Claspin is a newly identified protein that regulates Chk1 activation in Xenopus. In the present study we investigated the role of human Claspin in the DNA damage/replication checkpoint in mammalian cells. We observed that human Claspin is a cell cycle regulated protein that peaks at S/G2 phase. Claspin localizes in the nuclei, but it only associates with Chk1 following replication stress or other types of DNA damage. In addition, Claspin is phosphorylated in response to replication stress, and this phosphorylation appears to be required for its association with Chk1. Moreover, Claspin interacts with the checkpoint proteins ATR and Rad9. Given that both the ATR and Rad9-Rad1-Hus1 complexes are involved in Chk1 activation, it is possible that Claspin works as an adaptor molecule bringing these molecules together. Using small interfering RNA technology, we have shown that down-regulation of Claspin expression inhibits Chk1 activation in response to replication stress. More importantly, down-regulation of Claspin augments the premature chromatin condensation induced by hydroxyurea, inhibits the UV-induced reduction of DNA synthesis, and decreases cell survival. Taken together, these data imply a potential critical role for Claspin in replication checkpoint control in mammalian cells.

In response to DNA damage and DNA replication stresses, cells activate surveillance pathways called cell cycle checkpoints. Activation of these checkpoints slows or arrests cell cycle progression to ensure appropriate time for DNA repair. The components of mammalian checkpoint pathways include protein kinase cascades. At the top of these pathways are the phosphoinositide 3-kinase-related kinases ATM1 and ATR (1, 2). The ATM gene is mutated in patients with the syndrome ataxia telangiectasia. ATM mutant cells are defective in DNA damage checkpoints and are very sensitive to agents that promote DNA double strand breaks, such as γ-irradiation (3). In the case of ATR, disruption of this gene in mice results in embryonic lethality and fragmented chromosomes (4, 5). Furthermore, overexpression of a kinase-defective ATR mutant inhibits cell cycle arrest after DNA damage (6, 7), suggesting that ATR is involved in DNA damage checkpoints. In contrast to ATM, ATR is primarily involved in cellular responses to unreplicated DNA (induced by agents such as hydroxyurea (HU)) and to certain DNA-damaging agents, including UV. Cells overexpressing the kinase-inactive ATR are sensitive to HU and UV in addition to γ-irradiation (6, 7).

Downstream of ATM and ATR in the DNA damage pathway are the protein kinases Chk1 and Chk2. Although Chk2 requires ATM for its activation in response to γ-irradiation (8, 9), Chk1 activation by UV and HU requires ATR (10–12). ATR has been shown to phosphorylate mammalian Chk1 on serines 317 and 345 in vitro. In addition, phosphorylation of these sites in vivo was shown to be ATR-dependent (13). Like ATR, Chk1 is an essential gene. Chk1 deficiency leads to cell death in embryonic stem cells and embryonic lethality in mice (11, 14). In addition, embryos and conditional embryonic stem cells lacking Chk1 have defective checkpoint responses (11, 14). It appears that Chk1 is involved in the intra-S-phase checkpoint and is also responsible for the initiation of G2 arrest in response to DNA damage (11, 14–16).

Along with ATR, two protein complexes, Rad17-replication factor C (RFC) and Rad9-Rad1-Hus1, are also required for Chk1 activation (17, 18). These complexes have been implicated as sensors of DNA damage and replication stress (19, 20), but it is not known how they regulate Chk1 activation. Recently, a novel protein called Claspin was also shown to be involved in Chk1 activation in Xenopus (21). Claspin was isolated from Xenopus extracts as a Chk1-binding protein. Binding of Claspin to Chk1 was elevated in the presence of DNA templates that trigger cell cycle checkpoint arrest (21). Immunodepletion of Claspin from Xenopus egg extracts abolished the activation of Chk1 induced by unreplicated or UV-damaged DNA. Furthermore, Claspin-depleted extracts were unable to arrest the cell cycle in response to DNA replication blocks (21). These results suggest that Claspin is an upstream regulator of Chk1 in Xenopus.

