

Ionizing Radiation Induces Rapid Tyrosine Phosphorylation of p34^{cdc2}¹

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Abstract

Eukaryotic cells respond to ionizing radiation exposure with cell cycle arrest. However, little is known about the signaling mechanisms responsible for this effect. The present work has asked whether ionizing radiation exposure is associated with changes in phosphorylation of proteins in HL-60 myeloid leukemia cells. The results demonstrate increased tyrosine phosphorylation of a M_r 34,000 substrate. This effect was detectable at 1 to 10 min after irradiation and was induced by doses of 50 to 500 cGy. Immunoprecipitation studies further suggest that this substrate is the serine/threonine p34^{cdc2} protein kinase. Since p34^{cdc2} is required for entry into mitosis, these findings support the posttranslational modification of a cell cycle regulatory protein in the response to ionizing radiation.

Introduction

The response of eukaryotic cells to ionizing radiation includes cell cycle arrest, activation of DNA repair mechanisms, and lethality. These effects are probably related in part to the direct interaction of γ -rays with DNA or through the production of reactive oxygen intermediates (1). However, the molecular mechanisms that constitute the response of cells to ionizing radiation are unknown. Several checkpoints in cell cycle progression control growth in response to diverse positive and negative regulatory signals (2). Ionizing radiation, for example, slows growth by inducing delays in G₁-S and G₂ phases of the cell cycle. The available evidence suggests that G₂ arrest is necessary for repair of DNA damage before entry into mitosis (3, 4). Genetic studies with *Saccharomyces cerevisiae* have demonstrated that the RAD9 protein controls G₂ arrest induced by DNA damage (5, 6). Mutants of the *rad9* locus are unable to delay entry into mitosis following exposure to genotoxic agents and thereby replicate damaged DNA. Although the mammalian homologue of *rad9* remains unidentified, other studies in various eukaryotic cells have demonstrated that entry into mitosis is regulated by a M_r 34,000 serine/threonine protein kinase designated p34^{cdc2} (7-9).

While yeast have been studied extensively, little is known about the regulation of p34^{cdc2} in mammalian cells treated with DNA-damaging agents. The present studies have examined the effects of ionizing radiation on phosphorylation of p34^{cdc2}. The results indicate that ionizing radiation rapidly induces transient tyrosine phosphorylation of p34^{cdc2}.

Materials and Methods

Cell Culture. HL-60 cells were grown in RPMI 1640 containing 15% heat-inactivated fetal bovine serum supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine. Exponentially growing cells were suspended in serum-free media 18 h prior to irradiation. Irradiation was performed at room temperature using a Gammacell 40 (Nordion International,

Kanata, Ontario, Canada) with a ¹³⁷Cs source emitting at a fixed dose rate of 79.5 cGy/min as determined by dosimetry.

Immunoblot Analysis. Cells were washed twice with ice-cold PBS³ and lysed in buffer A (10 mM Tris (pH 7.4), 1 mM EGTA, 1 mM EDTA, 50 mM NaCl, 5 mM β -glycerophosphate, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM sodium vanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml of leupeptin and aprotinin). Insoluble material was removed by centrifugation at 14,000 rpm for 5 min at 4°C. Protein concentration was determined by Coomassie blue staining using bovine serum albumin as a standard. Soluble proteins (50 μ g) were separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to nitrocellulose paper. The residual binding sites were blocked by incubating the filter in 5% dry milk in PBS-0.05% Tween 20 for 1 h at room temperature. The filters were then incubated for 1 h with either mouse anti-P-Tyr monoclonal antibody (4G10; UBI, Lake Placid, NY) or a mouse anti-p34^{cdc2} monoclonal antibody, which is unreactive with other cyclin-dependent kinases (sc-54; Santa Cruz Biotechnology, Santa Cruz, CA). After washing twice with PBS-0.05% Tween 20, the blots were incubated with anti-mouse IgG peroxidase conjugate (Sigma Chemical Co., St. Louis, MO). The antigen-antibody complexes were visualized by chemiluminescence (ECL detection system; Amersham, Arlington Heights, IL).

Immunoprecipitation. Immunoprecipitations were performed with anti-P-Tyr or anti-p34^{cdc2} at 5 μ g/ml cell lysate. Immune complexes were collected with protein A-Sepharose (Pharmacia), and immunoprecipitates were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transfer to nitrocellulose and blocking, immunoblot analysis was performed with either anti-p34^{cdc2} or anti-P-Tyr and detected with the appropriate horseradish peroxidase-conjugated second antibody using the ECL system.

