Abolishment of the Tyr-15 Inhibitory Phosphorylation Site on cdc2 Reduces the Radiation-induced G2 Delay, Revealing a Potential Checkpoint in Early Mitosis

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ABSTRACT

cdc2 is inactivated before mitosis by phosphorylation at its inhibitory sites, Thr-14 and Tyr-15. Irradiation prevents HeLa cells from completing the G2-M transition, and they arrest in G2. Whereas phosphorylation at both of these sites occurs during the G2 arrest, the individual role of each site in the G2 delay has not previously been investigated. We have shown that the radiation-induced G2 delay is preserved in wild-type or cdc2-AY-transfected cells (which retain Tyr-15); this delay is abolished in cdc2-TF- or cdc2-AF-transfected cells (which lack Tyr-15). Thus Tyr-15, but not Thr-14, appears to be essential for development of a G2 delay after radiation. Abolishment of the G2 delay by mutation at Tyr-15 resulted in the accumulation of cells with condensed chromatin and disrupted lamina B, suggesting that these cells may be blocked at a second G2-M checkpoint in early mitosis (i.e., prophase). These data suggest (a) that the two inhibitory phosphorylation sites have distinct functions and that Tyr-15 phosphorylation, in particular, has a key role in the radiation-induced G2 delay, and (b) that a second G2-M checkpoint exists in early mitosis and that activation of this checkpoint by radiation prevents cells that enter mitosis from progressing further.

INTRODUCTION

Cyclin-dependent kinases and cyclins are important regulators of the eukaryotic cell cycle. Progression from the G2 phase of the cell cycle into mitosis requires the binding of cyclin B1 to cdc2, a series of phosphorylation and dephosphorylation events on both of these subunits, and translocation of the cdc2-cyclin B complex to the nucleus (1). Three residues, Thr-14, Tyr-15, and Thr-161, are involved in the phosphorylation/dephosphorylation events on cdc2 (2). Thr-161 phosphorylation by the cdc2-activating kinase is essential for activation of cdc2, whereas the Thr-14 and Tyr-15 residues act as inhibitory phosphorylation sites. Before mitosis, active cdc2 (phosphorylated at Thr-161 and bound to cyclin B1) is held in an inactive state by phosphorylation at these sites (3, 4). Inhibitory phosphorylation of cdc2 is regulated by the membrane-associated Myt 1 kinase, which preferentially phosphorylates cdc2 on its Thr-14 residue, although low levels of Tyr-15 phosphorylation are also detected (5). Myt1 kinase phosphorylates the Tyr-15 residue exclusively in the nucleus (6–8). The Src family protein tyrosine kinase Lyn has also been shown to phosphorylate the Tyr-15 site in human B-cell precursors (9). Despite the fact that different kinases are responsible for phosphorylating these two sites, the functional differences between Thr-14 and Tyr-15 have not previously been defined.

At the end of G2, rapid dephosphorylation of cdc2 at Thr-14 and Tyr-15 results in entry into mitosis (10, 11). The rapid dephosphorylation of cdc2 probably involves simultaneous inactivation of the Wee 1/Myt 1 kinases together with the activation of the dual specificity cdc25C phosphatase, which sequentially dephosphorylates cdc2 at Thr-14 followed by Tyr-15 (12). These mitotic changes are related to a positive feedback loop involving phosphorylation of these enzymes by the cdc2-cyclin B1 complex (13–15). More recently, a role for cdc25B as a putative trigger for this positive feedback loop has been proposed (16).

In addition to these phosphorylation/dephosphorylation events, the nuclear localization of the cdc2-cyclin B1 complex also plays an important role in mitotic timing (17). For example, the mitosis-promoting activity of cyclin B1 in frog eggs is abolished by expression of a mutant form of cyclin B1, which is unable to localize to the nucleus (18). Because cdc2 does not possess a NLS and is not able to bind to nuclear import proteins, it is thought that cyclin B1 acts as a nuclear targeting subunit for cdc2 (19). Nuclear import of cyclin B1 arises from cyclin B1 interaction with importin β (19). During interphase, cdc2-cyclin B1 shuttles between the nucleus and cytoplasm, but the rate of nuclear export exceeds the rate of nuclear import, and hence cyclin B1 is predominantly located in the cytoplasm (20–22). At prophase, more complex is found in the nucleus. Phosphorylation of a NES within the cytoplasmic retention sequence on cyclin B1 is thought to inhibit nuclear export (18, 23). In late prophase, phosphorylation of the NES may interfere with binding of the nuclear export receptor CRM1 to cyclin B1 (22), leading to the nuclear accumulation of cdc2-cyclin B1 complexes.

Exposure to DNA-damaging agents, such as ionizing radiation, results in cell cycle arrest, thereby allowing the damaged chromosomes to be repaired before the cell progresses to mitosis. Many reports indicate that DNA damage causes prolonged inhibitory phosphorylation of cdc2 at Thr-14 and Tyr-15 and that this is responsible for the G2 delays in the fission yeast Schizosaccharomyces pombe (24, 25), in Aspergillus nidulans (26), and in mammalian cells (27–29). Stabilization of these inhibitory phosphorylation sites may be linked to the inactivation of the cdc25C phosphatase because DNA damage inhibits cdc25C activation in human cells by activation of Chk1 and Chk2 (30). Both Chk1 and Chk2 functionally inactivate cdc25C by phosphorylation on the Ser-216 residue. Phosphorylation at this residue creates a binding site for 14-3-3 proteins, which then sequester cdc25C in the cytoplasm, thereby preventing cdc25C from interacting with cdc2 in the nucleus (31–34). In the fission yeast, cdc25C is exported from the nucleus into the cytoplasm in response to DNA damage (35).