In the present study we investigated the role of Claspin in the DNA damage and replication stress pathways in mammalian cells. We report here that Claspin expression is cell cycle-regulated and that Claspin binds to several checkpoint proteins, including Chk1, ATR, and Rad9. Suppression of Claspin expression results in defective Chk1 activation and defective S-phase checkpoint in response to DNA damage. Down-regulation of Claspin expression also increases premature chromatin condensation (PCC) induced by HU. In combination, our results suggest that Claspin participates in DNA damage and/or replication stress pathways in mammalian cells.

MATERIALS AND METHODS

**Cell Culture**—Human cell lines T24, K562, 293T, and HeLa were grown in RPMI 1640 supplemented with 10% fetal bovine serum. For cell cycle experiments T24 cells were grown until confluent and arrested by contact inhibition for 3 days as described by Yu and Baer (22). Cells were then released into the cell cycle by replating at low densities.

**Antibodies**—Mouse monoclonal anti-Claspin antibody was raised

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The abbreviations used are: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia mutated- and Rad3-related; siRNA, small interfering RNA; HU, hydroxyurea; PCC, premature chromatin condensation; GST, glutathione S-transferase.
against a mixture of four GST fusion proteins covering the entire length of Claspin. This anti-Claspin monoclonal antibody was used in Western blots and immunofluorescence experiments. Anti-Claspin antibody for immunoprecipitation was a polyclonal antibody raised against a GST fusion protein encoding residues 636–1003 of Claspin. Chk1 was immunoprecipitated with a polyclonal antibody raised against a GST-Chk1 fusion protein. Anti-Chk1 antibodies for Western blot were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-phospho-Chk1 (Ser-317) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-ATR rabbit polyclonal antibodies were generated against an N-terminal peptide (for immunoprecipitation) and a C-terminal peptide (for Western blot) of ATR. Rad9 antibodies for immunoprecipitation and Western blot were a gift of Dr. L. M. Karnitz. Anti-Chk2 antibodies were described previously (22).

Immunostaining—Cells grown on coverslips were fixed for 15 min with 3% paraformaldehyde solution and then permeabilized with 0.5% Triton X-100 for 5 min. Slides were incubated with primary antibodies for 20 min at 37 °C. Fluorescein isothiocyanate-conjugated goat anti-mouse and/or rhodamine-conjugated goat anti-rabbit serum (Jackson Immunoresearch) were used as secondary antibodies. All antibodies were diluted in 5% goat serum. Cells were counterstained with 4–6-diamidino-2-phenylindole dye for 30 s.

Small Interfering RNA (siRNA) Transfection—siRNA duplexes were 21 base pairs including a 2-deoxynucleotide overhang (24). The coding strand of Claspin siRNA was CUUGCUUAGAGGUGACUdTdT, and the control siRNA was UCAGUGAAUCUGUGGUGdTdT. Chk1 siRNA was designed as described previously (25). For transfections, 293T or HeLa cells were plated in 6-well plates and were transfected at 21% confluency with the siRNA duplex and Oligofectamine (Invitrogen). Transfection was repeated 24 h later, and cells were harvested 72 h after the first transfection.

Immunoprecipitation Experiments—293T cells were treated with 10 mM HU for 1.5 h and then lysed in NETN lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8), 0.5% Nonidet P-40). Cleared lysates (1–2 mg protein) were immunoprecipitated with the indicated polyclonal antibodies and protein A-Sepharose for 1 h. In phosphatease experiments, Claspin immunoprecipitates were treated with 400 units of λ-phosphatase in the accompanying buffer (New England Biolabs) for 1 h at 30 °C. Proteins were separated on 7.5% SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted with anti-Claspin antibody.

Mitotic Spreads—HeLa cells were transfected with siRNAs as described above. 54 h after the first transfection, 2 mM HU plus nocodazole (200 ng/ml) were added. Cells were harvested 20 h later, and mitotic spreads were processed as described previously (26).