Results and Discussion

HL-60 cells were exposed to 200 cGy ionizing radiation and monitored for proteins with increased levels of phosphotyrosine. Using an anti-P-Tyr antibody in immunoblot analyses, reactivity with a protein of approximately M_r 34,000 was increased at 1 min after ionizing radiation treatment (Fig. 1A). Similar findings were obtained at 5 and 10 min, while reactivity was decreased at 15 min (Fig. 1A). The filters were washed and re probed with an anti-p34^{cdc2} antibody. The anti-P-Tyr and anti-p34^{cdc2} signals were superimposable. Moreover, there was little detectable change in p34^{cdc2} protein levels following exposure to ionizing radiation (Fig. 1B). Similar findings were obtained with doses of ionizing radiation from 50 to 500 cGy (Fig. 2A). The finding that the signals obtained with the anti-p34^{cdc2} antibody (Fig. 2B) were also superimposable over those found with anti-P-Tyr suggested that p34^{cdc2} may undergo phosphorylation on tyrosine following ionizing radiation treatment.

In order to further define the identity of the protein phosphorylated in irradiated cells, extracts were subjected to immunoprecipitation with anti-p34^{cdc2}. The immunoprecipitates were then monitored by immunoblotting with anti-P-Tyr. Using this approach, the signal for p34^{cdc2} was increased in irradiated as compared to control cells (Fig. 3A). While this result further supported increased tyrosine phosphorylation of p34^{cdc2}, the filter was washed and re probed with anti-p34^{cdc2} to assay for levels of p34^{cdc2} protein. The finding that the anti-p34^{cdc2} signals were similar in control and irradiated cells

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³ The abbreviations used are: PBS, phosphate-buffered saline; P-Tyr, phosphotyrosine.

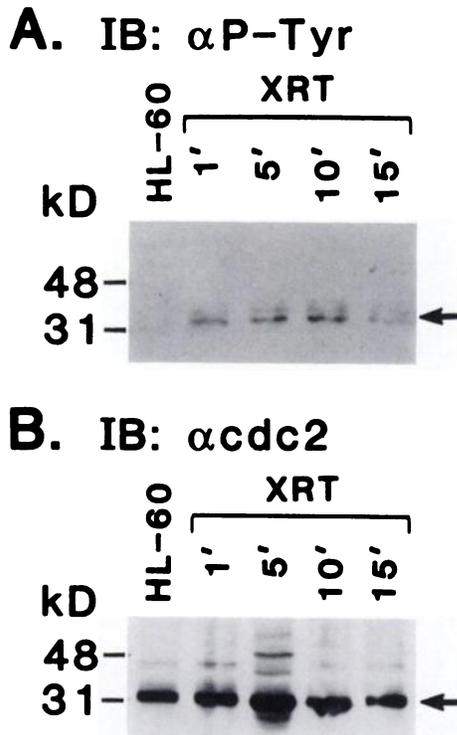


Fig. 1. Ionizing radiation exposure induces tyrosine phosphorylation of a *M*, 34,000 substrate. HL-60 cells were exposed to 200 cGy ionizing radiation (XRT) and harvested at the indicated times. Soluble proteins were subjected to immunoblot (IB) analysis with anti-P-Tyr (A) and anti-p34^{cdc2} (B) antibodies. Arrows, position of *M*, 34,000 signals.

(Fig. 3B) indicated that p34^{cdc2} undergoes increased phosphorylation on tyrosine following ionizing radiation exposure.

Activation of p34^{cdc2} requires association with cyclin B (8, 9) and certain posttranslational modifications. In *Schizosaccharomyces pombe*, the p34^{cdc2}/cyclin B complex is inactivated by phosphorylation of p34^{cdc2} on tyrosine 15 by Wee1 (10–13). Dephosphorylation of p34^{cdc2} on Tyr-15 by the *cdc25* gene product is necessary for activation of p34^{cdc2} and entry into mitosis (14, 15). The *wee1* and *cdc25* gene products thus determine the timing of entry into mitosis by a series of phosphorylations and dephosphorylations of p34^{cdc2}. Other work in *S. pombe* has demonstrated that mitotic checkpoints monitor DNA synthesis and the presence of DNA damage (16–18). The DNA damage checkpoint evidently regulates p34^{cdc2} by mechanisms distinct from those induced by the replication checkpoint (17, 18). Other studies have demonstrated that p34^{cdc2} kinase activity is decreased when CHO cells are exposed to 8 Gy ionizing radiation (19). While these findings suggested that loss of p34^{cdc2} kinase activity is one of the early responses to induction of DNA damage, the events responsible for this effect have remained unknown. The present results support tyrosine phosphorylation of p34^{cdc2} as a rapid response to ionizing radiation and provide an opportunity to define signaling events associated with activation of the DNA damage checkpoint.