The importance of inhibitory phosphorylation of cdc2 in the G2 arrest has been demonstrated using cdc2-AY, a mutant form of cdc2 that cannot be phosphorylated at either its Thr-14 residue (mutated to alanine) or its Tyr-15 residue (mutated to phenylalanine). cdc2-AY induces limited premature mitotic events when transiently overexpressed in mammalian cells (4, 6), and when expressed at normal levels (using a tetracycline-repressible promoter) in HeLa cells (36), suggesting that inhibitory phosphorylation at these sites is essential for regulating normal mitotic timing. In HeLa cells, overexpression of...
cdc2-AF is reported to decrease the radiation-induced G₂ arrest (36). It also results in a significant loss of cell viability after exposure to DNA-damaging reagents (37), indicating that inhibitory phosphorylation has a key role in establishing the DNA damage-induced G₂ arrest. However, whether both inhibitory phosphorylation sites are necessary for this DNA damage-induced response and, furthermore, whether Thr-14 and Tyr-15 have the same or distinct roles in mediating normal cell cycle progression have yet to be determined.

The subcellular localization of cyclin B1 and/or cdc2 has also been implicated in establishing a G₂ arrest after DNA damage (21, 38). Cyclin B1 is cytoplasmic in cells arrested in G₂, by DNA damage (36, 39). Expression of a nuclear cyclin B1 partially overrides the G₂ arrest that occurs after DNA damage, suggesting that cyclin B1 is a rate-limiting step in the initiation of mitosis (38). Interestingly, expression of nuclear cyclin B1 together with the nonphosphorylatable cdc2-AF under normal growth conditions results in a premature mitotic phenotype (38). More recently, overproduction of Myt 1 kinase is reported to induce a G₂ delay in normal cycling HeLa cells by interfering with cdc2-cyclin B1 trafficking to the nucleus (40).

The literature thus indicates significant roles for inhibitory phosphorylation of cdc2 and subcellular localization of the cdc2-cyclin B1 complex in maintaining a G₂ delay after DNA damage. However, the individual roles of the Thr-14 and Tyr-15 inhibitory phosphorylation sites have not previously been examined. In this study, we investigate the individual roles of these sites, using cdc2 mutants that lack either Thr-14 (cdc2-AY) or Tyr-15 (cdc2-TF) or both (cdc2-AF) of these sites. We show that the Thr-14 and Tyr-15 phosphorylation sites have distinct roles in regulating the G₂-M transition. In particular, the Tyr-15 but not the Thr-14 phosphorylation site is necessary for maintaining a G₂ delay after irradiation because this G₂ arrest is abolished in HeLa cells expressing mutants that lack the Tyr-15 phosphorylation site (i.e., cdc2-TF and cdc2-AF). In cells lacking the Tyr-15 residue, an increase in chromatin condensation corresponds to abolishment of the G₂ delay. This phenotype presumably arises when cells enter mitosis prematurely, with either incompletely replicated or damaged DNA. Thus, a second mitotic checkpoint (possibly in prophase) maybe activated in response to irradiation, resulting in abnormal chromatin condensation followed by entry into mitotic catastrophe and subsequent cell death. Coexpression of cyclin B1 with all of the cdc2 mutants leads to increased mitotic catastrophe and apoptosis in both irradiated and nonirradiated cells. The different responses between cells expressing wild-type and mutated cdc2 clearly indicates that the Thr-14 site also has a significant but distinct role from Tyr-15 in mediating the G₂-M transition in normal cycling cells. The role of nuclear localization of cdc2 in the radiation-induced G₂ delay has also been addressed by attaching an NLS to wild-type cdc2 and its mutants. Forced nuclear localization of the mutants has no significant effect on their response to radiation.

**MATERIALS AND METHODS**

**Construction of cdc2 Mutant Plasmids**

**Construction of the cdc2-AY and cdc2-TF Mutants.** To construct cdc2-AY, an oligonucleotide containing the base change that would mutate Thr-14 to Ala-14 was synthesized and amplified by PCR (5'-GTG-TCT-ACC-CTT-ATA-CAC-AAC-TCC-ATA-TGC-ACC-TTC-AAT-TTT-C-3'). Similarly, the cdc2-TF mutant was constructed by synthesis and amplification of an oligonucleotide that would mutate Tyr-15 to Phe-15 (5'-GTG-TCT-ACC-CTT-ATA-CAC-AAC-TCC-GAA-GGT-ACC-TTC-AAT-TTT-C-3'). In each case, the PCR product was ligated into the cdc2-AF-pECE vector between the AgeI sites. The mutations were then confirmed by sequencing.

