</sup>-agarose precipitates from extracts of γ-irradiation treated logarithmically growing populations of NHF1 cells as a percentage of histone H1 kinase activity associated with p13<sup>SUCl</sup>-agarose precipitates from sham-treated NHF1 cells at various times after treatment.
observed following exposure of diploid human fibroblasts to IR could be attributed to inhibition of the kinase activity of p34\textsuperscript{CDC2}/cyclin B1 protein complexes.

Since the mitotic delay following IR was very rapid, we sought to determine whether the arrest was exclusively a G2 response or whether there was also a component of inactivation of mitotic progression and MPF activity even after cells had entered mitosis. A population of NHF1 cells enriched in cells blocked in metaphase of mitosis was generated by replating from confluency-induced arrest and subsequently culturing in nocodazole for 18 h. These cells were exposed to 6-Gy γ-radiation and, after a subsequent 1-h incubation with nocodazole still present, were analyzed for histone H1 kinase activity relative to sham-treated control cells. We found little reduction in the histone H1 kinase activity in the irradiated population (reduced to only 80% of sham-treated controls; data not shown), indicating that the mechanism of mitotic delay does not work through an inactivation of active p34\textsuperscript{CDC2}/cyclin B complexes but that it functions prior to the activation of MPF.

Defective Early G2 Checkpoint Response to IR in Fibroblasts from Patients with Ataxia Telangiectasia Syndrome. Cells from individuals with AT have been reported to be defective in their G2 delay immediately following DNA damage (19). We examined the early G2 checkpoint response in low passage AT fibroblasts following exposure to 3-Gy γ-radiation. In contrast to the sharp reduction in mitotic index in NHF1 and another normal human fibroblast strain, NHF3, irradiation of AT fibroblasts produced much less inhibition. Two h after irradiation of four independent AT fibroblast strains with 3 Gy, the level of inhibition of mitotic index ranged from 51 to 84% of sham-treated control cells (Fig. 2A), in agreement with what has been reported previously (19). We found that the inhibition of histone H1 kinase activity was similarly defective following irradiation, with the kinase activity being reduced to only 68–100% of sham-treated controls (Fig. 2A). Thus, as measured by both mitotic indexes and histone H1 kinase activity, AT fibroblasts showed reduced capacity for an immediate G2 delay in response to γ-radiation.

p34\textsuperscript{CDC2} Molecules Accumulate in Inactive Forms following IR Exposure in NHF Cells but Not in AT Cells. When p34\textsuperscript{CDC2} proteins were analyzed by immunoblot analysis with use of a carboxyl-terminal specific anti-CDC2 antibody (Upstate Biotechnology, Inc.) diluted to 2 μg IgG/ml in 1% Biotin/Tris-buffered saline (TBS). Proteins were visualized by indirect immunofluorescence using an anti-rabbit IgG-peroxidase conjugated antibody (Boehringer Mannheim) diluted 1:5000, followed by a 1-min reaction with ECL reagent (Amersham). Lanes 1 and 2, 10-min exposure, 1 h after the initiating of the ECL reaction; Lanes 3 and 4, 2-h exposure, again 1 h after initiating the ECL reaction. These exposures were chosen to optimally visualize the three forms of the CDC2 proteins at approximately comparable intensities of the fastest migrating p34\textsuperscript{CDC2} form.

