Activation of the G2/M cell cycle checkpoint by DNA damage prevents cells from entering mitosis. Centrosome separation is initiated in G2 phase and completed in M phase. This critical process for cell division is targeted by G2/M checkpoint. Here we show that Plk1 signaling plays an important role in regulation of centrosome separation after DNA damage. Constitutively active Plk1 overrides the inhibition of centrosome separation induced by DNA damage. This inhibition is dependent on ATM, but not on Chk2 or Chk1. Nek2 is a key regulator of centrosome separation and is a target of Plk1 in blocking centrosome separation. We found that Plk1 can phosphorylate Nek2 in vitro and interacts with Nek2 in vivo. Down-regulation of Plk1 with RNA interference prevents Nek2-induced centrosome splitting. DNA damage is known to inhibit Plk1 activity. We propose that the DNA damage-induced inhibition of Plk1 leads to inhibition of Nek2 activity and thus prevents centrosome separation.

To maintain the integrity of the genome, mammalian cells have developed surveillance mechanisms called checkpoints that arrest the cell cycle after activation by unreplicated or damaged DNA (1, 2). Damage by ionizing radiation may activate G1, S, and G2/M checkpoints (3). The G2/M checkpoint prevents mitotic entry after DNA is damaged (4). The activation of this checkpoint requires the functions of checkpoint kinases ATM and ATR (ATM- and Rad3-related). Upon activation, ATM or ATR can phosphorylate and activate many proteins involved in cell cycle regulation, such as checkpoint kinases Chk1 and Chk2, P53 and BRCA1, therefore elicit a subsequent cell cycle arrest (5–8).

The centrosome is the major microtubule-organizing center of animal cells (9). It consists of a pair of centrioles surrounded by a protein matrix called pericentriolar material (10). During the cell cycle, centrosomes are duplicated and segregated along with the genome. The centrosome cycle has been divided into several steps including centrosome duplication, centrosome maturation, centrosome separation, and centriole disorientation (11). Centrosome separation is initiated in G2 phase and is completed in mitosis, leading to the formation of bipolar mitotic spindle (12).

The centrosomal cycle is closely integrated into the cell cycle, and the DNA damage checkpoints may regulate the centrosomal cycle. In Drosophila, a variety of DNA damaging agents trigger centrosome inactivation (13, 14). We have demonstrated that centrosomal separation is inhibited by exposure to DNA-damaging agents including IR, indicating that the centrosome may be implicated in a checkpoint function during cell cycle progression (15). However, the checkpoint pathways and genes involved in this cellular response to DNA damage are not known.

Polo-like kinase 1 (Plk1) is a serine/threonine protein kinase that is involved at several points in mitotic progression (16–18). Plk1 shows cell cycle dependent expression, accumulating to maximal levels during G2 and M phases (19). At mitosis, Plk1 activates phosphatase cdc25C, a positive regulator of cdcc2/cyclin B (20, 21). cdc2/cyclin B is the major cyclin that regulates G2/M phase transition (22, 23). Plk1 also contributes to the mitotic exit by regulating the anaphase-promoting complex (APC) (24, 25). Furthermore, Plk1 has a role in centrosome maturation and separation. Inhibition of Plk1 by anti-Plk1 antibody causes duplicated, but unseparated, centrosomes (26). In addition, the centrosomes were reduced in size, and the expected accumulation of γ-tubulin and MPM-2 immunoreactivity was impaired (26). Plk1 kinase activity is inhibited by DNA damage in G2 and in mitosis in an ATM- or ATR-dependent manner (27, 28). Moreover, overexpression of mutant Plk1s whose activities are constitutively active can override the G2 arrest induced by DNA damage. Therefore the evidence supports the contention that Plk1 is a target of the DNA damage response (27). The information that Plk1 regulates centrosome function and its activity is down-regulated after irradiation led us to explore its effects on radiation-induced inhibition of centrosome separation.

