**Abstract**

We have previously reported that the immediate G₂ checkpoint delay of normal human fibroblasts in response to ionizing radiation is correlated with inhibition of p34CDC²/cyclin B kinase activity. Here, we observed increased amounts of the cyclin-dependent protein kinase inhibitor p21cip₁ associated with p34CDC²/cyclin B protein complexes from irradiated normal human fibroblasts. Since wild-type p53 function is not required for the early G₂ checkpoint response to ionizing radiation, we investigated whether a p53-independent induction of p21cip₁ was required for the G₂ checkpoint. Early passage human fibroblasts expressing the E6 oncoprotein of human papilloma virus-type 16 (NHF E6) were analyzed. It has been demonstrated earlier that inactivation of wild-type p53 function in these cells by E6 protein does not alter their intact early G₂ checkpoint response to γ-rays. p21cip₁ was found to be undetectable in p34CDC²/cyclin B protein complexes and in total extracts from the E6-expressing cells, with or without exposure to ionizing radiation. These data indicate that p21cip₁ is not required for the immediate G₂ checkpoint response and is not induced by a p53-independent pathway in G₂ phase following exposure to γ-rays.

**Introduction**

DNA damage caused by IR² activates signal transduction pathways that lead to arrest in G₁ and G₂. The G₂ checkpoint response to IR requires the product(s) of the ataxia telangiectasia gene(s) and the p53 gene product (1). Following exposure to IR, p53 levels are elevated, as are the levels of expression of several other genes, including the growth arrest and DNA damage genes (1) and a protein that migrates in SDS-PAGE with an apparent relative molecular mass of 21 kDa known variably as p21 (2), WAF1 (3), CIP1 (4), CAP20 (5), or SDH (6), and referred to here as p21CIPI. p21CIPI was first identified as a component of complexes containing CDK, cyclin, and proliferating cell nuclear antigen proteins in normal cells, but not in transformed cells (7). p21CIPI has been found to inhibit the catalytic activity of each member of the CDK family, although with varying affinities (4, 5, 8). Recently, it has been reported that p21CIPI exists in both active and inactive CDK complexes depending on the stoichiometry of the complex components (9). The expression of p21CIPI fluctuates throughout the cell cycle: p21CIPI mRNA peaks in early G₁, reaches its lowest level in S-phase, and then peaks again in G₂ (10). p21CIPI has been suggested to be involved in the G₁ arrest in response to IR by inhibiting p34CDC²/cyclin E protein complexes (11). It has been previously reported that wild-type p53 function is not required for the rapid G₂ checkpoint response to IR (12–15). Therefore, any requirement for induction of p21CIPI in the G₂ checkpoint after exposure to γ-rays would occur through a p53-independent pathway. Recently, p21CIPI has been reported to be induced by p53-independent mechanisms in response to serum stimulation (16) as well as transforming growth factor β treatment (17). Since an early G₂ checkpoint response in normal human cells has been associated with inhibition of p34CDC²/cyclin B kinase activity (12), we analyzed p34CDC²/cyclin B immunocomplexes and total protein extracts from normal human fibroblasts as well as cells lacking the G₂ checkpoint response when the wild-type p53 function was determined whether quantitative changes in p21CIPI protein immediately following exposure to γ-rays corresponded to the integrity of the early G₂ checkpoint response.

**Materials and Methods**

**Cell Culture and Synchronization.** NHF1, NHF E6, and NHF4 neo cells were used at low passages as described previously (12). All cultures were grown at 37°C with 5% CO₂ in minimal essential medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. Synchronized populations of NHF1 cells predominately in either G₁ or G₂ were obtained as reported before (18), with some modifications: cells that had been maintained arrested by contact inhibition for 7 days were stimulated to enter the cell cycle by replating at lower density (1 X 10⁶ cells/100-mm dish). G₁ cells were obtained 6 h after resuming. For G₂-enriched populations, cells arrested as described above were reseded in the presence of 2 μg/ml aphidicolin and incubated for 24 h to arrest the cells at the beginning of the S-phase. Seven h after aphidicolin removal, approximately 50% of the cells were in G₂. Synchronization of the cell populations was monitored by flow cytometry (see below). Mitotic fractions were determined as previously reported (12).