Fig. 2. G2 phase response of AT fibroblasts to exposure to γ-radiation is defective. A, AT fibroblasts are defective in their inhibition of entrance into mitosis (mitotic index) and CDC2/cyclin in vitro histone H1 kinase activity, as compared with normal human fibroblast cultures, at 2 h following exposure to 3-Gy γ-radiation. The values shown for the mitotic index and histone H1 kinase activity of NHF1 cells treated with 3-Gy γ-radiation as a percentage of sham-treated cells, with SD and number of independent experiments, were 7.2 ± 5.2% (n = 7) and 15.0 ± 8.5% (n = 9), respectively. The values shown for the relative mitotic index and histone H1 kinase activity for NHF3 cells represent the average of 2 independent experiments. Results with AT fibroblasts represent single experiments with low passage cultures (P < 25) from four different individuals. A replicate experiment with GM8391 yielded a reduction in mitotic index similar to that shown. B, normal human fibroblasts show a shift in the proportion of the slowest migrating, tyrosine-phosphorylated forms of p34\textsuperscript{CDC2} molecules relative to the fastest migrating forms after γ-radiation while AT fibroblasts show no shift following exposure to ionizing radiation. NHF1 cells (P19; Lanes 1 and 2) and AT fibroblasts (Strain AG03058, P22; Lanes 3 and 4) were either sham treated (−, Lanes 1 and 3) or exposed to 3-Gy γ-radiation (+, Lanes 2 and 4); 2 h later, protein extracts were prepared and analyzed (25 μg of total cellular proteins) by 10% SDS-PAGE, followed by p34\textsuperscript{CDC2} immunoblot analysis with use of a carboxyl-terminal specific antiserum that recognizes only this member of the cdk family. Results with NHF3 cells treated with 3-Gy γ-irradiation as a percentage of sham-treated cells, with SD and number of independent experiments, were 7.2 ± 5.2% (n = 7) and 15.0 ± 8.5% (n = 9), respectively. The values shown for the relative mitotic index and histone H1 kinase activity for NHF3 cells represent the average of 2 independent experiments. Results with AT fibroblasts represent single experiments with low passage cultures (P < 25) from four different individuals. A replicate experiment with GM8391 yielded a reduction in mitotic index similar to that shown. B, normal human fibroblasts show a shift in the proportion of the slowest migrating, tyrosine-phosphorylated forms of p34\textsuperscript{CDC2} molecules relative to the fastest migrating forms after γ-radiation while AT fibroblasts show no shift following exposure to ionizing radiation. NHF1 cells (P19; Lanes 1 and 2) and AT fibroblasts (Strain AG03058, P22; Lanes 3 and 4) were either sham treated (−, Lanes 1 and 3) or exposed to 3-Gy γ-radiation (+, Lanes 2 and 4); 2 h later, protein extracts were prepared and analyzed (25 μg of total cellular proteins) by 10% SDS-PAGE, followed by p34\textsuperscript{CDC2} immunoblot analysis with use of a carboxyl-terminal specific anti-CDC2 antibody (Upstate Biotechnology, Inc.) diluted to 2 μg IgG/ml in 1% Biotin/Tris-buffered saline (TBS). Proteins were visualized by indirect immunofluorescence using an anti-rabbit IgG-peroxidase conjugated antibody (Boehringer Mannheim) diluted 1:5000, followed by a 1-min reaction with ECL reagent (Amersham). Lanes 1 and 2, 10-min exposure, 1 h after initiating the ECL reaction; Lanes 3 and 4, 2-h exposure, again 1 h after initiating the ECL reaction. These exposures were chosen to optimally visualize the three forms of the CDC2 proteins at approximately comparable intensities of the fastest migrating p34\textsuperscript{CDC2} form.
MDAH174 (passage 10, 11, and 9, respectively), that were heterozygous for wild-type and mutant p53 alleles. The MDAH041 line was determined to be heterozygous for a frame shift mutation in codon 184 of the p53 gene when we first established into culture (11). The MDAH087 cell line was found to be heterozygous for a base substitution mutation in codon 248 of the p53 gene when established in culture (11). We found a rapid G2 checkpoint response that was similar to that of NHFs following exposure to γ-radiation, with a reduction of the mitotic index to 3% and 5% of sham-treated controls, respectively, by 2 h after exposure (Table 1). These results were confirmed with an additional strain, MDAH174, that was assayed at early passage when it was still heterozygous for one wild-type p53 allele and one codon 175 mutant allele. Again, the low passage MDAH174 cells, assayed at passage 9, responded similarly to normal fibroblasts to irradiation with 3 Gy with a rapid reduction in mitotic index to 4% of sham-treated controls (Table 1), and a rapid reduction in cyclin B1-associated histone H1 kinase activity to 6% of sham-treated controls.