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Claspin Regulates Replication Checkpoint

RESULTS

Claspin Expression Is Cell Cycle-regulated—Because Chk1 is a cell cycle-regulated protein (27), we first investigated whether the expression of human Claspin is cell cycle-dependent. For this purpose we evaluated the levels of Claspin protein in synchronized T24 bladder carcinoma cells. T24 cells were arrested in G0 by contact inhibition and then were harvested after the first cell cycle by being replated at low densities. Cells were then harvested at different time points. For each time point the cell cycle distribution was determined by fluorescence-activated cell sorter analysis. Western blot analyses were performed with either a mouse monoclonal or rabbit polyclonal antibody raised against human Claspin. We observed that the levels of Claspin were undetectable in G0 cells and increased at late G1/S phase (Fig. 1A). The protein level of Claspin peaks at S/G2 phase similar to Chk1. In contrast, Chk2 protein levels remain constant throughout the cell cycle (Fig. 1A). These results indicate that, like Chk1, Claspin is a cell cycle-regulated protein and may have a function in S phase cells.

To investigate the cellular localization of Claspin, we performed immunofluorescence staining experiments using anti-Claspin antibodies. In unsynchronized HeLa cells we observed nuclear localization of Claspin. HU treatment did not change Claspin cellular localization but induced Chk1 activation as detected by staining with anti-phospho-Chk1 (Ser-317) antibody (Fig. 1B). Only cells that stained positive with Claspin antibodies stained positive with the anti-phospho-Chk1 antibody after HU treatment (Fig. 1B). This result is consistent
with the observations made by Western blot, demonstrating that Chk1 and Claspin are both expressed during the same cell cycle stages.

Claspin Is Phosphorylated following Replication Stress and DNA Damage—In Xenopus, Claspin appears to be phosphorylated, and this phosphorylation is required for its interaction with Chk1. Therefore, we investigated whether human Claspin is phosphorylated following replication stress. In cells treated with HU, the migration of Claspin in SDS-PAGE was retarded compared with that in untreated cells (Fig. 2A). Similar mobility shift was observed when cells were treated with UV (data not shown). The mobility shift was reversed by phosphatase treatment (Fig. 2A), suggesting that Claspin is hyperphosphorylated in mammalian cells in response to DNA damage. We also investigated whether caffeine, an inhibitor of phosphoinositide 3-kinase-related kinase kinases (28), affected Claspin phosphorylation. Pretreatment of cells with caffeine before HU treatment inhibited the HU-induced mobility shift of Claspin (Fig. 2B; also see Fig. 3D), suggesting that ATR or other phosphoinositide 3-kinase-related kinase kinases could be involved in the regulation of Claspin phosphorylation following replication stress.

Claspin Associates with Chk1 upon Replication Stress and DNA Damage—To determine whether Claspin associates with Chk1, we tested whether Claspin coimmunoprecipitates with Chk1 in 293T cells before and after replication stress. 293T cells were incubated in the presence and absence of 3 mM caffeine for 20 min prior to HU treatment. Cell lysates were immunoprecipitated with anti-Chk1 antibodies. Western blots were performed with anti-Claspin and anti-Chk1 antibodies.

was required for Claspin-Chk1 interaction. Treatment of cell lysates with λ phosphatase prior to pull-down assay prevented the HU-induced association between Claspin and Chk1 (Fig. 3C). Claspin levels remained unchanged after phosphatase treatment (Fig. 3C, lower panel), suggesting that Claspin phosphorylation is required for Claspin binding to Chk1. Similarly, the association between Claspin and Chk1 was also inhibited by pretreatment of cells with caffeine (Fig. 3D). Taken together, these data indicate that the damage-dependent phosphorylation of Claspin is required for the inducible Claspin-Chk1 association in mammalian cells.

Claspin Associates with ATR and Rad9—Because Chk1 activation depends on ATR and the Rad9-Rad1-Hus1 (9-1-1) complex (10, 17, 29, 30), we investigated whether Claspin could also associate with the ATR and the 9-1-1 complexes. We immunoprecipitated ATR from extracts prepared from 293T cells treated with or without 10 mM HU and performed Western blots with anti-Claspin antibodies. We observed that Claspin associates with ATR (Fig. 4A). As a control, no Claspin was immunoprecipitated with normal rabbit serum (Fig. 4A). Similarly, Claspin co-immunoprecipitated with Rad9 (Fig. 4B). No association was observed between Claspin and ATM or Chk2 (data not shown). It also appears that the ATR-Claspin and the Rad9-Claspin interactions decreased slightly following replication stress, whereas Claspin levels did not change (Fig. 2) The significance of these modest reductions in association is not yet clear. It is possible that Claspin may dissociate from ATR and/or Rad9 following replication stress and then associate with Chk1 to facilitate Chk1 activation.