**Protein Analysis and Histone H1 Kinase Assay.** After irradiation, cells were harvested and frozen cell pellets were lysed as described before (12). Protein concentrations of the clarified lysates were determined using the detergent-compatible protein assay kit (Bio-Rad Laboratories, Inc., Melville, NY). These lysates were then used for Western blot analysis, immunoprecipitations, and histone H1 kinase assays. For Western blot analysis, 50 or 100 μg protein cellular were analyzed on a 12% or 15% SDS-polyacrylamide gel that was further processed as reported previously (12). Antibodies used were antihuman p21WAF1 (mAb, 100 μg/ml; Oncogene Science, Uniondale, NY) and antihuman p34CDC² (carboxy-terminal specific polyclonal antibody, 1 μg/μl; Upstate Biotechnology Incorporated, Lake Placid, NY) at dilutions of 1:200 to 1:1,000, respectively. Immunoprecipitations were performed as described before (12) using 2 mg total protein, and the antibody incubations were carried out using 10 μg/ml antihuman cyclin B1 (mAb, 1 μg/μl; Upstate Biotechnology Incorporated) at 4°C overnight. In vitro histone H1 kinase assays were performed previously (12, 19) using 200 μg total protein for immunoprecipitations with 2 μg/ml antihuman cyclin B1 antibody.

**Flow Cytometry.** For flow cytometric analysis, 2 X 10⁶ cells resuspended in 2 ml PBS were fixed by addition of 4 ml methanol and processed for propidium iodide staining according to the instructions provided by the cellular DNA flow cytometry kit.
cytometric analysis reagent kit (Boehringer Mannheim, Indianapolis, IN). The samples were analyzed using a FACSscan (Becton Dickinson, San Jose, CA).

Results and Discussion

We previously reported that the immediate G2 checkpoint response of NHFs to IR is characterized by rapid reduction of the mitotic fraction accompanied by inhibition of p34CDC2/cyclin B in vitro histone H1 kinase activity (12). In this report the role of the CDK-inhibitor p21CIP1 in the G2 checkpoint response was investigated.

NHF1 cell cultures were assayed for p21CIP1 protein levels by Western blot analysis with antihuman p21CIP1 antibody (Fig. 1A). Senescent NHF1 cells that were used as a positive control of p21CIP1 (Fig. 1A, Lane 1) showed relatively high levels of expression of p21CIP1, as expected (6). As a control of induction of p21CIP1 following IR, total protein extracts from NHF1 cells enriched for G2-G1 populations were analyzed (Fig. 1A, Lanes 2 and 3). p21CIP1 was substantially induced in G1 cells 6 h after irradiation (compare sham-treated with irradiated extracts; Fig. 1A, Lane 2 with Lane 3, respectively) as reported previously (11). NHF1 cultures enriched for early G2-M cells (46% in G2-M, 35% in G0-G1, and 19% in S-phase, as determined by flow cytometry) showed higher p21CIP1 levels just prior to IR (Fig. 1A, Lane 4) than late G2-M cells obtained 3.5 h later (sham-treated; Fig. 1A, Lane 5). Interestingly, there was an increase of p21CIP1 levels in the G2-M populations 3.5 h after IR (irradiated; Fig. 1A, Lane 6) when these cells were compared with their sham-treated controls (Fig. 1A, Lane 5). To determine whether increased p21CIP1 is associated with p34CDC2/cyclin B1 protein complexes following IR, cyclin B1 immunoprecipitates from logarithmically growing NHF1 sham-treated or irradiated cells (3 Gy), collected 2 h after treatment, were assayed for the presence of p21CIP1 protein (Fig. 1B). In this way, analysis of p21CIP1 was restricted to only that associated with p34CDC2/cyclin B1 protein complexes. Indeed, there was a significant increase of p21CIP1 binding to these complexes after irradiation as compared to complexes from sham-treated controls (Fig. 1B, Lanes 1 and 2). This p21CIP1 increase was not due to differences in the amounts of p34CDC2/cyclin B1 protein complexes, since equivalent amounts of p34CDC2 were coprecipitated with cyclin B1 from both sham-treated and irradiated samples, as demonstrated by reprobing the same blot for p34CDC2 protein levels (Fig. 1C). The cyclin B1-associated p34CDC2 was found to be, by its slower mobility in SDS-PAGE (Fig. 1C, Lanes 1 and 2), predominately in the tyrosine-phosphorylated form characteristic of cells in G2. Furthermore, the p34CDC2/cyclin B1 protein complexes that showed increased association with p21CIP1 after IR also showed a reduction in the in vitro histone H1 kinase activity to 17% of the activity of sham-treated controls (data not shown). Thus, in NHF1 cells, p21CIP1 protein was found to be in abundance in G2 and associated with inactive p34CDC2/cyclin B1 protein complexes following IR.