Nek2 is a cell cycle-regulated serinethreonine kinase of the NIMA (never in mitosis A) family (29). Nek2 is located at the centrosome and has been implicated in centrosome splitting in mammalian cells (30, 31). There are two alternatively spliced forms of Nek2, Nek2A, and Nek2B (32). Nek2A is elevated in the S and G2 phases of the cell cycle, whereas Nek2B is present during S, G2, and mitosis (32). Nek2A, but not Nek2B, stimulates centrosome disjunction and is required for the formation of bipolar mitotic spindles (33). Nek2A forms a complex with protein phosphatase 1 (PP1) and the centrosomal Nek2-associated protein, C-Nap1 (34, 35). Nek2A can phosphorylate itself, PP1, and C-Nap1, whereas PP1 can dephosphorylate both Nek2A and C-Nap1 (34). Coexpression of PP1 with Nek2A suppresses centrosome splitting, whereas ectopic expression of inhibitor protein 2, a physiological inhibitor of PP1, stimulates splitting (36, 37). We have shown that irradiated cells fail to activate Nek2 and that radiation inhibits the centrosome splitting induced by overexpression of Nek2, suggesting that inhibition of centrosome separation as a DNA damage response is mechanistically linked to Nek2 (15). However, the upstream regulator of Nek2 in this DNA damage checkpoint pathway remained to be determined.

Here we show that a constitutively active mutant of Plk1 can override the inhibition of centrosome separation after ionizing radiation. The inhibition of centrosome separation is dependent on ATM but not on Chk2 or Chk1. Plk1 interacts with Nek2 in vivo and can phosphorylate telangiectasia mutated; ATR, ATM- and Rad3-related; APC, anaphase-promoting complex; PP1, protein phosphatase 1; siRNA, small interfering RNA; GFP, green fluorescent protein; wt, wild type.
Nek2 in vitro. Furthermore, inhibition of Plk1 by siRNA prevents Nek2-induced centrosome splitting. Our data indicate that Plk1 acts upstream of Nek2 in the signaling pathway, which leads to the inhibition of centrosome separation after IR.

MATERIALS AND METHODS

Cell Culture and Cell Treatment—HeLa and U2OS cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin. Centrin-GFP-Hela cell line was maintained in selective media containing G418 (200 μg/ml, Mediatech). Tetracycline-inducible GFP-NeK2A-U2OS cell line is a gift from Dr. Andrew Fry (University of Leicester, UK), and maintained in selective media containing Zeocin (200 μg/ml, Invitrogen) and hygromycin B (100 μg/ml, Roche Applied Science) (33). Tetracycline (2 μg/ml) was added to the culture medium to induce and maintain expression of GFP-NeK2A.

Cells were synchronized in G1/S using a thymidine block (2 mM, Sigma) for 16 h. Cells were then released from the block by washing three times with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and refed with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and thymidine/deoxyctyline (2.5 × 10⁻⁵ M). After 3 h, cells were irradiated using a Shepherd Mark 1 Model 68A cesium irradiator. Caffeine (Sigma) was added at a concentration of 3 mM. Cycloheximide was added at a concentration of 10 μg/ml. Proteasome inhibitor MG132 (Sigma) was added at a concentration of 20 μM.

Flow Cytometry Analysis—Cell pellets were resuspended in citrate buffer and stained with propidium iodide as described (38). Data were acquired with Becton Dickinson FACScan flow cytometer and analyzed using CellQuest (Version 2.01.2).

Transfection—Myc-tagged wild type (wt) Plk1, kinase inactive mutant K82R and constitutively active mutant T210D Plk1 were cloned into a PRCCMV vector and obtained from Dr. Eric Nigg’s laboratory. HeLa cells were seeded at a concentration of 1 × 10⁵/ml in 6-well plates (35 mm/well) and transfected using FuGENE 6 (Roche Applied Science) with GFP-centrin plasmid (0.5 μg) and wt Plk1 or T210D mutant Plk1 (0.5 μg). GFP-NeK2 U2OS cells or U2OS cells were seeded in 10-cm dishes, transfected with wt Plk1 or T210D Plk1 plasmids (4 μg). After 24–48 h, cells are subjected for analysis.

siRNA Interference—Double stranded ATM, Chk2 or Plk1 SMART-POOL siRNA oligonucleotides were purchased from Dharmacon Research (Lafayette, CO). Double-stranded siRNA oligonucleotides corresponding to firefly luciferase (Dharmacon) were used as controls. Oligofectamine (Invitrogen) was used to transfect HeLa and U2OS cells as specified by the manufacturers.

