Oncogene (2006) 25, 4165–4171

www.nature.com/onc

ORIGINAL ARTICLE

Chk1 is required to maintain Claspin stability

CCS Chini, J Wood and J Chen

Department of Oncology, Mayo Clinic and Foundation, Rochester, MN, USA

Claspin is a Chk1-interacting protein that participates in the DNA replication checkpoint. Expression of Claspin fluctuates in a cell cycle-dependent manner, but the mechanisms involved in the regulation of Claspin protein levels have not been explored. In this study, we show that Claspin expression is downregulated by the proteasome-mediated degradation pathway and that Chk1 is required to maintain Claspin stability. Downregulation of Chk1 expression by siRNA or inhibition of Chk1 activity by UCN01 decreases Claspin levels in cells. Conversely, overexpression of Chk1 increases Claspin levels. These data indicate a role of Chk1 in regulating Claspin stability in the cell. Since Claspin has also been shown to participate in Chk1 activation following DNA damage, we further explored the exact role of Claspin during Chk1 activation following replication stress. We observed that while Rad17 is required for early Chk1 activation after hydroxyurea treatment, Claspin is only required to sustain Chk1 activation. Based on these findings, we propose that Claspin functions at late stages of Chk1 activation following DNA damage. Once Chk1 is activated, it stabilizes Claspin, which in turn helps to maintain Chk1 activation during replication stress. In summary, these data indicate that the interaction between Claspin and Chk1 is complex. These proteins regulate each other and thus ensure the proper cell cycle progression and replication checkpoint control.
Oncogene (2006) **25,** 4165–4171. doi:10.1038/sj.onc.1209447; published online 27 February 2006

Keywords: Claspin; Chk1; protein stability; cell cycle

Introduction

In order to maintain genomic stability cells have to ensure fidelity of events, such as DNA replication and chromosome segregation. When DNA is damaged or a replication fork stalled, cells activate mechanisms known as checkpoints. These cell cycle checkpoints involve a network of signal transduction pathways containing sensors, transducers and effector proteins.

One pathway that plays a central role in response to several types of DNA damage is the ATR-Chk1 pathway (Schechter *et al*., 2004). Ataxia telangiectasia mutated- and Rad3-related (ATR) is activated in response to the formation of single-stranded DNA following DNA damage. Ataxia telangiectasia mutated- and Rad3-related phosphorylates several targets including the effector kinase Chk1. Phosphorylation of Chk1 by ATR is required for Chk1 activation and the subsequent phosphorylation of Chk1 substrates. Chk1 activation may also regulate the cellular distribution of Chk1 (Chen and Sanchez, 2004). Besides ATR, several proteins called adaptors are also required for Chk1 activation and phosphorylation (Chen and Sanchez, 2004). These proteins are not kinases, but they may contribute to the recruitment or phosphorylation of Chk1. One of these proteins is Claspin (Chini and Chen, 2004). In Xenopus (Kumagai and Dunphy, 2000, 2003) and human cells (Chini and Chen, 2003), Claspin is phosphorylated and associates with Chk1 in response to DNA damage and replication stress. Downregulation of human Claspin (Chini and Chen, 2003; Lin *et al*., 2004) decreases Chk1 activation in response to hydroxyurea (HU), ionizing radiation (IR) and UV, while immunodepletion of Xenopus Claspin (Kumagai and Dunphy, 2000) inhibits Chk1 activation induced by DNA templates in egg extracts. In addition, it has been shown *in vitro* for both Xenopus (Kumagai *et al*., 2004) and human systems (Clarke and Clarke, 2005) that the presence of Claspin is required for the ATR/ATM-dependent phosphorylation of Chk1. Furthermore, Claspin has been shown to be involved in regulation of the S-phase checkpoint, cell survival, and prevention of premature mitosis (Chini and Chen, 2003; Lin *et al*., 2004). All of these findings indicate a role for Claspin in the ATR-Chk1-dependent replication checkpoint control.

Claspin is a S-phase-specific protein (Chini and Chen, 2003) and levels of Claspin appear to be tightly regulated. However, the mechanisms involved in this regulation have not been addressed. In the present study we explored the regulation of Claspin protein levels and the role of Chk1 in this process. We observed that Claspin levels are regulated by the proteasome-mediated degradation pathway and surprisingly that Chk1 is required for Claspin stability. These results indicate that the interaction between Claspin and Chk1 is complex. Both proteins regulate each other in order to properly control the replication checkpoint in the cell.