**Early Immediate G2 Checkpoint Response to IR Does Not Directly Require Wild-type p53.** To determine whether the G2 checkpoint response requires the wild-type p53 function, we investigated the G2 delay in response to γ-radiation in both normal diploid fibroblasts expressing the HPV16 E6 gene product (60) and in embryonic cell cultures from mice in which both of their p53 alleles had been disrupted through homologous recombination (62, 63). NHF1 cells and cells from another normal human neonatal foreskin culture, NHF4, that had been infected with a defective retroviral expression vector containing the human papilloma virus type 16 E6 gene to functionally inactivate the endogenous p53 gene product (NHF1 E6 and NHF4 E6) or infected with the vector alone (NHF1 neo and NHF4 neo; Ref. 60), were examined for their early G2 checkpoint response to IR. We found that both low passage NHF1 E6 cells (passage 4 from infection) and NHF4 E6 cells (passage 3 from infection) responded to exposure to 3-Gy γ-radiation with a strong and rapid G2 checkpoint response, showing a reduction 2 h later in their mitotic index to 3% and 2%, respectively, of sham-treated controls (Table 2). These analyses also were done following exposure to 1.5-Gy γ-radiation, and we found both NHF6 E6 cultures responded with a rapid and strong G2 delay that was similar to the response of NHF neo cultures to exposure to IR (data not shown). Furthermore, we found the cyclin B1-associated in vitro histone H1 kinase activity of NHF4 E6 cells, passage 7, to be reduced to 8% of the activity of sham-treated controls at 2 h following exposure to 3-Gy γ-radiation, which was a reduction equivalent to that observed with the irradiated NHF4 neo cells of 10% of sham-treated levels. Interestingly, exposure of E6-expressing NHF1 cells at higher passages (passage 14) to 3-Gy γ-radiation resulted 2 h later in a reduction of the mitotic index to only 28% of sham-treated controls (Table 2). This attenuated early G2 delay in response to IR was also observed in NHF1 E6 cultures at passage levels 17 and 20.

**Table 1.** G2 delay of entry into mitosis following IR was normal in low passage, precrisis Li-Fraumeni fibroblasts with one wild-type and one mutant p53 allele

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Mitotic index*</th>
<th>Relative mitotic index %</th>
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<tbody>
<tr>
<td>NHF1</td>
<td>1.9 (14/6000)</td>
<td>0.1 (6/6000)</td>
</tr>
<tr>
<td>NHF3</td>
<td>1.0 (43/4500)</td>
<td>0.02 (1/4500)</td>
</tr>
<tr>
<td>MDAH041 (P10)</td>
<td>0.8 (32/4000)</td>
<td>0.025 (1/4000)</td>
</tr>
<tr>
<td>MDAH087 (P11)</td>
<td>1.3 (9/6000)</td>
<td>0.07 (2/6000)</td>
</tr>
<tr>
<td>MDAH174 (P9)</td>
<td>1.5 (25/5000)</td>
<td>0.06 (2/5000)</td>
</tr>
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* The percentage of cells in the logarithmically growing population that were in mitosis 2 h after either sham treatment or exposure to 3-Gy γ-radiation (number mitotic cells/total number cells counted).

* The relative mitotic index represents the mitotic index of the γ-irradiated cell culture as a percentage of the mitotic index of the sham-treated cell culture.

When we examined low passage mouse embryonic cell cultures that fail to express wild-type p53 protein due to homologous recombination disruption of their p53 gene (62, 63), referred to as p53-/- cells, we found that they had a G2 delay following exposure to 6 Gy of γ-radiation that was similar to low passage embryonic cells from wild-type mice (Table 2). However, high passage, postcrisis p53-/- cells (culture 14) were clearly defective in their initial G2 delay following IR, as reflected by less reduction in the mitotic index (to only 31% of sham-treated controls; Table 2) and less reduction in histone H1 kinase activity (not shown). When these experiments were analyzed as averages of the individual experiments instead of cumulatively as shown in Table 2, we obtained a reduction of entry into mitosis relative to sham-treated controls to 7 ± 3% for the p53-/- culture 56, 16 ± 7% for the low passage p53-/- culture 32, and 30 ± 3% for the high passage p53-/- culture 14. Analysis of the data by unpaired Student’s t test showed that the means of the low passage p53-/- cells (culture 56) and the low passage p53-/- cells (culture 32) were not significant for relative mitotic index (two-tailed P value = 0.06). However, the differences between the means of the low passage p53-/- cells (culture 32) and the high passage postcrisis p53-/- cells (culture 14) were very significant for relative mitotic indexes (two-tailed P value = 0.025); the differences between the means of the low passage p53-/- cells (culture 56) and the high passage postcrisis p53-/- cells (culture 14) were extremely significant for relative mitotic indexes (two-tailed P value = 0.0001). These results, together with those obtained with the NHF1 E6 cells, support the interpretation that lack of wild-type p53 does not directly result in defective early immediate G2 checkpoint response to IR but more likely results in genetic instability as has been described (10, 11, 63), with subsequent alterations causing reduced capacity to delay entry into mitosis following DNA damage.