Immunoprecipitation and Immunoblotting—Transfected cells were lysed in radioimmune precipitation assay buffer supplemented with proteinase inhibitors (20 mM p-nitrophenyl phosphate, 1 mM Pefabloc, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 5 μg/ml aprotonin). Cell lysates were incubated with anti-Myc antibody (Santa Cruz) or anti-GFP antibody (University of Alberta, Canada) overnight at 4 °C, followed by an additional 1-h incubation with Protein A-Sepharose beads (Santa Cruz). Immunocomplexes were washed three times with 1X phosphate-buffered saline. Western blot analysis was performed by loading samples on a 10% SDS/PAGE and transferring to nitrocellulose membranes (Invitrogen). Membranes were incubated with various antibodies, horseradish peroxidase-linked secondary antibody, and visualized by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech). Rabbit polyclonal antibody against ATM and goat polyclonal antibody against Plk1 were purchased from Santa Cruz Biotechnology.

RESULTS

To evaluate the role of Plk1 in the failure of centrosome separation after IR, we used a constitutively active mutant of Plk1 that is not inhibited after DNA damage. In this mutant, Thr-210 was changed to Asp (T210D) (39). HeLa cells were transfected with myc-tagged wild type or constitutively active mutant (T210D) Plk1 plasmids and irradiated 24 h after transfection. Four hours after IR, cell lysates were assayed for Plk1 activity using β-casein as a substrate. Although the activity of wild type Plk1 decreased by 61%, the activity of the T210D Plk1 mutant remained unchanged, confirming that the activity of the T210D Plk1 mutant was not inhibited by IR (Fig. 1A) (27). To test the effect of Plk1 activity after IR on centrosome separation, we co-transfected wild type Plk1 or T210D Plk1 with GFP-centrin into HeLa cells. The GFP-centrin was expressed to allow easy visualization of the centrosomes and as a transfection marker. These cells were then synchronized with a thymidine block, followed by irradiated with 6 grays. Cells transfected with GFP-centrin alone were used as a control. Centrosome separation was assayed by microscopic evaluation of GFP-centrin in the transfected cells 12 h after release from the thymidine block (Fig. 1B). Centrosomes were classified as separated if they were >2 μm apart from one another. After partial synchronization, 40% of the cells in G2/M based on fluorescence-activated cell sorter analysis, and 58% of all cells had separated centrosomes (Fig. 1C). However, 60% of the irradiated, synchronized

Immunofluorescence Staining—The cells were grown on coverslips, transected, and cultured for 24–48 h before fixation. Cells were fixed and permeabilized with methanol for 2 min. After washing three times with 1X phosphate-buffered saline, coverslips were stained with mouse anti-myc antibody for 1 h at room temperature, followed by incubation with rhodamine-conjugated anti-mouse IgG secondary antibody for 1 h. Samples were mounted in 4',6-diamidino-2-phenylindole vectashield mounting medium (Vector Laboratories) and analyzed using fluorescence microscopy. Images were collected using a Hamamatsu digital camera and OpenLab software. Centrosome separation was assayed by counting GFP-centrin. Centrosomes were classified as separated if they were >2 μm apart from one another.

In Vitro Kinase Assay—For Plk1 kinase assay, anti-myc immunoprecipitates were incubated with 0.5 mg/ml dephosphorylated casein in Plk1 washing buffer (20 mM Hepes, 150 mM KCl, 10 mM MgCl₂, 1 mM EGTA, 0.5 mM dithiothreitol, 5 mM NaF) supplemented with 10 μM ATP and 1 μCi of [γ-32P]ATP. For in vitro phosphorylation assay, anti-Plk1 immunoprecipitates were incubated with 100 ng of recombinant Nek2 protein in Plk1 washing buffer with 10 μM ATP and 1 μCi of [γ-32P]ATP. The reaction mixtures were incubated at 30 °C for 30 min and resolved on SDS-polyacrylamide gels. The gels were dried and subjected to autoradiography. The intensity of the bands was measured by Alphalager 2200 program.

PP1 Activity Assay—PP1 was immunoprecipitated with 5 μg of anti-PP1α monoclonal antibody (Santa Cruz) from protein samples. The immune complexes were then incubated with 30 μl of protein G plus agarose beads (Santa Cruz) washed three times with lysis buffer followed by Ser/Thr assay buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM CaCl₂). PP1 activity was assayed using a Ser/Thr phosphatase assay kit (Upstate Cell Signaling Solutions). The PP1 immune complex beads in 50 μl of Ser/Thr assay buffer were incubated with the phosphopeptide KRpTIRR at 30 °C for 30 min. The beads were pelleted, and 25 μl of supernatant was analyzed for free phosphate in the malachite green assay by dilution with 100 μl of developing solution (Malachite Green). After incubation for 15 min, the release of phosphate was quantified by measuring the absorbance at 620 nm in a microtiter plate reader.
Role of Plk1 in Failure of Centrosome Separation