Correspondence: Dr J Chen, Department of Oncology Research, Mayo Clinic College of Medicine, 200 First St. SW, Rochester, MN 55905, USA.
E-mail: Chen.Junjie@mayo.edu
Received 28 October 2005; revised 30 December 2005; accepted 13 January 2006; published online 27 February 2006

Results

Claspin degradation is mediated by the proteasome pathway

We previously reported that Claspin expression fluctuates during the cell cycle (Chini and Chen, 2003). Whereas levels of Claspin are low in resting cells (G0) and G1 cycling cells, they increase considerably as cells enter S-phase. As cells progress from S-phase, Claspin levels start to decrease (Chini and Chen, 2003). The mechanisms underlying the regulation of Claspin protein levels have not yet been examined. To test whether Claspin is actively degraded in cells, we expressed exogenous S-FLAG-tagged Claspin in asynchronous HeLa cells. Cells were then treated with cycloheximide, an inhibitor of protein synthesis. As shown in Figure 1a, Claspin levels declined quickly, and displayed much shorter half-life than another protein BACH1, which is also involved in DNA damage checkpoint control. This same phenomenon was observed when we analysed levels of endogenous Claspin after cycloheximide treatment (Figure 1b). We also determined Claspin levels after treatment of cells with different concentrations of cycloheximide. Concentrations as low as 10 μg/ml of cycloheximide inhibit Claspin levels by the same degree as higher concentrations. These results together imply that cells have an active mechanism to regulate Claspin degradation.

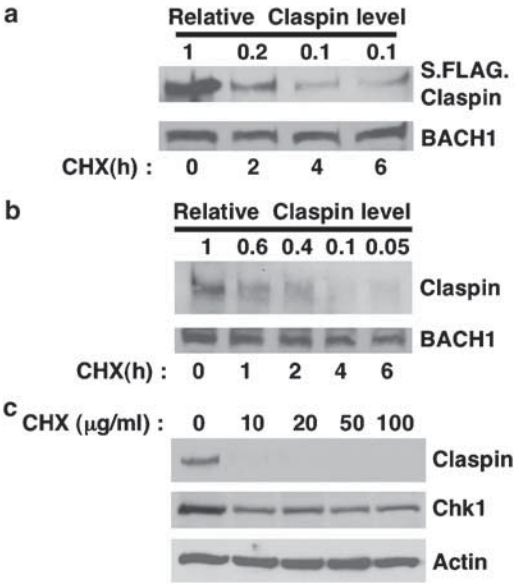

Figure 1 Claspin is rapidly degraded in the cell. (**a**), Hela cells were transfected with S-FLAG-tagged Claspin. Cells were incubated with 100 μg/ml cycloheximide (CHX) for the indicated periods. Western blots were performed on whole cell lysates using anti-FLAG and anti-BACH1 antibodies. (**b**) HeLa cells were treated with 100 μg/ml cycloheximide for various lengths of time. Western blots were performed on whole cell lysates using anti-Claspin and anti-BACH1 antibodies. Relative Claspin protein levels were determined by densitometry and were normalized to that obtained in nontreated samples (0 h control). (**c**) HeLa cells were incubated with different doses of cycloheximide (CHX) for 5 h. Western blots were performed on whole cell lysates using anti-Claspin, anti-Chk1 and anti-Actin antibodies.

To determine whether Claspin degradation is mediated by the proteasome pathway, we analysed Claspin levels after treatment of HeLa cells with the proteasome inhibitor MG132. We found that 5-h treatment of HeLa cells with MG132 caused a drastic increase in Claspin levels (Figure 2a). To determine whether this effect was specific for HeLa cells, we also analysed Claspin levels in HT1080 and U20S cells. Similar to that observed in HeLa cells, there were marked increases of Claspin levels in these cells following treatment with MG132 (Figure 2b and c). In addition, the expression of an epitope-tagged Claspin also increased following treatment with MG132 (Figure 2b). These data support that Claspin degradation is a general phenomenon and is mediated through the proteasome-dependent pathway in several cell types.

We further investigated whether proteasome inhibition was indeed affecting Claspin half-life. We treated cells for different periods of time with cycloheximide in the presence and absence of MG132. In the absence of MG132, Claspin displayed a much shorter half-life (Figure 2d), confirming that Claspin is actively degraded by the proteasome pathway in cells.

Chk1 regulates Claspin levels

Claspin was isolated as a Chk1-interacting protein and was shown to be required for Chk1 activation in Xenopus (Kumagai and Dunphy, 2000) and human cells (Chini and Chen, 2003; Lin *et al*., 2004). It is believed that Claspin functions upstream of Chk1 in the ATR-Chk1 pathway, possibly facilitating activation of Chk1 by ATR. Interestingly, when we investigated the effect of Chk1 small interfering RNA (siRNA) in HeLa cells, we observed that Chk1 siRNA suppresses the expression of both Chk1 and Claspin, while Claspin siRNA only suppressed Claspin expression (Figure 3a). This surprising result suggests that the presence of Chk1 is required for the maintenance of Claspin levels in cells.

In order to confirm the effect of Chk1 on Claspin regulation, we determined Claspin levels in cells treated with the Chk1 inhibitor UCN01 (Busby *et al*., 2000). In the presence of UCN01, Chk1 is still present but its kinase activity is inhibited. As shown in Figure 3b, treatment of HeLa cells with 300 nM UCN01 inhibited Claspin expression. Caffeine, which inhibits the ATR pathway and consequently Chk1 activation, has similar effects on Claspin levels (Figure 3b). Inhibition of Claspin levels was also seen using a lower concentration of UCN01 (100 nM) (Figure 3c). These results indicate that Chk1 kinase activity is required for maintaining the steady level of Claspin *in vivo*.