**Defective Early G2 Checkpoint Response to IR in Immortal Li-Fraumeni Syndrome Fibroblasts That Lack Wild-type p53.** Immortal LFS fibroblast derivative cultures that had lost their wild-type p53 alleles (11) were examined for their early G2 checkpoint response to IR. Both the MDAH041(high) and MDAH087(high) immortal LFS fibroblast lines showed less delay than did the two normal diploid fibroblast strains, NHF1 and NHF3, as was reflected in both the histone H1 kinase activities and mitotic indexes (Fig. 3). By 2 h following exposure to 6 Gy of γ-radiation, the NHF1 and NHF3
62.3 ± 2.9% (n = 4), respectively. Similarly, the values for the histone H1 kinase activities from γ-irradiated MDAH041(high) and MDAH087(high) cells relative to sham-treated cells, with SD and number of independent experiments, were 6.7 ± 1.7% (n = 6) and 13.9 ± 4.6% (n = 7), respectively. The values shown for the relative mitotic index and histone H1 kinase activity of NHF1 cells treated with 6-Gy γ-radiation as a percentage of sham-treated cells, with SD and number of independent experiments, were 6.7 ± 1.7% (n = 6) and 13.9 ± 4.6% (n = 7), respectively. The values shown for the mitotic index and histone H1 kinase activity of NHF1 and MDAH041 cells at 2 h after treatment with 6-Gy γ-radiation as a percentage of sham-treated cells, with SD and number of independent experiments, were 55.7 ± 14.2% (n = 4) and 62.3 ± 2.9% (n = 4), respectively. Similarly, the values for the histone H1 kinase activities from γ-irradiated MDAH041(high) and MDAH087(high) cells relative to sham-treated controls were 57.0 ± 25.7% (n = 4) and 62.4 ± 6.4% (n = 4), respectively.

Wild-Type p53 Genotype:

<table>
<thead>
<tr>
<th>Relative Mitotic Index</th>
<th>Relative Histone H1 Kinase Activity</th>
</tr>
</thead>
<tbody>
<tr>
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<td>-</td>
</tr>
<tr>
<td>NHF3</td>
<td>+</td>
</tr>
<tr>
<td>MDAH041 (high)</td>
<td>+/ +</td>
</tr>
<tr>
<td>MDAH087 (high)</td>
<td>-/ -</td>
</tr>
</tbody>
</table>

Human Fibroblast Cultures

Fig. 3. Postcrisis, high passage LFS fibroblasts are defective in their inhibition of entrance into mitosis (mitotic index) and CDK/cyclin in vitro histone H1 kinase activity compared with normal human fibroblast cultures at 2 h following exposure to 6-Gy γ-radiation. The values shown for the mitotic index and histone H1 kinase activity of NHF1 cells treated with 6-Gy γ-radiation as a percentage of sham-treated cells, with SD and number of independent experiments, were 6.7 ± 1.7% (n = 6) and 13.9 ± 4.6% (n = 7), respectively. The values shown for the relative mitotic index and histone H1 kinase activity of NHF1 cells represent the average of 2 independent experiments. Two spontaneously immortalized lines of fibroblasts from two different individuals with LFS were derived independently and both were found to have lost their one wild-type p53 allele. The values shown for the mitotic index of MDAH041(high) and MDAH087(high) cells at 2 h after treatment with 6-Gy γ-radiation as a percentage of sham-treated cells, with SD and number of independent experiments, were 55.7 ± 14.2% (n = 4) and 62.3 ± 2.9% (n = 4), respectively. Similarly, the values for the histone H1 kinase activities from γ-irradiated MDAH041(high) and MDAH087(high) cells relative to sham-treated controls were 57.0 ± 25.7% (n = 4) and 62.4 ± 6.4% (n = 4), respectively.