**Construction of the cdc2-GFP and cdc2-NLS-GFP Mutants.** The cdc2-pECE vector described above was digested with RsaI to obtain a 387-bp fragment containing the 3' terminal of cdc2 (containing the BulII site). An oligonucleotide containing the NLS sequence (AVKRPATATKQQAGKKKLD) flanked by two NruI restriction sites on the 5' and 3' sites and containing the 5' terminal of the GFP sequence was synthesized (5'-AAT-CAG-ATT-AAG-AGT-TGC-CCA-CCC-GTC-AAG-AAG-CGA-GAA-GTC-ACC-AAG-GCT-GGT-CAC-GCC-GGT-ACC-CTC-ACT-CCT-GCG-CCC-GGT-CAC-GGG-GCC-GGC-GGC-ACC-ATG-AGT-AAG-GAC-AAAG-GAT-CTC-ATC-ATA-CTA-3'). The DNA fragment and oligonucleotide were ligated together and amplified by the same PCR reaction. The PCR product was ligated into the NH₂-terminal of each of the wild-type and mutant cdc2-pECE vectors (between BulII and SacI). The cdc2-NLS sequence was then cut out of the pECE vector (between SacI and Bsu36I) and ligated into the pLEGFP-N1 vector (Clontech) to produce cdc2-NLS-GFP. The NLS sequence was removed by NruI digestion, and the plasmid was religated to produce cdc2-GFP. This was repeated for each of the cdc2 mutants described above to construct the following plasmids: (a) cdc2-TF-GFP; (b) cdc2-AY-GFP; (c) cdc2-TF-GFP; (d) cdc2-AF-GFP; (e) cdc2-TF-NLS-GFP; (f) cdc2-AY-NLS-GFP; (g) cdc2-TF-NLS-GFP; and (h) cdc2-AF-NLS-GFP. The DNA sequences of all these constructs were confirmed by sequencing.

**Cell Culture and Nocodazole Trapping**

HeLa cells were grown in DMEM supplemented with 10% FCS, penicillin, and streptomycin. Cells were transfected with the cdc2-GFP plasmids (1 μg DNA/10⁵ cells) using Fugene 6 (Boehringer Mannheim) 24 h before irradiation. Transfected cells were washed twice and irradiated with 4 or 6 Gy using a Shepherd Mark 1 Model 68A cesium irradiator. Nocodazole (0.04 μg/ml) was added to each well immediately after irradiation, and samples were collected by trypsinization at subsequent time points (i.e., 9.5 and 12 h after irradiation), fixed in 4% paraformaldehyde for 10 min, and stored at 4°C in PBS to await further examination.

rTA HeLa cells were grown under Geneticin (G418; 400 μg/ml) selection in DMEM supplemented with 10% FCS, penicillin, and streptomycin. Cells were transfected with the cdc2-GFP plasmids as described above, and 18 h before irradiation, rTA HeLa cells were infected with 100 pfu/cell cyclin B1 adenovirus (David Morgan, University of California, San Francisco, CA; Ref. 38). Cells were irradiated, treated with nocodazole, and fixed as described above.

**Immunofluorescence Analysis**

The fixed, transfected HeLa cells were stained with Hoechst 33342 (100 μg/ml) for 10 min, resuspended in PBS, and examined with a fluorescence microscope. The number of mitotic, interphase, and apoptotic cells and the number of cells with condensed chromatin were counted in either nontransfected or GFP-expressing cells. The expression of GFP was related to the expression of the cdc2-GFP wild-type and mutant plasmids.

In addition, transfected HeLa cells were stained for lamin B using a mouse monoclonal antibody (OncoGene Research Products) diluted to 0.1 μg/ml in diluent (0.1% Triton X-100, 4% bovine serum albumin, and 2% normal goat serum in PBS) and a secondary antibody (goat anti-mouse IgG Rhodamine Red; Jackson Immunoresearch Laboratories) diluted 1:1000 in diluent. The fixed, transfected rTA HeLa cells were first stained with anti-Myc monoclonal antibody (Oncogene Research Products) diluted to 0.1 μg/ml in diluent (0.1% Triton X-100, 4% bovine serum albumin, and 2% normal goat serum in PBS) and a secondary antibody (goat anti-mouse IgG Rhodamine Red; Jackson Immunoresearch Laboratories) diluted 1:1000 in diluent to detect the cyclin B1-expressing cells. The cells were then stained with Hoescht 33342 and examined by fluorescence as described above. Only the nuclei of cells that were positive for both GFP (i.e., expressed the cdc2-GFP plasmids) and rhodamine (i.e., expressed the myc-tagged cyclin B1 adenovirus) were counted for this study.

**Immunoprecipitation and Histone H1 Kinase Assay**

Cells were solubilized in lysis buffer [50 mM Tris (pH 7.4), 100 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM sodium orthovanadate, and 1 μM protease inhibitor mixture (Pharmigen)] for 10 min at 4°C. Insoluble material was removed by centrifugation at 14,000 rpm for 15 min at 4°C. The supernatant was transferred to a fresh tube containing 50 μl of protein A-agarose beads and incubated for 1 h at 4°C to

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preclear the lysate. The lysate was centrifuged at 2,500 rpm for 10 min at 4°C to remove the beads. One μl of polyclonal anti-GFP (Luc Berthiaume; University of Alberta, Alberta, Canada) was added to the lysate and incubated for 1 h on a rotator at 4°C. Protein A-agarose beads (50 μl) were added to the lysate, and it was incubated for an additional 2 h at 4°C. The immunoprecipitates were washed twice with lysis buffer and once with kinase buffer KAB [50 mM Tris (pH 7.4), 10 mM MgCl₂, and 1× protease inhibitor mixture]. Immunoprecipitates were incubated with KAB containing 50 μM cold ATP, 1 mg/ml histone H1, and 12.5 μCi of [γ-³²P]ATP for 20 min at 37°C. Then 2× SDS loading buffer was added, and the reactions were incubated for 5 min at 100°C. Reaction products were separated by 10% SDS-PAGE and detected by autoradiography.