As Claspin was also shown to interact with BRCA1 and regulate BRCA1 phosphorylation (Lin *et al*., 2004), we investigated whether BRCA1 would also be involved in Claspin regulation. We compared Claspin levels in HCC1937, which only express a truncated form of BRCA1 (Tomlinson *et al*., 1998), with HCC1937 cells reconstituted with wild-type myc-epitope-tagged BRCA1. We found that Claspin levels are lower in HCC1937 cells than that in HCC1937 cells which are reconstituted with wild-type BRCA1 (Figure 3d),

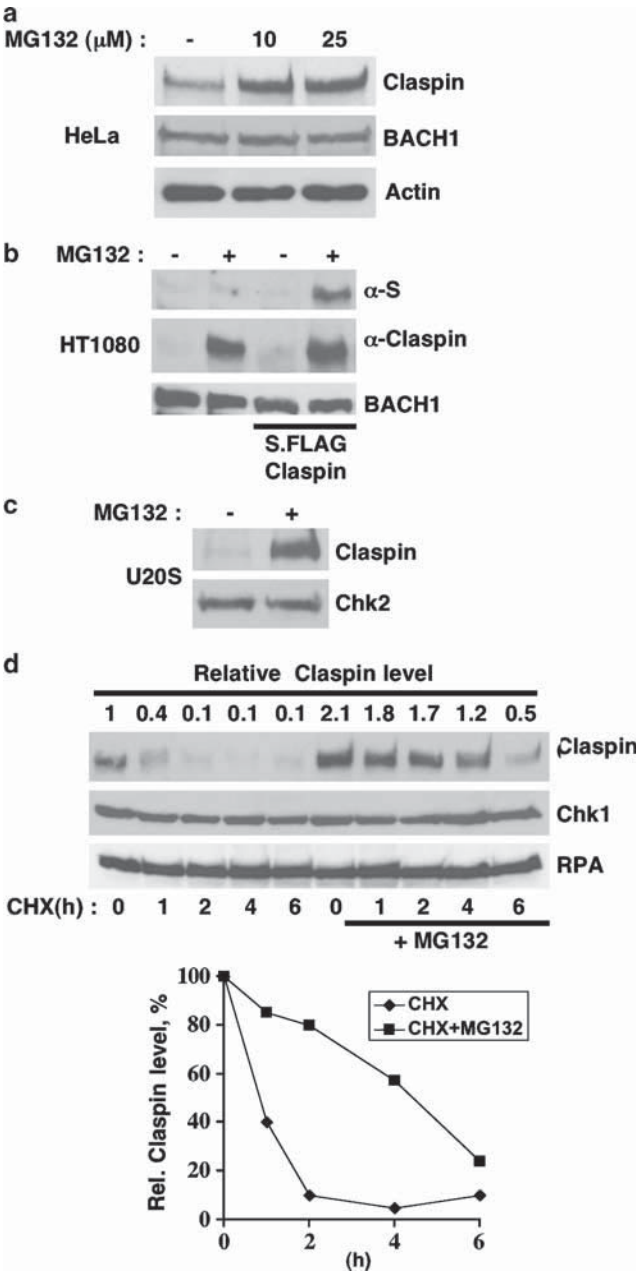

Figure 2 Claspin is degraded by the proteasome-dependent pathway. (**a**) HeLa cells were treated with 10 or 25 μM of the proteasome inhibitor MG132 for 5 h. Whole cell lysates were subjected to Western blots with anti-Claspin, anti-BACH1, and anti-Actin antibodies. (**b**) HT1080 cells and HT1080 cells transfected with S-FLAG-tagged Claspin were treated for 5 h with 10 μM MG132. Whole cell lysates were subjected to Western blots with anti-S and anti-Claspin antibodies. (**c**) U20S cells were treated with 10 μM MG132 for 5 h. Whole cell lysates were subjected to Western blots with anti-Claspin and anti-Chk2 antibodies. (**d**) HeLa cells were treated with cycloheximide (CHX) for the indicated time points in the presence or absence of 10 μM MG132. MG132 was added 4 h before cells were lysed. Western blots were performed on whole cell lysates using anti-Claspin, anti-Chk1 and anti-RPA antibodies. Relative Claspin protein levels were determined by densitometry and were normalized to that obtained in nontreated samples (0 h control, no MG132). In the graph, samples with and without MG132 were normalized to their own control (0 h, 100%).

indicating that BRCA1 participates, either directly or indirectly, in Claspin regulation.

HCC1937 cells have a defect in Chk1 activation (Yarden *et al*., 2002), since BRCA1 is required for efficient Chk1 activation following DNA damage. One possible explanation is that the reduced levels of Claspin in HCC1937 cells are due to the lower levels of activated Chk1 in these cells. To investigate this possibility, we treated wild-type BRCA1 reconstituted HCC1937 cells with UCN01 to inhibit Chk1 activity. As shown in Figure 3d, UCN01 decreased Claspin expression in HCC1937 + BRCA1 cells, suggesting that the reduced levels of Claspin in BRCA1-deficient cells may be due to a defect in Chk1 activity in these cells.

As inhibition of both Chk1 levels and kinase activity decreases Claspin expression, it is possible that Chk1 regulates Claspin levels through phosphorylation of Claspin. As shown in Figure 3e, UCN01 inhibits Claspin hyperphosphorylation in response to hydroxyurea, suggesting that Chk1 may regulate Claspin phosphorylation.