Previous work in B-lymphocyte precursors has demonstrated that ionizing radiation exposure is associated with increased protein tyrosine kinase activity (20, 21). Assays of Src-family tyrosine kinases (p59^{fyn}, p56/53^{lyn}, p55^{blk}, and p56^{ck}) indicate a lack of their involvement in this response to ionizing radiation (20). While the present work has not addressed the specific tyrosine kinases activated by ionizing radiation, our results indicate that p34^{cdc2} acts as a substrate

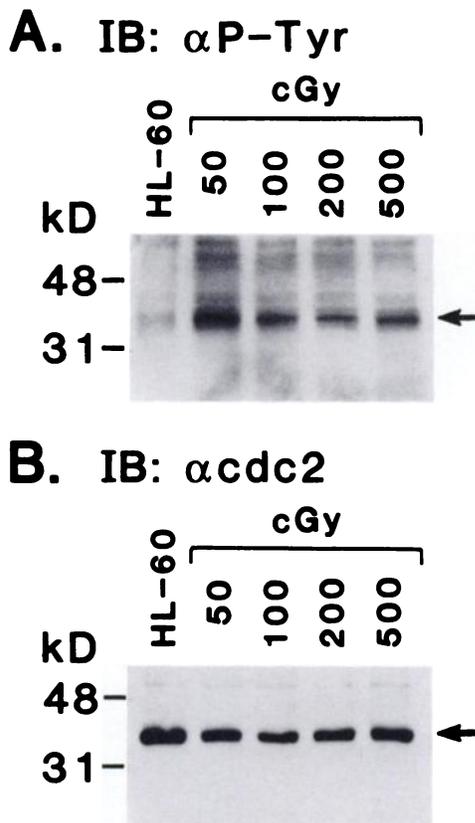


Fig. 2. Different doses of ionizing radiation induce tyrosine phosphorylation of the *M*, 34,000 protein. HL-60 cells were exposed to the indicated doses of ionizing radiation and then harvested at 5 min. Soluble proteins were subjected to immunoblot (IB) analysis with the anti-P-Tyr (A) and p34^{cdc2} (B) antibodies. Arrows, position of *M*, 34,000 signals.

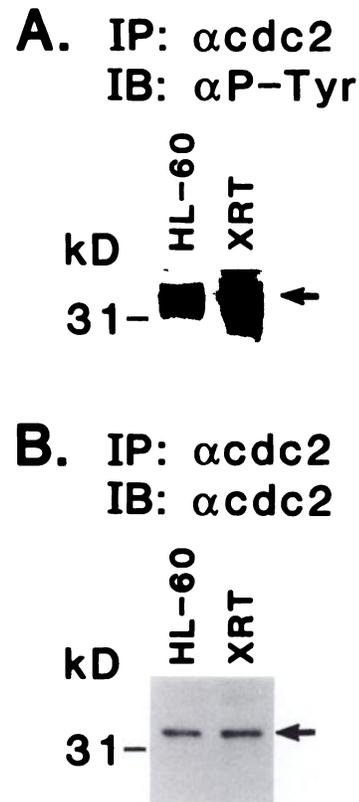


Fig. 3. Ionizing radiation induces tyrosine phosphorylation of p34^{cdc2}. HL-60 cells were exposed to 50 cGy ionizing radiation (XRT) and harvested at 5 min. Cell lysates from control and irradiated cells were subjected to immunoprecipitation (IP) with p34^{cdc2} antiserum and protein A-Sepharose. The immunoprecipitates were analyzed by immunoblotting (IB) with anti-P-Tyr (A) and anti-p34^{cdc2} (B).

for increased tyrosine phosphorylation following exposure to ionizing radiation. Of interest, recent studies have demonstrated that ionizing radiation exposure of *S. pombe* cells with a defective or missing *wee1* gene retain the capacity to undergo cell cycle arrest (22). Thus, tyrosine kinases other than Wee1 may be activated during the response of mammalian cells to DNA damage. In this context, studies in irradiated HL-60 cells have demonstrated activation of p56/p53^{lyn} and association of this tyrosine kinase with p34^{cdc2}.⁴ These findings, taken together with the present results, may contribute to our understanding of how the DNA damage checkpoint transduces signals to the mitotic machinery.