To demonstrate that cdc2-GFP fusion proteins interact with cyclin B1, GFP immunoprecipitates were also separated by SDS-PAGE directly, and Western blots were performed using a monoclonal antibody to cyclin B1 (Upstate Biotechnology). Interactions between cdc2-GFP fusion proteins and cyclin B1 were further demonstrated by immunoprecipititating cyclin B1. Briefly, cells were solubilized in lysis buffer for 15 min at 4°C. Insoluble material was removed by centrifugation at 14,000 rpm for 15 min at 4°C. The supernatant was transferred to a fresh tube containing 50 μl of protein G-agarose beads and incubated for 1 h at 4°C to preclear the lysate. The lysate was centrifuged at 2,500 rpm for 10 min at 4°C to remove the beads. Two μg of monoclonal cyclin B1 antibody (Upstate Biotechnology) was added to the lysate and incubated overnight on a rotator at 4°C. Protein G-agarose beads (50 μl) were added to the lysate, and it was incubated for an additional 2 h at 4°C. The immunoprecipitates were washed three times with lysis buffer and resuspended in electrophoresis buffer. Then 2× SDS loading buffer was added, and the reactions were incubated for 5 min at 100°C. Reaction products were separated by 10% SDS-PAGE and detected by autoradiography using a monoclonal antibody to cdc2 (Sigma Chemical Co.).

RESULTS

Generation of cdc2 with Mutations at the Inhibitory Phosphorylation Sites. To examine the role of the individual inhibitory phosphorylation sites of cdc2 during the radiation-induced G2 delay in HeLa cells, we generated expression vectors for cdc2 mutants lacking one [Thr-14 (cdc2-AY) or Tyr-15 (cdc2-TF)] or both (cdc2-AF) of these inhibitory phosphorylation sites. The wild-type cdc2 (cdc2-TY-GFP) and its mutants were constructed as GFP fusion proteins to enable transfected cells to be detected quickly and reproducibly. The transfection efficiency of cdc2-GFP plasmids into HeLa cells ranged between 15% and 20%. As shown in Fig. 1, A and B, cdc2-TY-GFP was present in both the cytoplasm and nucleus of the interphase cells. Each cdc2-GFP mutant was also distributed throughout the cytoplasm and nucleus (data not shown). To establish that cdc2-GFP fusion proteins were functionally active, we first showed that cyclin B1 complexed with the cdc2-GFP fusion proteins. Cells transfected with the cdc2-GFP fusion proteins were trapped in mitosis by nocodazole treatment 14 h before immunoprecipitation.

The immunoprecipitation was performed using either (a) an antibody to cyclin B1 (Fig. 1E) followed by Western blot analysis with a monoclonal antibody to cdc2 or (b) an antibody to GFP (Fig. 1F) followed by Western blot analysis using an antibody to cyclin B1. In Fig. 1E, a band is observed at 61 kDa corresponding to cdc2-GFP. Two bands representing the active and inactive forms of endogenous cdc2 were also observed in both the nontransfected and transfected samples at approximately 34 kDa after cyclin B1 immunoprecipitation (data not shown). Similar cdc2 protein levels were observed in both the nontransfected and transfected samples, indicating that transfection of cdc2-GFP vectors does not alter the expression of endogenous cdc2 and cyclin B1. In Fig. 1F, a band is observed at 59 kDa, corresponding to cyclin B1. In both figures, the lower band represents the specific immunoglobulin band at 55 kDa. These data indicate that the GFP tag does not interfere with the interaction between cdc2 and cyclin B1. In addition, we performed histone H1 kinase assays in asynchronous, nonirradiated and irradiated samples to demonstrate that the cdc2-GFP-cyclin B1 complex (i.e. the M phase-promoting factor) is functionally active (Fig. 1G). In these cases, we immunoprecipitated the cdc2-GFP-cyclin B1 complex using a polyclonal antibody to GFP. The irradiated and nonirradiated samples were treated with nocodazole 14 h before immunoprecipitation to trap cells that had entered mitosis. The lanes labeled NT correspond to immunoprecipitates from nontransfected cells and hence show background levels. As shown in Fig. 1G, the anti-GFP immunocomplexes for wild-type cdc2-GFP and its nonphosphorylatable/partially phosphorylatable mutants all have increased histone H1 kinase activity after 14 h of treatment with nocodazole, compared with the corresponding asynchronous samples. This shows that the cdc2-GFP proteins are functionally active and that the M phase-promoting factor activity is maintained in cells expressing mutant cdc2-GFP. After exposure to irradiation, HeLa cells arrest in G2. This involves inactivation of the cdc2-cyclin B1 complex via several mechanisms including inhibitory phosphorylation of cdc2 at Thr-14 and Tyr-15 (27–29), in addition to decreased cyclin B1 expression (41). In Fig. 1G, the lanes labeled ionizing radiation correspond to GFP immuno-
complexes from cells exposed to 6 Gy before nocodazole treatment for 14 h. As expected, cdc2-TY-GFP histone H1 kinase activity was decreased in irradiated cells compared with the nonirradiated cdc2-TY-GFP sample. GFP immunocomplexes obtained for each of the mutants after irradiation were increased compared with the wild-type cdc2-GFP histone H1 kinase activity. These findings agree with the data from Fig. 3, which shows that in cells transfected with these nonphosphorylatable/partially phosphorylatable mutants, the radiation-induced G2 arrest is reduced after 12 h, although it is still observed in the cdc2 wild-type-transfected cells.