Many experiments in this study were performed in the absence of DNA damage. Several studies indicate that Chk1 has basal activity and phosphorylates its target proteins, such as Cdc25A, even in the absence of exogenous DNA damage and/or replication stress (Kaneko *et al*., 1999; Sorensen *et al*., 2003). This is because Chk1 is required for the maintenance of normal DNA replication. The key function of Chk1 is to monitor normal DNA replication and its function is augmented when cells encounter endogenous DNA lesions. So, it is reasonable that Chk1 regulates Claspin stability and probably Claspin phosphorylation even in the absence of DNA damage.

In order to understand how Chk1 affects Claspin levels, we investigated whether Chk1 inhibition in HeLa cells would lead to an increase in Claspin degradation. We treated HeLa cells with Chk1 siRNA and UCN01 in the presence and absence of the proteasome inhibitor MG132 (Figure 4a and b). The effect of both Chk1 siRNA and UCN01 were reversed by proteasome inhibition, indicating that Chk1 inhibition promotes Claspin protein degradation. To confirm the specificity of the effect of Chk1siRNA and UCN01 on Claspin levels, we determined whether these treatments would affect normal cell cycle distribution and thus indirectly influence Claspin expression. As shown in Figure 4c, Chk1 siRNA did not alter the normal cell cycle distribution. UCN01 treatment did cause a small decrease in S-phase cells. However, this small decrease in S-phase could not account for the dramatically reduced Claspin levels seen in cells treated with UCN01 (Figure 4b). These results together support that Chk1 regulates Claspin protein stability.

Chk1 overexpression stabilizes Claspin

As inhibition of Chk1 expression or activity reduced Claspin levels, we expected that Chk1 overexpression would have the opposite effect on Claspin protein levels. Indeed, overexpression of HA-Chk1 in HeLa cells increased Claspin levels (Figure 5a). However,

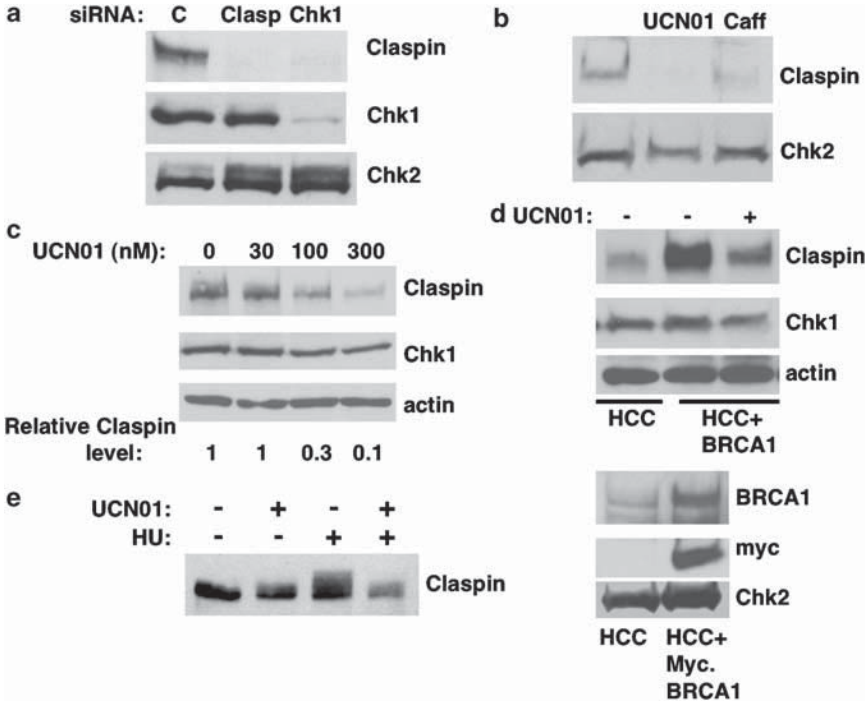

Figure 3 Inhibition of Chk1 expression and activity decreases Claspin levels in the cell. (**a**) HeLa cells were treated with control, Claspin and Chk1 siRNA. Whole cell lysates were subjected to Western blots with anti-Claspin, anti-Chk1 and anti-Chk2 antibodies. (**b**) HeLa cells were treated with 300 nM UCN01 or 3 mM caffeine for 6 h. Western blots were performed in whole cell lysates using anti-Claspin and anti-Chk2 antibodies. (**c**) HeLa cells were treated with different doses of UCN01 for 5 h. Western blots were performed in whole cell lysates using anti-Claspin, anti-Chk1, and anti-Actin antibodies. Relative Claspin protein levels were determined by densitometry and were normalized to the Actin levels. (**d**) upper panel, HCC1937 cells and HCC1937 cells reconstituted with myc-tagged BRCA1 were treated with or without UCN01 for 16 h as indicated. Whole cell lysates were subjected to Western blottingh with anti-Claspin and anti-Chk1 antibodies. Lower panel, expression of BRCA1 in HCC1937 cells. BRCA1 was immunoprecipitated from HCC1937 and HCC1937 reconstituted with myc-epitope-tagged BRCA1 and blotted with either anti-BRCA1 or anti-myc antibodies. Western blot with anti-Chk2 antibody was included as a control. (**e**) HeLa cells were treated with hydroxyurea in the presence or absence of 300 nM UCN01. Claspin was immunoprecipitated and Western blots were performed using anti-Claspin antibody.