Claspin Is Required for Chk1 Phosphorylation—To investigate whether Claspin is required for Chk1 activation in mammalian cells, we used the siRNA technique to suppress Claspin expression (24). As shown in Fig. 5, transfection of a siRNA targeted to Claspin suppressed Claspin expression in 293T cells. Similar effects were observed in HeLa cells (data not shown). As a control, an unrelated control siRNA did not affect Claspin levels (Fig. 5). To analyze the involvement of Claspin in Chk1 activation we transfected 293T cells with control and Claspin siRNA. After 72 h, cells were treated with 10 mM HU. Phosphorylation of Chk1 was determined by Western blot per-
formed with anti-phospho-Chk1 antibodies (Ser-317). Although transfection of Claspin siRNA decreased Chk1 activation, transfection of a control siRNA failed to do so (Fig. 5). As a control, siRNA transfections did not change Chk1 protein levels, as judged by immunoblotting with anti-Chk1 antibody. Thus, Claspin is involved in the Chk1 activation following DNA damage.

**Claspin Prevents PCC**—PCC is a hallmark of mammalian cells that begin mitosis before completing DNA replication (26). This is a lethal event that is induced by agents like HU that promote DNA damage or prolong S phase and is prevented by a conserved checkpoint pathway involving ATR and Chk1 (26). Caffeine and overexpression of ATR-kinase dead (ATR-kd) or Chk1-kd augmented the PCC induced by HU or UV (26). To examine whether Claspin also participates in this ATR-Chk1-dependent checkpoint control, we transfected HeLa cells with a Claspin or a control siRNA. Cells were then treated with 2 mM HU for 20 h. Mitotic spreads were prepared and counted. Cells with fragmented chromosomes were judged as cells with PCC. We found that transfection with Claspin siRNA, but not with control siRNA, increased the HU-induced PCC (Fig. 6A). Similar to earlier reports (26), we observed increased PCC in cells transfected with Chk1 siRNA following HU treatment (Fig 6A). There were also modest increases of PCC in cells transfected with Claspin or Chk1 siRNA without HU treatment, indicating that PCC may occur in untreated control cells in the absence of Claspin or Chk1. In conclusion, these data suggest that like ATR and Chk1, Claspin is involved in the pathway that prevents the premature chromatin condensation.

**Claspin Is Involved in the UV-induced S Phase Checkpoint**—In addition to ionizing radiation, other DNA-damaging agents such as UV and some carcinogens also lead to reduction of DNA synthesis and induce the S phase checkpoint. This...
Claspin Regulates Replication Checkpoint

phenomenon is believed to be a checkpoint response regulate by the ATR/Chk1 pathway, because it is blocked by caffeine, UCN-01 (Chk1 inhibitor (8, 31–33), or overexpression of the kinase-inactive forms of Chk1 or ATR (33). To determine whether Claspin participates in the regulation of this S phase checkpoint, we examined the rate of DNA synthesis in cells transfected with control or Claspin siRNAs. There was a 30–40% reduction in DNA synthesis in normal cells treated with 10 J/m² UV (Fig. 6B). This inhibition was reversed by caffeine, demonstrating that this is a checkpoint response. Similarly, Chkl siRNA almost completely reversed the inhibition of DNA synthesis induced by UV (data not shown), indicating that this response depends on Chkl in the cell. As a control, cells transfected with an unrelated siRNA showed an inhibition of DNA synthesis (about 30%) similar to untransfected cells. In cells transfected with Claspin siRNA, we observed a reversal, albeit modest, of DNA synthesis inhibition (~15%) following UV radiation, suggesting that Claspin is involved in this S phase checkpoint.

Claspin Is Required for Cell Survival—Both ATR and Chk1 are essential for normal cell survival (4, 5, 11, 13, 34). Given that Claspin is required for full Chk1 activation and Chk1-dependent checkpoint regulation, we investigated whether Claspin would be similarly required for cell survival. As shown in Fig. 6C, inhibition of Claspin expression by siRNA greatly reduced the numbers of colonies formed, implying a potential critical role for Claspin in replication checkpoint control.