These data show that (a) cdc2-GFP fusion proteins could be easily and reproducibly detected by immunofluorescence (Fig. 1, A and B), (b) cdc2-GFP fusion proteins interact with cyclin B1 (Fig. 1, E and F), and (c) the GFP tag does not interfere with the kinase activity of cdc2 (Fig. 1G). Indeed, distinctive biological effects could be discerned after expression of each cdc2-GFP, further demonstrating their activity (see below). Hence, cdc2-GFP fusion proteins were used for the remainder of this study.

It is worth noting that in subsequent experiments, which compared nontransfected, GFP-transfected, and cdc2-GFP-transfected cells, cells transfected with either the GFP vector alone or the cdc2-GFP fusion proteins entered mitosis at a slower rate than nontransfected cells. This was true for different GFP vectors in several cell lines.4

The Tyr-15 but not the Thr-14 Phosphorylation Site Is Essential for Maintaining the G2 Delay after Irradiation. Because HeLa cells express the papillomavirus E6 protein that enhances degradation of p53 (42), they lack the G1 checkpoint in response to radiation. Consequently, HeLa cells accumulate mainly in G2 in response to DNA damage (43). This is illustrated in Fig. 2A. HeLa cells were irradiated and treated with nocodazole, and at various time points after irradiation, the cells were stained with Hoescht dye to enable determination of the percentage of mitotic cells or cells with condensed chromatin. Fig. 2, B–D, shows the various nuclear phenotypes encountered during this study [i.e., mitotic (arrowhead) and interphase (Fig. 2B), condensed chromatin (Fig. 2C), and undergoing mitotic catastrophe (Fig. 2D)]. Fig. 2A shows that a significant G2 delay occurred up to 10 h after exposure of cells to 4 and 6 Gy. By 12 h, the irradiated cells began to exit the G2 arrest, and by 24 h after irradiation, the G2 delay was no longer present. Based on this timing, we decided to examine the effects of the cdc2-GFP mutants 9.5 and 12 h after irradiation because significant G2 delays were observed at these time points.

In transfected populations of cells, the nuclear phenotypes of GFP-positive cells were assessed (as shown in Fig. 2, B–D) and counted 9.5 and 12 h after irradiation (Fig. 3). The distribution of wild-type cdc2-GFP and its nonphosphorylatable/partially phosphorylatable mutants in both the cytoplasm and nucleus was not altered in irradiated cells (data not shown).

In a manner similar to that of nontransfected HeLa cells, a radiation-induced G2 delay was observed 9.5 and 12 h after irradiation in cells transfected with wild-type cdc2-TY-GFP; i.e., the total percentage of cells in mitosis was lower in cells exposed to 4 or 6 Gy than in nonirradiated cells (Fig. 3, A and D). The percentage of cells with condensed chromatin was virtually undetectable in cdc2-TY-GFP (wild-type)-transfected cells (Fig. 3, B and E), indicating that these cells progressed from G2 through prophase to the later stages of mitosis in a normal manner.

Transfection with cdc2-AY-GFP led to a pattern similar to that of the wild type at 9.5 h, with a slight reduction in the mitotic delay (Fig. 3A). At 12 h, the mitotic delay induced by radiation was substantially reduced compared with cdc2-TY-GFP (wild-type; Fig. 3A). No increase in the number of cells with condensed chromatin was observed at either time (Fig. 3, A and E), indicating that cells progress normally through G2 into mitosis.

Transfection with either cdc2-TF-GFP or cdc2-AF-GFP also resulted in a slight reduction in the time required for the appearance of mitotic cells after radiation, similar to the effect of cdc2-AY-GFP (Fig. 3A). In contrast, the percentage of cdc2-TF-GFP- or cdc2-AF-GFP-expressing cells that had condensed chromatin was markedly higher than the percentage of cells transfected with cdc2-TY-GFP (wild-type) or cdc2-AY-GFP (Fig. 3, B and E) that had condensed chromatin. The total percentage of cells in mitosis or with condensed chromatin was similar in irradiated and nonirradiated cells for both the cdc2-TF-GFP and cdc2-AF-GFP samples (Fig. 3, C and F), indicating that a G2 delay was not maintained 9.5 or 12 h after irradiation in cells transfected with mutants that lack the Tyr-15 residue. Abolishment of the G2 delay was concomitant with an increase in condensed chromatin.

Cells with Condensed Chromatin Arrest in Early Mitosis. To demonstrate that cells with condensed chromatin had entered mitosis, we examined the distribution of lamin B in cdc2-AF-GFP-expressing cells (Fig. 4). Lamins are nuclear envelope proteins that can serve as

4 Unpublished data.
markers for different phases of the cell cycle. When a cell enters mitosis, nuclear envelope breakdown occurs. This process begins in early prophase and involves depolymerization of the nuclear lamin, disassembly of the nuclear pores, and dispersion of the nuclear membranes (44, 45). Studies examining the disassembly patterns of lamins show that during early prophase, B-type lamins still associated with the inner nuclear membrane exhibit focal indentations or deep invaginations (46). At the prophase/prometaphase transition, the B-type lamina is fragmented but is not completely dissolved. During metaphase, the lamin B polymer breaks down into small pieces, which tend to concentrate around the mitotic spindle. As shown in Fig. 4, lamin B is localized around the nucleus in interphase cells, demonstrating that the nuclear envelope is intact. Lamin B staining is diffuse in metaphase cells, indicating that nuclear envelope breakdown has occurred (data not shown). In cells with condensed chromatin, the distribution of lamin B is fragmented and in many cases (indicated by the arrowhead in Fig. 4) still appears to be localized to the inner nuclear membrane, although the nuclear envelope is no longer intact. This suggests that cells with condensed chromatin have entered mitosis but have arrested during an early mitotic phase, possibly early prophase.