FIGURE 1. A constitutively active Plk1 mutant overrides IR-induced inhibition of centrosome separation. A, HeLa cells were transfected with myc-tagged wt or T210D Plk1 plasmids and irradiated (6 grays). A shows the Plk1 kinase assay using β-casein as a substrate. B, HeLa cells were cotransfected with myc-tagged wt or T210D Plk1 and GFP-centrin plasmids, synchronized at G1/S by thymidine block, and irradiated (6 grays) after release. Transfected cells were fixed 12 h after release from the thymidine block and stained with anti-myc Ab (shown in yellow). Centrosomes were stained by expression of GFP-centrin (green). Panels a and b show typical cells transfected with wt Plk1 in which IR has inhibited centrosome separation. Panels c and d show cells transfected with T210D Plk1 in which inhibition of centrosome separation has been released. C, quantitation of the experiment outlined in B showing the percentage of cells with separated centrosomes after IR. Centrosomes >2 μm were considered as separated. Around 200 cells were counted for each experiment and results are from three independent experiments. * t test, p < 0.05.

Previously, we have shown that the kinase activity of Nek2 decreased in response to IR (15). This raised the possibility that Plk1 might be an upstream regulator of Nek2 and that inhibition of Plk1 activity by IR might lead to a decrease in Nek2 activity. Because Nek2 phosphorylation is associated with its kinase activity (34), we tested whether Plk1 could phosphorylate Nek2 in vitro. We immunoprecipitated Plk1 in cells transfected with the kinase inactive K82R
levels decreased more rapidly in Plk1-transfected cells, indicating that Plk1 was involved in the degradation of Nek2 (Fig. 3B). This is consistent with published results showing that Plk1 activates APC activity and reports showing that Nek2 is degraded by the APC (24, 42). Thus, to test the interaction between Plk1 and Nek2, it was necessary to prevent Nek2A protein degradation. We added the proteasome inhibitor MG132 after GFP-Nek2A had been induced. The protein samples from GFP-Nek2A U2OS cells transfected with myc-tagged Plk1 plasmids were immunoprecipitated with either GFP antibody or myc antibody. The presence of associated proteins was determined by immunoblotting using either myc antibody or GFP antibody. As shown in Fig. 3C, Nek2A was coimmunoprecipitated with Plk1 and vise versa, indicating that Plk1 and Nek2 interact with each other in vivo.

Because Nek2 forms a complex with PP1 in vivo (34), and PP1 can dephosphorylate and inactivate Nek2 (34), we examined the possibility that Plk1 was acting through phosphorylation to inactivate PP1, leading to the increase of Nek2 kinase activity. We transfected wild type or T210D mutant Plk1 into U2OS cells, and immunoprecipitated cells with antibody against PP1α. A phosphatase assay was performed using a Ser/Thr phosphatase assay kit (Upstate Cell Signaling Solutions). There was no difference in PP1α phosphatase activity between Plk1 transfected cells and control cells (Fig. 3D), indicating that Plk1 did not change the phosphatase activity of PP1α.

Finally, we tested whether suppression of Plk1 could inhibit Nek2 induced centrosome separation. To address this question, we treated inducible GFP-Nek2A-U2OS cells with siRNA against Plk1. Decreased Plk1 expression was determined by Western blot analysis. We then induced Nek2A expression by using tetracycline and studied centrosome separation in the cells. Overexpression of GFP-Nek2A stimulated a premature splitting of centrioles in 70% of cells, which is consistent with previous observations (33). However, the splitting of centrioles was suppressed in the cells treated with siRNA against Plk1 by 30% (Fig. 4), supporting the notion that Plk1 is involved in the regulation of Nek2 induced centrosome separation.

**DISCUSSION**

Previously we have proposed that DNA damage checkpoints regulate the centrosomal cycle, and that inhibition of centrosome separation is a cellular response to DNA damage (15). DNA damage inhibits Plk1 kinase activity in an ATM/ATR-dependent manner; constitutively active mutants of Plk1 abrogate the DNA damage-induced G2 arrest; therefore Plk1 is a target for DNA damage checkpoint regulation (27). In this study, we have shown that a constitutively active mutant of Plk1 overrides IR-induced inhibition of centrosome separation, suggesting that a DNA damage checkpoint regulates centrosome separation by targeting Plk1.