DISCUSSION

The results presented here demonstrate that Claspin is one of the key regulators involved in Chk1 activation in mammalian cells. Using siRNA to down-regulate Claspin expression, we observed that Claspin is required for full Chk1 activation in response to replication stress. We have also shown that the association between Claspin and Chkl is induced by replication stress and other types of DNA damage and appears to be phosphorylation-dependent similar to observations in Xenopus (21). These studies taken together suggest that the mechanisms by which Claspin regulates Chkl activation are conserved in vertebrates.

Our study provides additional information on the possible mechanism by which Claspin regulates Chk1 activation. Our working hypothesis is that Claspin associates with ATR and is possibly phosphorylated by ATR in response to replication stress. After phosphorylation, Claspin associates with Chkl and may facilitate Chk1 activation by ATR. Several observations suggest that ATR could be the kinase that phosphorylates Claspin. First, Claspin coimmunoprecipitates with ATR. Second, the migration of Claspin in SDS-PAGE and the association between Claspin and Chkl are inhibited by caffeine, an ATR inhibitor (28). Finally, Claspin has several S(Q/T)Q motifs, which are potential substrates for kinases such as ATM and ATR (3, 35). Because Claspin interacts with both Chkl and ATR, Claspin may act as an adaptor molecule responsible for bringing ATR and Chkl together. Because Chkl is phosphorylated by ATR (11, 13, 30), by bringing these two kinases in proximity Claspin may facilitate the phosphorylation of Chkl by ATR. However, we were unable to detect any association between ATR and Chkl in our co-immunoprecipitation experiments. It is possible that the ATR/Claspin/Chkl interaction is transient and difficult to detect by the co-immunoprecipitation technique used in this study. Given that we observed an interaction between ATR and Claspin, our study supports a direct role of ATR in damage-induced phosphorylation of Claspin. However, a recent study had identified phosphopeptide motifs in Xenopus Claspin required for Claspin/Chkl interaction (36). These phosphopeptide sequences do not resemble the consensus ATR phosphorylation site sequences (S/T/Q). Thus it remains to be determined whether ATR directly phosphorylates Claspin following DNA damage.

We have also shown here that Claspin associates with the Rad9-Rad1-Hus1 complex. This complex interacts with the Rad17 complex (37) and has been postulated to be the sensor of the DNA damage/reparation stress (20). The current model is that ATR and Rad17-RFC are two independent protein complexes that recognize DNA damage and/or replication forks (19). Although the Rad17 complex is required for chromatid loading of the 9-1-1 complex (a proliferating cell nuclear antigen [PCNA]-like structure) onto DNA, it is dispensable for the recruitment of ATR to sites of DNA damage (38). Similarly, ATR is not required for the chromatin loading of the 9-1-1 complex (18). Although Claspin, ATR, and Rad17 each bind independently to chromatin, they appear to have some functional interactions on chromatin, as all of them are required for Chk1 activation in Xenopus. Given that our results demonstrate that Claspin associates with Chkl, ATR, and the Rad9-Rad1-Hus1 complex, Claspin likely works by bridging both the 9-1-1 and ATR complexes to Chkl.

Our data suggest that Claspin regulates replication checkpoints in mammalian cells. Claspin expression is cell cycle-regulated, and like Chkl, its protein level peaks in the S phase (27). Claspin is involved in the regulation of PCC and DNA synthesis in response to DNA damage, two phenomena that are also regulated by ATR and Chkl (6, 32, 33). Furthermore, like Chkl and ATR (5, 13), Claspin is required for cell survival. Thus, our results strongly suggest that Claspin is a mediator in the ATR-Chkl signaling pathway.

We consistently observed a lesser effect by the Claspin siRNA than the Chkl siRNA on PCC and S-phase checkpoint regulation, although they inhibited the expression of their target genes to a similar extent. These observations agree with our finding that the Claspin siRNA does not completely block Chkl activation following DNA damage. It is possible that there are additional signaling molecules besides Claspin required for full Chkl activation. Future studies will reveal the exact mechanism underlying the activation of Chkl by these multiple protein complexes following DNA damage.

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