Nuclear Localization of cdc2 and Its Mutants Does Not Alter Their Response to Radiation. In addition to the inhibitory phosphorylation of cdc2, the nuclear localization of the cdc2-cyclin B1 complex also plays a significant role in mitotic timing (17). cdc2 does not have a NLS, nor can it bind to nuclear import proteins. Thus, it has been suggested that cyclin B1 acts as a nuclear targeting subunit for cdc2 (19). To investigate the role of nuclear translocation of cdc2 in the radiation-induced G2 delay, the NLS of SV40 was ligated to the NH2 terminus of cdc2-TY and its nonphosphorylatable/partially phosphorylatable mutants. The cdc2-NLS-GFP wild-type (Fig. 1, C and D)
and mutants (data not shown) localized to the nucleus. Forced nuclear localization of cdc2-TY-NLS-GFP (i.e., wild-type) and its mutants resulted in a slight but nonsignificant decrease in the percentage of mitotic cells and/or cells with condensed chromatin at both 9.5 and 12 h after nocodazole trapping (compare Figs. 3C and 5A; compare Figs. 3F and 5B). Despite these decreased levels of mitotic activity, a similar pattern was observed between cdc2 mutants expressing NLS and cdc2 mutants lacking NLS in response to irradiation (compare Figs. 3C and 5A; compare Figs. 3F and 5B); thus, cells expressing cdc2 with an intact Tyr-15 phosphorylation state (cdc2-TY-NLS-GFP and cdc2-AF-NLS-GFP) were arrested in G2 after exposure to 4 and 6 Gy, whereas the G2 delay was abolished in cells expressing cdc2 without the Tyr-15 phosphorylation site (cdc2-TF-NLS-GFP and cdc2-AF-NLS-GFP). These data indicate that the forced nuclear localization of cdc2 does not significantly alter its response to irradiation.

Coexpression of Cyclin B1 with cdc2 Mutants Reveals an Important Role for Thr-14 Distinct from That of Tyr-15. Although interesting results were obtained from the studies comparing the effects of irradiation on different cdc2 mutants, it was possible that these observed responses were limited by decreased levels of cyclin B1 in response to the irradiation. Previous studies report that under some conditions, DNA damage transiently decreases cyclin B1 mRNA expression in HeLa cells (41). Furthermore, overexpression of cyclin B1 decreases the damage-induced G2 arrest (38, 47). Thus, we attempted to address these issues by coexpressing cyclin B1 with cdc2 and its nonphosphorylatable or partially phosphorylatable mutants. The differences in the percentage of cells in mitosis, cells with condensed chromatin, or cells undergoing cell death via mitotic catastrophe and apoptosis between wild-type cdc2 and the mutants in the presence of excess cyclin B1 are shown in Fig. 6, A–C.

As shown in Fig. 6A, overexpression of cyclin B1 alone did not affect the response of HeLa cells to irradiation as compared with nontransfected cells (Fig. 2A). Thus, 9.5 h after exposure of cells to 4 and 6 Gy, the percentage of cells in mitosis was significantly lower than that in nonirradiated cells. The percentage of cells with condensed chromatin (Fig. 6B) or cells undergoing mitotic catastrophe or apoptosis (Fig. 6C) was very low, and no differences were observed between nonirradiated and irradiated samples. Coexpression of cyclin B1 with cdc2-TY-GFP significantly increased the percentage of nonirradiated cells in mitosis above the levels observed in cells transfected with cdc2-TY-GFP alone (compare Figs. 6A and 3A). However, the percentage of mitotic cells was significantly decreased in cells coexpressing cyclin B1 and cdc2-TY-GFP at 9.5 h after exposure to 4 and 6 Gy, indicating that a G2 delay was still maintained in response to irradiation (Fig. 6A). These data show that normal cell cycle regulation and responses to DNA damage occur when cyclin B1 is coexpressed with wild-type cdc2-TY-GFP.

In cells coexpressing cyclin B1 with cdc2-AY-GFP, the percentage of mitotic cells was decreased compared with that of cells coexpressing cdc2-TY-GFP with cyclin B1 (Fig. 6A). In addition, an increase in the percentage of cells with condensed chromatin (Fig. 6B) or cells undergoing mitotic catastrophe or apoptosis (Fig. 6C) occurred compared with cells expressing cdc2-AY-GFP alone. Furthermore, no significant differences were observed between irradiated and nonirradiated samples for each of the nuclear phenotypes examined. Thus, it would appear that coexpression of cyclin B1 with cdc2-AY-GFP abolishes the radiation-induced G2 arrest (Fig. 6A), whereas expression of cdc2-AY-GFP alone sustains a G2 arrest in response to irradiation (Fig. 3A). The difference between the radiation-induced response in cells transfected with wild-type cdc2-TY-GFP (which retains both THR-14 and TYR-15 sites) augmented with cyclin B1 and the radiation-induced response in cells transfected with the single mutant cdc2-AY-GFP augmented with cyclin B1 reveals that THR-14 has a significant role distinct from that of TYR-15 in mediating the G2-M transition.