HA-Chk2, which was expressed at higher levels than HA-Chk1, did not have the same effect on Claspin levels (Figure 5a). The same increase was also observed when Chk1 was co-expressed with exogenous Claspin (Figure 5b). In conclusion, our results show that Claspin can be stabilized when Chk1 expression is increased *in vivo*.

As a control, we established that overexpression of either Chk1 and Chk2 did not significantly change cell cycle distribution (Figure 5c). We also attempted to determine whether Chk1 overexpression may increase Claspin half-life, but these experiments were not conclusive since overexpressed Chk1 levels also decreased following cycloheximide treatment (data not shown).

Claspin is necessary to sustain Chk1 activation

If Chk1 activity is required for maintaining Claspin protein levels, why would Claspin in turn act upstream of Chk1 and be required for Chk1 activation? To resolve this dilemma, we performed a time course of Chk1 activation in the presence or the absence of Claspin siRNA. Chk1 activation was monitored by immunoblotting using anti-phospho-S317 antibody.

In HeLa cells treated with Claspin siRNA, basal Chk1 levels are not altered (Figure 3a). When we analysed Chk1 activation in the presence of Claspin siRNA, we observed a decrease in Chk1 activation only at later time points after 10 mM hydroxyurea treatment. Early Chk1 activation following hydroxyurea treatment was not decreased by Claspin siRNA (Figure 6a).

We also compared the effect of the Claspin siRNA with that of Rad17 siRNA. Like Claspin, Rad17 has also been shown to be required for Chk1 activation (Wang *et al*., 2003). In contrast to Claspin siRNA, Rad17 siRNA inhibited Chk1 activation at early time points following hydroxyurea treatment (Figure 6a). Thus, it appears that Claspin is not involved in initial Chk1 activation, but may play a role in sustaining or enhancing Chk1 activation at later time points.

In order to confirm this possibility, we tested the effect of Claspin siRNA on late Chk1 activation in response to lower concentration of hydroxyurea (1 mM) in both HeLa and U20S cell lines. In HeLa, a 50% decrease in Chk1 activation was only observed at 4 and 6 h after hydroxyurea treatment. In U20S Chk1 activation was more sensitive to Claspin inhibition, and a higher degree of inhibition was observed at 4 and 6 h following hydroxyurea treatment. These data together suggest that Claspin does play a role in late Chk1 activation.

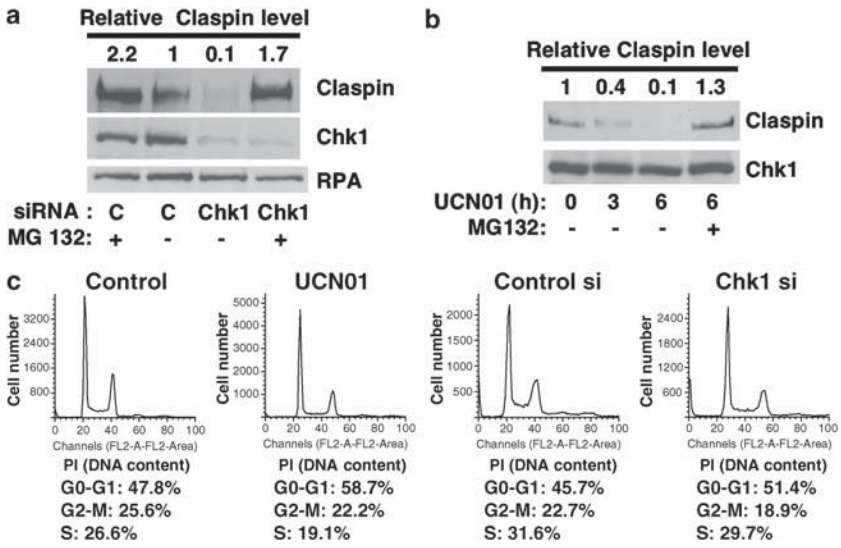

Figure 4 Inhibition of Claspin expression by Chk1 siRNA and UCN01 is reversed by proteasome inhibition. (**a**) HeLa cells were treated with control and Chk1 siRNA. 67 h after the first siRNA transfection cells were treated with MG132 and 5 h later cells were collected and lysed. Whole cell lysates were subjected to Western blots with anti-Claspin, anti-Chk1 and anti-RPA antibodies. (**b**) HeLa cells were treated with 300 nM UCN01 for 3 or 6 h. MG132 was added for the last 4 h of UCN01 treatment. Western blots were performed in whole cell lysates using anti-Claspin and anti-Chk1 antibodies. (**c**) Cell cycle distributions after siRNA treatments (72 h) and after 6 h incubation with UCN01 were determined by FACS analysis and summarized below. Relative Claspin protein levels were determined by densitometry and were normalized to that obtained in non-treated samples (control) or samples transfected with control siRNA.