We found that inhibition of centrosome separation by IR was overridden by addition of caffeine or inhibition of ATM expression using siRNA, indicating that ATM mediates the inhibition of centrosome separation by IR. Because the inhibition of Plk1 kinase activity by DNA damage is ATM/ATR-dependent, it is possible that ATM functions through the inhibition of Plk1 kinase activity. Chk2 is an important downstream mediator of ATM (6). It has been shown that DNA damage leads to increased Drosophila checkpoint kinase 2 (DmChk2) localization to the centrosome and a null mutation in DmChk2 suppresses the mitotic response to DNA damage. Therefore, a DmChk2-dependent pathway is activated to disrupt spindle assembly and chromosome segregation in response to DNA damage in Drosophila (14). However, suppression of Chk2 expression by siRNA had no effect on the inhibition of centrosome separation after IR, excluding its role in regulation of cen-

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**FIGURE 2. Inhibition of centrosome separation by IR depends on ATM not Chk2 or Chk1.** GFP-centrin HeLa cells were treated with caffeine (A), siRNA against ATM (B), Chk1 or Chk2 (C), or synchronized with a thymidine block, irradiated after release. Centrosome separation was determined by counting centrosomes stained with GFP-centrin. Centrosomes >2 μm were scored as separated. Values are given as the percentage of the cells with separated centrosomes ± S.D. for three independent experiments. Around 200 cells/experiment were counted. D. Western blot shows ATM, Chk2, and Chk1 expression after siRNA treatment. siRNA against firefly luciferase GL3 was used as a control.

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mutant, wild type, or T210D Plk1 plasmids using antibody against Plk1. The immunocomplex was incubated with recombinant Nek2 protein in kinase buffer containing γ-32P. Incorporation of γ-32P was seen as a band at 52 kDa in a 10% SDS-polyacrylamide gel detected by autoradiography, indicative of Nek2 phosphorylation. The intensity of the bands was measured by Alphalager 2200 program and plotted (Fig. 3A). Although Nek2 is autophosphorylated, expression of wild type or kinase active Plk1 increased Nek2 phosphorylation in vitro as seen in Fig. 3A.

Plk1 and Nek2 are both found in the centrosome (19, 30). We asked whether they could interact with each other. When we transfected wild type or T210D mutant Plk1 into inducible GFP-Nek2A U2OS cells where GFP-Nek2A was expressed upon tetracycline addition, we found that Nek2A protein levels decreased after Plk1 transfection. In the presence of cycloheximide, a protein synthesis inhibitor, Nek2A protein
It is interesting to note that Chk2 coimmunoprecipitates with Plk1 and Plk1 phosphorylates Chk2 at Thr-68 (43). Chk2 phosphorylated at Thr-68 is localized to the centrosomes and midbodies in the absence of DNA damage (43). Whether phosphorylation of Chk2 at this site functions on centrosome regulation remains unknown.

We have found that Plk1 interacts with Nek2 in vivo and can phosphorylate Plk1 in vitro, suggesting that Plk1 is an upstream regulator of Nek2. In response to IR, both Plk1 and Nek2 have decreased kinase activity. It is possible that inhibition of Plk1 kinase activity leads to a decreased Nek2 activity. This decreased activity of Nek2 is at least partially responsible for IR-induced inhibition of centrosome separation (15). Nek2 also forms a complex with PP1 (34, 35). PP1 can dephosphorylate Nek2 and inactivate it (34). Radiation increases PP1 activity in an ATM-dependent manner (34). Alterations in PP1 activity could also be a mechanism that results in a decreased Nek2 activity and inhibition of centrosome separation. However, in our work, Plk1 did not activate PP1, indicating that Plk1 and PP1 regulate Nek2 independently. It seems intriguing that Plk1 is also involved in the degradation of Nek2. Destruction of Nek2 occurs in early mitosis mediated by the proteasome and is dependent on the APC/Cdc20 ubiquitin ligase (42). Plk1 activates APC activity (24, 25) and therefore indirectly promotes Nek2 degradation. It is likely that Plk1 mediates phosphorylation and activation of Nek2 prior to its induction of degradation. This sequence could be a mechanism to limit the effect of Nek2 activation.

In summary, we propose that there is a signaling pathway that leads to the inhibition of centrosome separation in response to IR. Upon radiation, the kinase activity of Plk1 decreases in an ATM-dependent manner, which results in a reduced activity of Nek2 and inhibition of centrosome separation.
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