p21\(^{\text{CIP1}}\) Is Not Required for the Early G\(_2\) Checkpoint Response to Ionizing Radiation

Eleni N. Levedakou, William K. Kaufmann, David A. Alcorta, Denise A. Galloway, and Richard S. Paules

Abstract

We have previously reported that the immediate G\(_2\) checkpoint delay of normal human fibroblasts in response to ionizing radiation is correlated with inhibition of p34\(^{\text{CDC2}}\)/cyclin B kinase activity. Here, we observed increased amounts of the cyclin-dependent protein kinase inhibitor p21\(^{\text{CIP1}}\) associated with p34\(^{\text{CDC2}}\)/cyclin B protein complexes from irradiated normal human fibroblasts. Since wild-type p53 function is not required for the early G\(_2\) checkpoint response to ionizing radiation, we investigated whether a p53-independent induction of p21\(^{\text{CIP1}}\) was required for the G\(_2\) checkpoint. Early passage human fibroblasts expressing the E6 oncoprotein of human papilloma virus type 16 (NHF4 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 G\(_2\) checkpoint response to \(\gamma\)-rays. p21\(^{\text{CIP1}}\) was found to be undetectable in p34\(^{\text{CDC2}}\)/cyclin B protein complexes and in total extracts from the E6-expressing cells, with or without exposure to ionizing radiation. These data indicate that p21\(^{\text{CIP1}}\) is not required for the immediate G\(_2\) checkpoint response and is not induced by a p53-independent pathway in G\(_2\) phase following exposure to \(\gamma\)-rays.

Introduction

DNA damage caused by IR\(^2\) activates signal transduction pathways that lead to arrest in G\(_1\) and G\(_2\). The G\(_2\) 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 SDI1 (6), and referred to here as p21\(^{\text{CIP1}}\). p21\(^{\text{CIP1}}\) 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). p21\(^{\text{CIP1}}\) has been found to inhibit the catalytic activity of each member of the CDK family, although with varying affinities (4, 5, 8).

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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-Gₐ populations were analyzed (Fig. 1A, Lanes 2 and 3). p21CIP1 was substantially induced in G₂ 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 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 histone 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 phospho-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


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