We have previously shown that wild-type p53 is not required for the rapid G2 arrest following IR (12). Nevertheless, the data described above demonstrated that after exposure of NHF1 cells to IR in G2, p21CIP1 protein levels increased, and the protein was associated with inhibited p34CDC2/cyclin B1 protein complexes. Therefore, we asked whether p21CIP1 was induced through a p53-independent mechanism as has been described before (16, 17) and was a critical component of the immediate G2 checkpoint response to IR. To address this question, NHF4 E6 cells expressing the human papilloma virus-type 16 E6 gene product, and hence lacking wild-type p53 function, along with NHF4 neo control cells were examined for p21CIP1 expression. The absence of p53 protein in these cells was verified by Western blot analysis, and the lack of G2 checkpoint arrest in response to IR, which requires p53 function, was confirmed by their ability to abrogate radiation-induced G1 growth arrest (data not shown). Since degeneration of the G2 checkpoint has been observed in high cell passages following p53 inactivation in both human and mouse cells (12), only early passage cells (passage 7 after retroviral infection) were used.

The integrity of the early G2 checkpoint in the NHF4 E6 cells was confirmed by analysis of mitotic fractions and in vitro histone H1 kinase activity associated with cyclin B1 immunocomplexes from sham-treated and irradiated cells as follows: NHF4 neo and NHF4 E6 cells growing logarithmically were exposed to 3 Gy of IR and 2 h later were either methanol fixed for determination of the mitotic fraction, or were harvested for histone H1 kinase assays. In irradiated cell populations, the relative mitotic fraction (expressed as a percentage of sham-treated values) was reduced to 2% for the NHF4 E6 cells and 1% for the NHF4 neo controls, indicating that the NHF4 E6 cells had an intact early G2 checkpoint. To assay for p34CDC2/cyclin B1-associated histone H1 kinase activity, cyclin B1 immunoprecipitates were used for in vitro histo H1 kinase reactions. The reactions were performed in duplicates and were analyzed by SDS-PAGE (Fig. 2A). The cyclin B1-associated histone H1 kinase activities of the irradiated NHF4 E6 and NHF4 neo cells (as quantitated by phosphor-imager counting) were reduced to 8 and 10% of sham-treated levels, respectively, again confirming the integrity of the G2 checkpoint of the NHF4 E6 cells.

When cyclin B1 immunoprecipitates from these cells were analyzed by Western blotting to determine the amount of associated p21CIP1 (Fig. 2B, Lanes 1–4), p21CIP1 protein was undetectable in p34CDC2/cyclin B1 protein complexes from both sham-treated and irradiated NHF4 E6 cells (Fig. 2B, Lanes 3 and 4, respectively). In contrast, an increase of p21CIP1 protein was observed in the complex from the irradiated NHF4 neo cells (Fig. 2B, Lane 2) as compared to their sham-treated controls (Fig. 2B, Lane 1). This increase was similar to that shown before with NHF1 immunoprecipitates (Fig. 1B, Lane 2). When total protein extracts of NHF4 E6 cells were examined, p21CIP1 levels were barely detectable and not significantly induced after irradiation (Fig. 2B, Lane 7, sham treated; Lane 8, irradiated). In contrast, abundant p21CIP1 protein was seen in the total...
however, of p21CIP1 involvement in later events in G2 delay remains open. Considering the rapidity by which normal cells in G2 respond to IR (within 1 h the cells cease entering mitosis (12, 20), early G2 checkpoint response is likely to be transmitted by a protein synthesis-independent signal transduction pathway. Recently, the role of an autoregulatory feedback-loop between CDC2 and CDC25C has been reported in the presence of DNA damage (21). The rapid G2 checkpoint response could be mediated through inhibition of the CDC25C phosphatase. Alternatively, it may involve specific inhibitors of G2 complexes other than p21CIP1, or some, as yet, unidentified enzyme activations that trigger the early G2 arrest upon exposure to IR.

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References


Fig. 2. Early passage NHF4 E6 cells exhibit an intact G2 checkpoint response to IR in the absence of p21CIP1. A, lysates (200 µg) from logarithmically growing NHF4 neo and NHF4 E6 cells (passage 7) were used for immunoprecipitations with antihuman cyclin B1 antibody to assay for cyclin B1-associated histone H1 kinase activity. The cells were harvested 2 h after irradiation (3 Gy), and the samples shown are sham treated (sh) and irradiated (ir). The histone H1 kinase reactions were performed in duplicates and analyzed on a 10% SDS gel. B, lysates (2 mg) from logarithmically growing NHF4 neo cells (Lanes 1, sham treated; Lane 2, irradiated) and NHF4 E6 cells (Lane 3, sham treated; Lane 4, irradiated) treated as described above were used for immunoprecipitations (IP) with antihuman cyclin B1 antibody and analyzed on a 15% SDS gel using Western blotting with antihuman p21CIP1 antibody. Total extracts (100 µg) from these cells were also analyzed (Lanes 5 and 6, sham-treated and irradiated NHF4 neo cells, respectively; Lanes 7 and 8, sham-treated and irradiated NHF4 E6 cells, respectively).

G2 CHECKPOINT IS p21CIP1 INDEPENDENT

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