Cells had reduced mitotic fractions to 7 and 10% of sham-treated controls, respectively, whereas the mitotic fractions of the immortal LFS cells were reduced to only 56 and 62% of sham-treated controls, respectively (Fig. 3). Assay of histone H1 kinase activity in irradiated NHF1 and NHF3 cells showed inhibition to ~15% of sham-treated controls in both strains but a reduction to only 57 and 62% of sham-treated controls with the high passage MDAH041 and MDAH087 cells, respectively (Fig. 3). Similar results were obtained with exposure of high passage MDAH041 and MDAH087 cells to 3 Gy of γ-radiation (not shown). In addition, the third immortal LFS cell line that lacked wild-type p53, postcrisis MDAH174 cells, again showed less of this rapid G2 delay following irradiation, with the mitotic index reduced to 21% of controls and cyclin B1-associated histone H1 kinase activity reduced to 37% of sham-treated controls.

To further characterize the apparent defective early G2 delay response of immortal LFS cells to IR, a time course study was done. In contrast to the very rapid inhibition of both mitotic index and histone H1 kinase activity seen with NHFs (Fig. 1), the irradiated immortal LFS fibroblasts displayed little to no inhibition of histone H1 kinase activity and mitotic index at 1 h after γ-irradiation (Fig. 4). The difference in the kinetics of the response of NHFs (Fig. 1) with both postcrisis LFS lines, which were very similar to each other (Fig. 4), could not be accounted for by differences in growth properties since NHF1 and MDAH041(high) cells have a doubling time that is similar (21 and 24 h, respectively), while the MDAH087(high) cells double at a much slower rate (~41 h).6 We did observe that with time, both immortal LFS cell lines showed inhibition of mitotic indexes, and to a lesser extent histone H1 kinase activities, albeit ~2–3 h later than were seen with normal fibroblasts. Thus, the immortal LFS fibroblasts were still capable of responding to DNA damage in G2 by gradually delaying entry into mitosis and activation of p34CDC2/cyclin B protein kinase complexes, albeit with impaired kinetics.

When p34CDC2 proteins from immortal LFS fibroblasts, along with normal and AT fibroblasts, were analyzed by immunoblots with use of the carboxyl terminal-specific antiserum at various times following exposure to 3-Gy γ-radiation, we again found that the strong and rapid G2 delay induced in the NHF1 following irradiation was accompanied by an accumulation of the slowly migrating forms of the protein (Fig. 5B, Lanes 2-5). In contrast, the p34CDC2 molecules from the γ-radiated immortal LFS cells, MDAH087(high), showed no significant alteration in the relative amounts of the different forms of the protein from 0 to 4 h following treatment (Fig. 5B, Lanes 6-9). As before, no significant shift was seen in the extracts from the AT cells (Fig. 5B, Lanes 10-13). As with the irradiated AT cells, we found good agreement between the lack of increase in the relative amounts of the phosphotyrosine-containing p34CDC2 molecules and the defective early G2 delay observed in these populations following γ-radiation. Also, while somewhat variable from experiment to experiment, we found that generally the unirradiated immortal LFS cells growing logarithmically contained a higher relative abundance of the slowest

6 W. K. Kaufmann, unpublished observations.
migrating p34CDC2 molecules than the unirradiated NHFs (compare Fig. 5B, Lanes 6 and 10, with Lane 2).

Reduced p34CDC2/cyclin B kinase activity following IR could be a consequence not only of inhibitory phosphorylation of p34CDC2 molecules but also a reduction of levels of cyclin B protein, as has been reported for X-irradiated HeLa cells (67, 68). We found no reduction but instead a slight increase in the levels of cyclin B proteins in total protein extracts from NHFs following exposure to IR (Fig. 5A). Furthermore, when p34CDC2 immune complexes were examined for cyclin B1, we found a similar slight increase in the amount of cyclin B1 protein associated with p34CDC2 from both NHF1 and NHF3 cells 2 h after exposure to IR, showing that the p34CDC2/cyclin B complexes were not dissociated in response to irradiation (Fig. 6A, Lanes 1-4). In agreement with a defective G2 delay, there was no significant difference between cyclin B1 levels in total protein extracts from unirradiated and irradiated MDAH087(high) or AT fibroblasts (Fig. 5A) or between cyclin B1 levels coprecipitated with p34CDC2 molecules from unirradiated or γ-irradiated immortal MDAH087 cells (Fig. 6A, Lanes 5 and 6). However, we consistently observed higher levels of cyclin B1 in the total protein extracts from unirradiated immortal LFS cells than in the diploid NHF1 cells (compare Fig. 5A, Lanes 2 and 6).