Discussion

In our previous study of human Claspin, we showed that Claspin is a cell cycle-regulated protein. Claspin levels are very low in resting cells and peak in S-phase (Chini and Chen, 2003). However, the mechanism involved in Claspin regulation was not investigated. In this study, we demonstrate that Claspin is a protein with a short half-life and its degradation is mediated through the proteasome pathway. Surprisingly, we also observe that the presence of kinase active Chk1 is required for maintaining Claspin stability. Inhibition of Chk1 expression by siRNA or inhibition of Chk1 kinase activity by UCN01 lead to a decrease in Claspin levels and these effects were reversed by proteasome inhibition. Consistent with these observations, overexpression of Chk1 increases Claspin levels in the cell. Therefore, it appears that the amount of Claspin is determined in cells by the level and activity of Chk1. Previously, we showed that Claspin expression during the cell cycle correlates with Chk1 expression (Chini and Chen, 2003). Based on the observations presented in this study, the increase of Claspin expression observed at the G1/S transition may be the result of an increase in Chk1 level and activity at these cell cycle phases.

Claspin expression appears to fluctuate during the cell cycle in a manner similar to BRCA1. BRCA1 levels are also low in resting and G1 cycling cells and its levels increase considerably as cells enter S-phase. Similar to Claspin, BRCA1 levels are regulated by the ubiquitin/proteasome pathway *in vivo* (Choudhury *et al*., 2004). Interestingly, Claspin levels are lower in BRCA1-deficient cells, indicating that the BRCA1-pathway may be involved in regulation of Claspin. As BRCA1-deficient cells have a defect in Chk1 activation (Yarden *et al*., 2002), the lower Claspin levels are possibly due to a defect in Chk1 activation. This agrees with our observation that blocking Chk1 activity in wild-type BRCA1 reconstituted cells inhibits the BRCA1-dependent increase of Claspin expression in these cells. However, since BRCA1 also interacts with Claspin (Lin *et al*., 2004), we should not rule out the possibility that BRCA1 may also regulate Claspin levels independent of Chk1.

The mechanism involved in the regulation of Claspin by Chk1 is not yet known. One possible mechanism is through phosphorylation of Claspin by Chk1. Chk1 exists in an active form during the S- to M-phase of the cell cycle (Kaneko *et al*., 1999), and it has been shown to phosphorylate substrates even in the absence of DNA damage (Sorensen *et al*., 2003, 2004). In addition, Chk1 is known to regulate the stability of several proteins through phosphorylation. Chk1 phosphorylates Cdc25A and targets it for ubiquitin-dependent degradation (Zhao *et al*., 2002; Sorensen *et al*., 2003). In contrast, the Chk1-dependent phosphorylation of Pds1 inhibits Pds1 ubiquitination and promotes its stabilization (Agarwal *et al*., 2003). Therefore, similar to Chk1/Pds1 regulation, Chk1 may phosphorylate Claspin and prevent its degradation. Further studies are needed to demonstrate that Chk1 can directly phosphorylate and regulate Claspin *in vivo*.

Our present and previous findings suggest that Claspin and Chk1 regulate each other during the cell

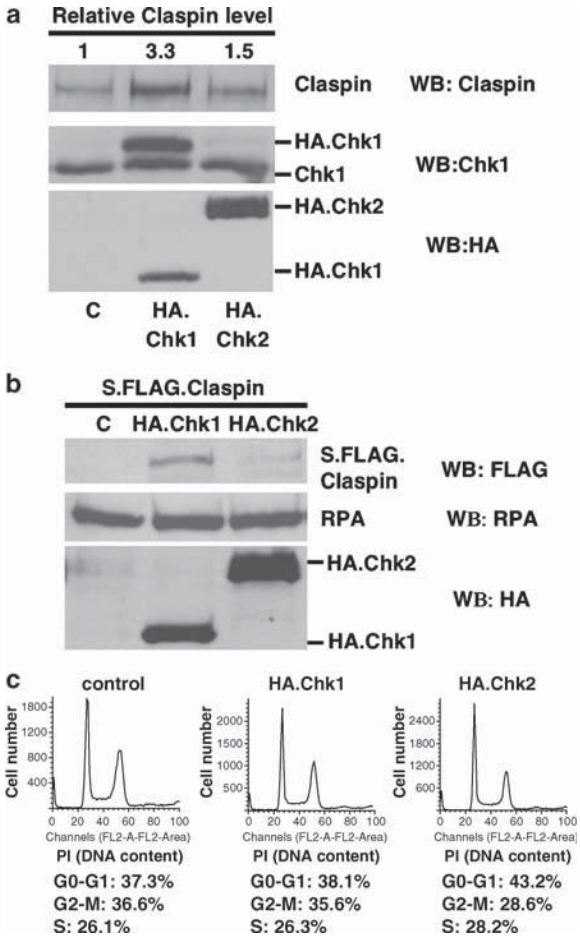

Figure 5 Overexpression of Chk1 increases Claspin stability. (**a**) HeLa cells were transfected with empty vector (C) or vectors encoding HA-tagged Chk1 or HA-tagged Chk2. Whole cell lysates were subjected to Western blots with anti-Claspin, anti-Chk1, and anti-HA antibodies. (**b**) Hela cells were transfected with vector encoding S-FLAG-tagged Claspin in combination with empty vector (C), or vectors encoding HA-tagged Chk1 or HA-tagged Chk2. Whole cell lysates were subjected to Western blots with anti-FLAG, anti-HA antibodies, and anti-RPA antibodies. (**c**) Cell cycle distributions in the presence of empty vector (control) and transfected HA-Chk1 and HA-Chk2 were determined by FACS analysis and summarized below. Relative Claspin protein levels were determined by densitometry and were normalized to that obtained in samples transfected with empty vector.