An increased percentage of cells in mitotic catastrophe and apoptosis was observed when cdc2-TF-GFP was coexpressed with cyclin B1 (Fig. 6C) compared with cells expressing cdc2-TF-GFP alone. Comparable with cells that were transfected with cdc2-TF-GFP alone, the radiation-induced G2 delay (measured by combining the percentage of cells in mitosis and cells with condensed chromatin) was abolished in the presence of cyclin B1 (Fig. 6, A and B). Coexpression of cdc2-AF-GFP with cyclin B1, however, had the most dramatic effect on cell viability because, in both nonirradiated and irradiated cells, at least half of the cells were undergoing mitotic catastrophe or apoptosis 9.5 h after irradiation (Fig. 6C). Jin et al. (38) have previously reported a moderate decrease in damage-induced arrest in cells coexpressing cyclin B1 and cdc2-AF.

**DISCUSSION**

This study has examined the role of the individual inhibitory phosphorylation sites of cdc2 and the nuclear localization of cdc2 during the radiation-induced G2 delay in HeLa cells. We have shown that Thr-14 and Tyr-15 have distinct roles during the G2-M transition and that Tyr-15 has a more significant role in maintaining a G2 arrest after DNA damage. Our data also indicate that a second checkpoint in early mitosis may exist that prevents DNA-damaged cells that have entered mitosis prematurely from completing mitosis.
Thr-14 and Tyr-15 Have Distinct Roles during the G2-M Transition. Construction of cdc2 mutants that lack one or both of the inhibitory phosphorylation sites enabled us to investigate the individual roles of Thr-14 and Tyr-15 in the G2-M transition. Our data indicate that the two sites have independent roles in regulating the progression from G2 into mitosis because the cdc2 mutants that lack one phosphorylation site [Thr-14 (cdc2-AF-GFP) or Tyr-15 (cdc2-TF-GFP)] give rise to different responses after exposure to irradiation. Cells expressing cdc2 that retain the Tyr-15 phosphorylation site (cdc2-AY-GFP) were arrested in G2 up to 9.5 h after irradiation. This G2 delay was associated with an increased incidence of chromatin condensation, indicating that inhibitory phosphorylation at these sites is essential for regulating normal mitotic timing. The effects of cdc2-AF-GFP on the radiation-induced G2 arrest were also examined. We showed that the G2 delay was abolished in cdc2-AF-GFP-expressing cells because the percentage of cells in mitosis and/or with condensed chromatin was similar between irradiated and nonirradiated cells. This inability to arrest in G2 was associated with an increased incidence of chromatin condensation. Thus, although cdc2-AF expression accelerates the entry of cells into prophase as defined by condensed chromatin and nuclear envelope breakdown, it does not result in an increased number of mitotic cells. In addition, we also found increased apoptosis and/or mitotic catastrophe after irradiation in cells expressing cdc2-AF-GFP. Previously, overexpression of cdc2-AF in HeLa cells has been shown to decrease the radiation-induced G2 arrest (36). cdc2-AF expression also results in a significant loss of cell viability after exposure to DNA-damaging reagents (37), indicating that inhibitory phosphorylation has a key role in establishing the DNA damage-induced G2 arrest. Taken together, the data from this study and previous studies indicate that inhibitory phosphorylation of cdc2 is essential for regulating entry into mitosis at the correct time point during the cell cycle and maintaining DNA-damaged cells in the G2 phase of the cell cycle until the damaged DNA has been repaired. Our study indicates, for the first time, that Thr-14 and Tyr-15 have distinct roles in mediating the G2-M transition and that the presence of Tyr-15 alone, but not Thr-14 alone, is able to sustain the radiation-induced G2 delay. It is interesting that our results show that Tyr-15 phosphorylation has a key role in maintaining the G2 delay in human HeLa cells because in other systems, for example, A. nidulans (26) and fission yeast (3), the DNA damage checkpoint is mediated through Tyr-15 phosphorylation alone.

The observation that Tyr-15 phosphorylation but not Thr-14 phosphorylation leads to a G2 arrest in response to irradiation may be linked to the subcellular localization of the enzymes responsible for phosphorylating and dephosphorylating these sites. Both Thr-14 and Tyr-15 can be phosphorylated in the cytoplasm by the cytoplasmically located Myt 1 kinase (5), whereas only the Tyr-15 residue can be phosphorylated in the nucleus by the Wee 1 kinase (6–8). Active Lyn kinase interacts with cdc2 in both the cytoplasm and nucleus, and evidence suggests that it is responsible for phosphorylating the Tyr-15 residue alone (9, 48, 49). Thus, the different responses to irradiation by cdc2-TF-GFP and cdc2-AY-GFP may be due to Thr-14 phosphorylation having an early cytoplasmic role in inhibiting premature entry into mitosis, and Tyr-15 having a later nuclear role in maintaining the cdc2-cyclin B1 complex in an inactive state.

In addition to inactivation of cdc2 by the Myt 1, Lyn, and Wee 1 kinases described above, cdc2 activity is also regulated by the family...
of cdc25 phosphatases, which have an important role in cdc2 activation. Both cdc25B and cdc25C are essential for the G$_2$-M transition (15, 16). Cdc25B activation occurs during late S phase and peaks during G$_2$ before the activation of cdc25C (16). During S phase, cdc25B is predominantly localized to the nucleus, but it translocates to the cytoplasm during G$_2$ (50), where it is believed to target the cdc2-cyclin B1 complex directly (16, 51). Cdc25C is translocated from the cytoplasm to the nucleus at the onset of mitosis by dephosphorylation at the Ser-216 residue. This signals the release of 14-3-3 proteins (which sequester cdc25C in the cytoplasm) and allows cdc25C to reenter the nucleus and activate the cdc2-cyclin B1 complex by dephosphorylating the Thr-14 and Tyr-15 residues (31–33).