**DISCUSSION**

In this study we have investigated the early immediate events involved in the G2 delay in response to γ-irradiation in normal human diploid foreskin fibroblasts. This response was compared with the early G2 checkpoint response of diploid skin fibroblast strains derived from AT and LFS patients, and low and high passage fibroblast cultures lacking wild-type p53 function. By analysis of mitotic frac-
tion and cyclin-dependent protein kinase activity from 0 to 4 h following exposure to IR, it was possible to define a rapid and stringent early G2 checkpoint response in cultures of NHFs that resulted in a reduction in the number of cells in mitosis by >90% within 2 h after irradiation. We found a strong correlation between the rapid inhibition of entry into mitosis and inhibition of p34<sup>cdc2</sup>/cyclin B1 protein kinase activity. The rapid G2 delay in normal diploid human fibroblasts in response to γ-irradiation was accompanied by an accumulation of phosphorylated forms of p34<sup>cdc2</sup> molecules. Low passage, diploid LFS fibroblasts with one wild-type p53 allele showed a rapid G2 checkpoint response to IR that was similar to normal fibroblasts, as did low passage, diploid fibroblasts expressing the HPV16 E6 gene. Additionally, studies with embryonic explants from mice deficient in wild-type p53 further confirmed that functional wild-type p53 was not required to elicit this immediate G2 delay in response to ionizing radiation-induced DNA damage, as was reported by Kastan et al. (24). However, G2 checkpoint function was degraded in diploid AT fibroblasts and in high passage mouse and human fibroblasts lacking wild-type p53. AT fibroblasts, high passage HPV16 E6-expressing human fibroblasts, postcrisis p53-deficient mouse fibroblasts, and postcrisis LFS fibroblasts all displayed less inhibition of entry into mitosis and less inhibition of p34<sup>cdc2</sup>/cyclin B protein kinase activity immediately after irradiation than did normal human or mouse fibroblasts.

We interpret the reduction in the number of cells in mitosis and the reduced levels of p34<sup>cdc2</sup>/cyclin B protein kinase activity shortly after exposure to IR to be due to a strong, intact G2 checkpoint response functioning properly in NHFs and that this initial G2 checkpoint function is impaired in AT and high passage p53-deficient fibroblasts. An alternative interpretation of our data is that cells in late G2 are more sensitive to the cytotoxic effects of IR than are cells in other phases of the cell cycle and therefore are depleted from the cell culture. While this is a formal possibility that we cannot rule out at this time, we do not feel that this is the mechanism underlying the events we are observing. This is because we do not observe any obvious rapid increase in cell death in either logarithmically growing or G2- or G1/M-enriched NHF cells compared with AT or immortal LFS cultures. In addition, significant rapid G2 delay can also be observed after low dose exposures to IR, in the range of 0.5–1.0 Gy, that produce only moderate reductions in the colony forming ability of the irradiated cultures (19, 69). Therefore, we conclude that we are in fact investigating the early events in a G2 checkpoint-induced delay of progression in the cell cycle in response to exposure to IR.

Earlier studies have linked DNA damage-induced G2 delay with p34<sup>cdc2</sup> protein kinase activity in immortal cell lines from human tumors and rodents (16, 30, 52, 53, 67, 68, 70, 71). However, those studies utilized cell cultures that had already lost some aspects of normal cell proliferation control in the process of having acquired the capacity to proliferate indefinitely in tissue culture. As such, those studies are limited in the information they can provide as to how normal cell cycle control pathways function. In addition, as mentioned earlier, Schimke et al. (55, 56) have demonstrated significant differences in cell cycle control mechanisms between rodent and human cell lines. Our results with normal diploid human fibroblasts support the interpretation that the majority of the G2 checkpoint response observed as reduced histone H1 kinase activity reflects reduced activity of mitotic p34<sup>cdc2</sup>/cyclin B1 complexes, with only a slight reduction of the kinase activity of CDK/cyclin A complexes. This is in agreement with a recent study by O’Connor et al. (53) with a human Burkitt’s lymphoma cell line that showed that CDK2/cyclin A complexes were not inhibited significantly in response to nitrogen mustard treatment but that CDC2/cyclin B1 and CDC2/cyclin A protein kinase complex activities were suppressed as cells delayed entry into mitosis.