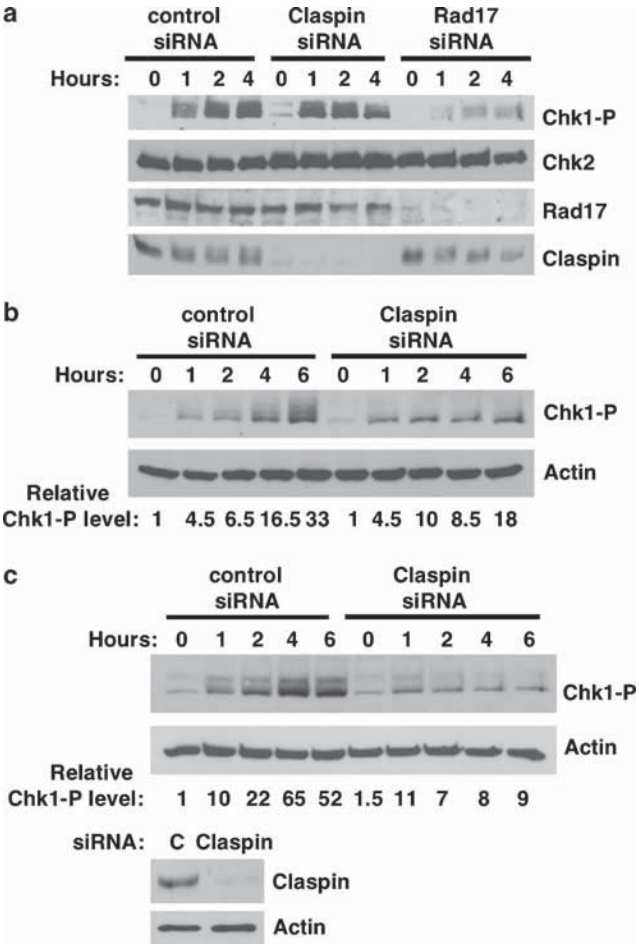

Figure 6 Claspin regulates late Chk1 activation following hydroxyurea treatment (**a**) HeLa cells were transfected with control, Claspin and Rad17 siRNA. After 72 h, cells were treated for 1, 2 or 4 h with 10 mM hydroxyurea. Whole cell lysates were subjected to Western blots with antibodies against S317-Chk1, Claspin, Rad17 and Chk2. (**b**) HeLa cells were transfected with control and Claspin siRNA. After 72 h, cells were treated for 1, 2, 4 or 6 h with 1 mM hydroxyurea. Whole cell lysates were subjected to Western blots with antibodies against S317-Chk1 and Actin. (**c**) upper panel, U20S cells were transfected with control and Claspin siRNA. After 72 h, cells were treated for 1, 2, 4 or 6 h with 1 mM hydroxyurea. Whole cell lysates were subjected to Western blots with antibodies against S317-Chk1 and Actin. Relative phosphorylated Chk1 levels were determined by densitometry and were normalized according to Actin levels. Lower panel, expression of Claspin in U20S cells transfected with control and Claspin siRNA. Claspin was immunoprecipitated from U20S cells and Western blots were performed with anti-Claspin and anti-Actin antibodies.

cycle. Claspin had originally being identified as a protein that interacts with Chk1 after DNA damage and regulates Chk1 activation. The original hypothesis is that Claspin may work as an adaptor molecule mediating ATR-dependent Chk1 activation (Kumagai *et al*., 2004). However, it is still not clear how Claspin contributes to Chk1 activation. Ataxia telangiectasia mutated- and Rad3-related (ATR), Claspin and Rad17 have all been shown to be important for Chk1 activation and they each bind independently to chromatin during S-phase (Lee *et al*., 2003). These proteins may be involved in recognizing different facets of stalled DNA replication forks and contribute differently in Chk1 activation. Our results suggest that while Rad17 appears to be required for the early phase of Chk1 activation, the role of Claspin is likely to be at the later stage of Chk1 activation. It is possible that Claspin may facilitate the amplification of Chk1 activation following the initial Chk1 phosphorylation by ATR. The exact mechanism by which Claspin and Chk1 regulate each other remains to be determined and will contribute to our understanding of the complex regulation of these proteins in replication checkpoint control.