The order of events that leads to the activation of cdc2 at the G$_2$-M transition is not clear, but evidence suggests that cdc2 binds to cyclin B1 before phosphorylation at its Thr-161 site (52). The active cdc2-cyclin B1 complex is then inactivated by phosphorylation at Thr-14 and Tyr-15 (5–8). During interphase, cdc2-cyclin B1 shuttles between the nucleus and the cytoplasm. Nuclear accumulation of cdc2-cyclin B1 complexes occurs during late prophase and is regulated by phosphorylation of the NES on cyclin B1 (22). It is not clear whether the cdc2-cyclin B1 complex passes into the nucleus in an active or inactive state. However, if it were to pass into the nucleus in an inactive state, then several questions would arise: for example, what would be the purpose of the nuclear Wee 1 kinase and why would cdc25B be translocated to the cytoplasm during G$_2$? Indeed, the fact that cdc25B shuts into the cytoplasm and targets cdc2-cyclin B1 during G$_2$ (16, 50, 51) implies that cdc2-cyclin B1 is active when it first accumulates in the nucleus and would remain in an active form if it were not for Tyr-15 phosphorylation by the nuclear Wee 1 kinase (6–8). This is supported by a recent report that shows that in irradiated HeLa cells, the cytoplasmic form of cdc2 is active, whereas the nuclear accumulated form is inactive (53). After DNA damage, cdc2 is held in inactive state by inhibitory phosphorylation at Tyr-15 in both A. nidulans (26) and fission yeast (3). This is presumably related to the inactivation and cytoplasmic sequestration of cdc25C in response to DNA damage (31–34, 54), which would prevent cdc25C from dephosphorylating cdc2 in the nucleus. Consequently, any cdc2-cyclin B1 complex activated by cdc25B in the cytoplasm would immediately be phosphorylated by Wee 1 at its Tyr-15 residue upon entry into the nucleus. Thus, the cell would be able to delay entry into mitosis until the damaged DNA has been repaired.

Our data support the hypothesis that cdc2-cyclin B enters the nucleus in an active state because cdc2-AY-GFP (which retains the Tyr-15 site) maintains a G$_2$ delay after irradiation, whereas cdc2-TF-GFP immediately be phosphorylated by Wee 1 at its Tyr-15 site upon entry into the nucleus. Meanwhile, inactivated cdc25C is translocated to the cytoplasm (where it cannot dephosphorylate the nuclear cdc2-AY) until the damaged DNA has been repaired. After DNA repair, the G$_2$-M transition is able to progress as cdc25C reenters the nucleus and dephosphorylates cdc2-AY at Tyr-15, thereby allowing an active cdc2-cyclin B1 complex to initiate normal entry into mitosis. Because cdc2-TF-GFP does not have the Tyr-15 phosphorylation site, when the activated cdc2-TF-cyclin B1 complex enters the nucleus, Wee 1 is unable to inactivate it by phosphorylation at Tyr-15, and, consequently, the cell enters mitosis prematurely, resulting in an increased level of chromatin condensation. A similar process occurs with cdc2-AF-GFP, which lacks both inhibitory phosphorylation sites, although in this case the level of chromatin condensation is higher than cdc2-TF-GFP. Thus, it is proposed that a second checkpoint in early mitosis exists to prevent the cell from entering mitosis prematurely.
inhibit mitotic progression of DNA-damaged cells that have overcome the first G2 block. Other studies show that in p53-positive cells, several redundant pathways exist that can initiate a G2 arrest regardless of the phosphorylation status of cdc2 (56–58). However, HeLa cells are p53 deficient and therefore do not have alternative p53-dependent mechanisms for inducing a G2 arrest. Cells that pass through this first Tyr-15-dependent checkpoint (i.e., cdc2-TF- and cdc2-AF-expressing cells) arrest in early mitosis with condensed mitotic spindle formation; Refs. 59 and 60). Thus, it is possible that the mammalian homologues of NIMA kinase have a similar role (61, 62) and could therefore be potential candidates for inducing a second alternative checkpoint.

In summary, it is proposed that two different but interacting pathways serve as G2-M checkpoints in human HeLa cells. The first checkpoint involves Tyr-15 phosphorylation of cdc2 and serves as a G2 checkpoint from which damaged DNA can be repaired and cell cycle progression can continue. The second checkpoint serves as a later irreversible checkpoint in early mitosis from which mitotic catastrophe and subsequent cell death occur.

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In summary, it is proposed that two different but interacting pathways serve as G2-M checkpoints in human HeLa cells. The first checkpoint involves Tyr-15 phosphorylation of cdc2 and serves as a G2 checkpoint from which damaged DNA can be repaired and cell cycle progression can continue. The second checkpoint serves as a later irreversible checkpoint in early mitosis from which mitotic catastrophe and subsequent cell death occur.


Abolishment of the Tyr-15 Inhibitory Phosphorylation Site on cdc2 Reduces the Radiation-induced G_{2} Delay, Revealing a Potential Checkpoint in Early Mitosis

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