The goal of our studies was to focus on the early signaling response to DNA damage induced in cells in G2. In most of the studies described here, we have exposed logarithmically growing populations of fibroblasts in culture to γ-radiation and then analyzed G2-M phase-specific cell cycle parameters between 1 and 4 h following treatment. This approach was designed to ensure that any altered mitotic phenotype that we observed was due cells that were in G2 or M at the time of irradiation and therefore could be attributed to a G2 or M phase-mediated response. Flow cytometric analysis of checkpoint response indicated at the most a 20% increase in the percentage of NHF1 cells in G2-M 2 h after IR, at a time when the mitotic index was reduced by ~95%. Thus, our approach of determining the number of cells actually in mitosis or determining the level of activity of cyclin B1/p34<sup>cdc2</sup> protein kinase complexes provided a very sensitive measure of G2 checkpoint response to IR, at a time when only subtle changes were detected by flow cytometric analysis. We also have confirmed differences in G2 checkpoint function between NHF and immortal LFS cells by exposure of synchronized populations to IR in early to mid G2 (data not shown). Other studies have looked at the effects of DNA-damaging treatments in such a way that they could not rule out contributions from S phase regulation (68, 71). In particular, those studies which describe lower levels of cyclin B protein (68, 71) were done in such a way that the cell cycle may have been delayed prior to the synthesis of cyclin B. We found no reduction in total cyclin B1 levels or cyclin B1 levels associated with p34<sup>cdc2</sup> following IR at times when the histone H1 kinase activity was maximally inhibited but in fact we detected a slight increase as cells accumulated in G2 (Fig. 5A, Lanes 2-5; Fig. 6, Lanes 1-4).

We found that diploid AT fibroblasts were clearly defective in their initial G2 checkpoint response to IR by failing to delay entry into mitosis and failing to inhibit the activation of p34<sup>cdc2</sup>/cyclin B protein kinase complexes in the same rapid manner as NHFs. Cells from individuals with AT syndrome have been reported to have both a decreased (19) and an increased (72) G2 delay following exposure to IR. However, as was demonstrated in a recent study (73), this apparent discrepancy in the literature can be resolved by differentiating between the initial failure of AT cells in G2 to respond to IR by delaying entry into mitosis and the increased accumulation of AT cells in G2-M at later times following exposure to IR, as compared with normal cells. Thus, it appears that AT cells irradiated in G1 and S accumulate such severe chromatin damage that successful progression through mitosis is impossible.

The early G2 delay defect in both AT and immortal LFS fibroblasts was not complete, and some delay in entry into mitosis did occur with time in response to radiation. This supports the idea that there is more than one pathway or multiple components in response to irradiation to arrest cell cycle progression. Multiple control points for p34<sup>cdc2</sup> kinase activity have been demonstrated so it is not unreasonable that this could be true. The rapidity of the response to irradiation, with mitotic fractions being reduced by 80–90% in normal fibroblasts within 1 h after irradiation, would seem to limit the number of steps in the initial signaling pathway. In addition, in Saccharomyces cerevisiae the radiation-induced G2 delay mediated through RAD9 does not require protein synthesis but is mediated through a rapid, post-translational signal (9). While the AT cells appear to resemble a rad9 checkpoint mutant, the immortal LFS cells seem to be defective in their ability to delay entry into mitosis as a consequence of genetic alterations that have occurred as a result of the genetic instability associated with the loss of wild-type p53 (10, 11, 63). We noted that these immortal LFS cells express a relatively high level of cyclin B1 proteins (Fig. 5A). Interestingly, another spontaneously immortalized human cell line has been reported to express elevated levels of cyclin B protein, among other alterations, following the transition through...
which can be expected to accelerate neoplastic evolution. The results with the NHF1 E6 cells, p53-/- mouse embryonic fibroblasts, and high passage LFS fibroblasts suggest an association between loss of p53 function and chromosomal instability (10, 11, 22, 63, 85), and both were found to have a pattern of p34CDC2 phosphorylation (12, 22, 76), and both display 10-fold increased frequencies of spontaneous and radiation-induced chromosomal aberrations (12, 17, 19, 76, 80, 81) and individuals with AT syndrome have 61-184-fold increased incidence rates of cancer (82). LFS cells and p53-/- mouse cells, even at early passages in secondary culture, display substantially increased frequencies of nondysjunction and chromosomal aberrations (22, 63). Interestingly, logarithmically growing cultures of both AT and immortal LFS fibroblasts carry abnormally high levels of chromatid damage (12, 22, 76), and both were found to have a pattern of p34CDC2 molecules that somewhat resembled the pattern of p34CDC2 proteins in normal fibroblasts after exposure to IR (Fig. 2B, Lanes 2 and 3; Fig. 5, Lanes 5, 6, and 10). That exponentially growing AT cells progress through G2 and M despite having a pattern of p34CDC2 phosphorylation that resembles the pattern seen in G2-delayed NHF cells is an apparent paradox that we are currently investigating in more detail. One possibility is that these cells are generating some signals in response to DNA damage but that they are unable to respond appropriately to those signals. In agreement with this idea, Lavin et al. (83) have observed a DNA-binding protein which normally translocates through G2 and M despite having a pattern of p34CDC2 proteins in normal fibroblasts after exposure to IR (Fig. 2B, Lanes 2 and 3; Fig. 5, Lanes 5, 6, and 10). That exponentially growing AT cells progress through G2 and M despite having a pattern of p34CDC2 phosphorylation that resembles the pattern seen in G2-delayed NHF cells is an apparent paradox that we are currently investigating in more detail. One possibility is that these cells are generating some signals in response to DNA damage but that they are unable to respond appropriately to those signals. In agreement with this idea, Lavin et al. (83) have observed a DNA-binding protein which normally translocates from the cytoplasm to the nucleus in response to IR. In AT cells this protein is constitutively present in the nuclei of AT cells (84). Those authors suggest that this may reflect a continuous state of stress or DNA damage in AT cells (84). As exemplified in studies of the RAD9 checkpoint in yeast, the G2 checkpoint function in response to DNA damage represents an important defense against spontaneous and induced chromosomal damage (6, 7).