References

- Hall, E. J. In: Hall, E. J. (ed.), *Radiobiology for the Radiologist*, pp. 128–135. Philadelphia, PA: J. B. Lippincott Co., 1988.
- Hartwell, L. H., and Weinert, T. A. Checkpoints: controls that ensure the order of cell cycle events. *Science (Washington DC)*, *246*: 629–634, 1989.
- Lau, C. C., and Pardee, A. B. Mechanism by which caffeine potentiates lethality of nitrogen mustard. *Proc. Natl. Acad. Sci. USA*, *79*: 2942–2946, 1982.
- Steinmann, K. E., Belinsky, G. S., Lee, D., and Schlegel, R. Chemically induced premature mitosis: differential response in rodent and human cells and the relationship to cyclin B synthesis and p34^{cdc2}/cyclin B complex formation. *Proc. Natl. Acad. Sci. USA*, *88*: 6843–6847, 1991.
- Weinert, T., and Hartwell, L. The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. *Science (Washington DC)*, *241*: 317–322, 1988.
- Schiestl, R. H., Reynolds, P., Prakash, S., and Prakash, L. Cloning and sequence analysis of the *Saccharomyces cerevisiae RAD9* gene and further evidence that its product is required for cell cycle arrest induced by DNA damage. *Mol. Cell. Biol.*, *9*: 1882–1896, 1989.
- Murray, A. The cell cycle as a *cdc2* cycle. *Nature (Lond.)*, *341*: 14–15, 1989.
- Nurse, P. Universal control mechanism regulating onset of M-phase. *Nature (Lond.)*, *344*: 503–507, 1990.
- Pines, J., and Hunter, T. Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34^{cdc2}. *Cell*, *58*: 833–846, 1989.
- Russell, P., and Nurse, P. Negative regulation of mitosis by *wee1+*, a gene encoding a protein kinase homolog. *Cell*, *45*: 559–567, 1987.
- Featherstone, C., and Russell, P. Fission yeast p107^{wee1} mitotic inhibitor is a tyrosine/serine kinase. *Nature (Lond.)*, *349*: 808–811, 1991.
- Parker, L. L., Atherton-Fessler, S., Lee, M. S., Ogg, S., Falk, F. L., Swenson, K. I., and Piwnica-Worms, H. Cyclin promotes the tyrosine phosphorylation of p34^{cdc2} in a *wee1+* dependent manner. *EMBO J.*, *10*: 1255–1263, 1991.
- Parker, L. L., Atherton-Fessler, S., and Piwnica-Worms, H. p107^{wee1} is a dual-specificity kinase that phosphorylates p34^{cdc2} on tyrosine 15. *Proc. Natl. Acad. Sci. USA*, *89*: 2917–2921, 1992.
- Gould, K., and Nurse, P. Tyrosine phosphorylation of the fission yeast *cdc2+* protein kinase regulates entry into mitosis. *Nature (Lond.)*, *342*: 39–44, 1989.
- Gould, K. L., Moreno, S., Tonks, N., and Nurse, P. Complementation of the mitotic activator p80^{cdc25}, by a human protein-tyrosine phosphatase. *Science (Washington DC)*, *250*: 1573–1576, 1990.
- Enoch, T., and Nurse, P. Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. *Cell*, *60*: 665–673, 1990.
- Al-Khodairy, F., and Carr, A. M. DNA repair mutants defining G2 checkpoint pathways in *Schizosaccharomyces pombe*. *EMBO J.*, *11*: 1343–1350, 1992.
- Rowley, R., Subramani, S., and Young, P. G. Checkpoint controls in *Schizosaccharomyces pombe*: *rad1*. *EMBO J.*, *11*: 1335–1342, 1992.
- Lock, R. B., and Ross, W. E. Inhibition of p34^{cdc2} kinase activity by etoposide or irradiation as a mechanism of G₂ arrest in Chinese hamster ovary cells. *Cancer Res.*, *50*: 3761–3766, 1990.
- Uckun, F. M., Tuel-Ahlgren, L., Song, C. W., Waddick, K., Myers, D. E., Kirihara, J., Ledbetter, J. A., and Schieven, G. L. Ionizing radiation stimulates unidentified tyrosine-specific protein kinases in human B-lymphocyte precursors, triggering apoptosis and clonogenic cell death. *Proc. Natl. Acad. Sci. USA*, *89*: 9005–9009, 1992.
- Uckun, F. M., Schieven, G. L., Tuel-Ahlgren, L. M., Dibirdik, I., Myers, D. E., Ledbetter, J. A., and Song, C. W. Tyrosine phosphorylation is a mandatory proximal step in radiation-induced activation of the protein kinase C signaling pathway in human B-lymphocyte precursors. *Proc. Natl. Acad. Sci. USA*, *90*: 252–256, 1993.
- Barbet, N. C., and Carr, A. M. Fission yeast *wee1* protein kinase is not required for DNA damage-dependent mitotic arrest. *Nature (Lond.)*, *364*: 824–827, 1993.

⁴ S. Kharbanda, Z-M. Yuan, R. Weichselbaum, and D. Kufe, unpublished data.

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