Reduced G2 checkpoint function in AT, LFS, and p53-/- cells has been associated with chromosomal instability (10, 11, 22, 63, 85), which can be expected to accelerate neoplastic evolution. The results with the NHF1 E6 cells, p53-/- mouse embryonic fibroblasts, and high passage LFS fibroblasts suggest an association between loss of wild-type p53 and loss of G2 checkpoint function. Wild-type p53 function did not appear to be required for the initial G2 checkpoint function, since both the low passage NHF E6 cells and p53-/- embryonic cultures retained a rapid G2 delay response to IR that was similar to normal controls. These same NHF1 E6 cell cultures were found to be defective in their G1 checkpoint response to both the metabolic poison N-(phosphonacetyl)-L-aspartate (60) and to IR (31).

Furthermore, primary embryonic cultures from these same p53-deficient mice, as well as another line of p53-deficient mice, have been found to lack a G1 arrest in response to the metabolic poison N-(phosphonacetyl)-L-aspartate (10, 11). However, we observed that accompanying the rapid genomic changes of these cultures that resulted in severe aneuploidization and subsequent acquisition of indefinite life span, these cells also displayed degradation of their G2 checkpoint function. Thus, under three circumstances in which p53 function and G1 checkpoint control was lost (i.e., high passage E6-expressing NHF1 cells, high passage p53-/- mouse embryonic cells, and postcrisis LFS cells), cells displayed a defective early G2 checkpoint response to IR. The attenuation of G2 checkpoint control in the immortal LFS cells would appear therefore to be a consequence of loss of p53 function. The implication of these observations is important for cancer prognosis and therapy. Mutations that inactivate p53 appear to have profound biological consequences not recognized previously. Not only do cells lose the ability to regulate the G1 checkpoint, they can also evolve to lose portions of G2 checkpoint control. The preservation of a functional G2 checkpoint in response to DNA damage should help to protect cells against severe chromosomal damage by preventing the entry into mitosis of cells with damaged chromatids. The loss of the G1 checkpoint function can be expected to increase the formation of chromatid breaks because cells would have less time for repair of damaged DNA before it is replicated. The proper functioning of the G2 checkpoint in cells that have lost G1 checkpoint function would prevent the division of these genetically abnormal cells. Even a partial degradation of G2 checkpoint function could release cells into mitosis with abnormally elevated levels of chromatin damage. Not only would these cells develop aneuploidy due to loss and gain of chromatid fragments, but they also could express a selective growth advantage over cells that retain normal G2 checkpoint function. This growth advantage would then favor the expansion of cells with degraded G2 checkpoint function subsequent to loss of normal p53 function and G1 checkpoint control.

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