Materials and methods

Cell culture, plasmids and antibodies
Human cell lines HeLa, U20S and HT1080 were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). HCC1937 cells and HCC1937 reconstituted with BRCA1 have been previously described (Tomlinson *et al*., 1998; Scully *et al*., 1999; Yu *et al*., 2003). Claspin was cloned into the pIRES2-EGFP vector containing S and FLAG tags. Mouse monoclonal and polyclonal anti-Claspin antibodies have been previously described (Chini and Chen, 2003). Anti-Chk1 antibodies for Western blot were form Santa Cruz Biothecnology (Santa Cruz, CA, USA) and anti-phospho-Chk1 (Ser317) antibody was from Cell Signaling Technology (Beverly, MA, USA). Anti-FLAG M2 antibody was from Sigma and anti-S antibody was a gift from SH Kaufmann (Hackbarth *et al*., 2004). Anti-RPA and anti-Rad17 antibodies were gifts from L Karnitz. UCN01 was a gift of JN Sarkaria (Busby *et al*., 2000). Anti-Chk2 (Ward *et al*., 2001) and anti BACH1 (Yu *et al*., 2003) antibodies were previously described.

Small interfering RNA transfection
Small interfering RNA duplexes were 21 base pairs including a 2-deoxynucleotide overhang. The target cDNA sequence for the Claspin siRNA was: AACCTTGCTTAGAGCTGAGTC; the control siRNAs were: AATTCAATAAATTCTTGAGGT (Lou *et al*., 2003; Kim *et al*., 2005) or siCONTROL (non-targeting siRNA #2, Dharmacon). Chk1 siRNA was designed as previously described (Zhao *et al*., 2002). Rad 17 siRNA was a gift from L Karnitz and had the target sequence AACAGACTGGGTTGACCCATC. For transfections, HeLa or U20S cells were plated in six-well plates and were transfected at 40% confluency with the siRNA duplex and oligofectamine. Transfection was repeated 24 h later and cells were analysed 72 h after the first transfection.

Protein stability experiments
To determine the half-life of Claspin, 100 μg/ml cycloheximide (Sigma, St Louis, MO, USA) was added to cells to inhibit protein synthesis. Cells were harvested in NETN lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8), 0.5% Nonidet P-40) supplemented with protease and phosphatase inhibitors. Proteins were subjected to Western blot with anti-Claspin and control antibodies. To determine the effects of proteasome inhibitors on Claspin stability cells were incubated with 10 μM MG132 for 5 h before lysis.

Western blots
Cells were harvested in NETN lysis buffer with protease and phosphatase inhibitors. Whole cell lysates were subjected to SDS–PAGE and blotted with specific antibodies as indicated.

Abbreviations

ATR, ataxia telangiectasia mutated- and Rad3-related; siRNA, small interfering RNA; HU, hydroxyurea; UV, ultra violet light; HA, hemagglutinin; EGFP, enhanced green fluorescent protein.

Acknowledgements

We thank members of the Chen laboratory for helpful discussions and technical support. This work was supported in part by grants from the National Institute of Health (CA89239, CA92312 and CA100109). JC is a recipient of the DOD breast cancer career development award (DAMD 17-02-1-0472).

References

Agarwal R, Tang Z, Yu H, Cohen-Fix O. (2003). *J Biol Chem* **278**: 45027–45033.
Busby EC, Leistritz DF, Abraham RT, Karnitz LM, Sarkaria JN. (2000). *Cancer Res* **60**: 2108–2112.
Chen Y, Sanchez Y. (2004). *DNA repair* **3**: 1025–1032.
Chini CCS, Chen J. (2003). *J Biol Chem* **278**: 30057–30062.
Chini CCS, Chen J. (2004). *DNA repair* **3**: 1033–1037.
Choudhury AD, Xu H, Baer R. (2004). *J Biol Chem* **279**: 33909–33918.
Clarke CA, Clarke PR. (2005). *Biochem J* **388**: 705–712.
Hackbarth JS, Lee SH, Meng XW, Vroman BT, Kaufmann SH, Karnitz LM. (2004). *Biotechnique's* **37**: 835–839.
Kaneko Y, Watanabe N, Morisaki H, Akita H, Fujimoto A, Tominaga K *et al*. (1999). *Oncogene* **18**: 3673–3681.
Kim JE, McAvoy SA, Smith DI, Chen J. (2005). *Mol Cell Biol* **25**: 10907–10915.
Kumagai A, Dunphy WG. (2000). *Mol Cell* **6**: 839–849.
Kumagai A, Dunphy WG. (2003). *Nat Cell Biol* **5**: 161–165.
Kumagai A, Kim S-M, Dunphy WG. (2004). *J Biol Chem* **279**: 49599–49608.
Lee J, Kumagai A, Dunphy WG. (2003). *Mol Cell* **11**: 329–340.
Lin S-Y, Li K, Stewart GS, Elledge SJ. (2004). *Proc Natl Acad Sci USA* **101**: 6484–6489.
Lou Z, Chini CC, Minter-Dykhouse K, Chen J. (2003). *J Biol Chem* **278**: 13599–13602.
Schechter D, Costanzo V, Gautier J. (2004). *DNA repair* **3**: 901–908.
Scully R, Ganesan S, Vlasakova K, Chen J, Socolovsky M, Livingston DM. (1999). *Mol Cell* **4**: 1093–1099.
Sorensen CS, Svljuasen RG, Falck J, Schroeder T, Ronnstrand L, Khanna KK *et al*. (2003). *Cancer Cell* **3**: 247–258.
Sorensen CS, Syljuasen RG, Lukas J, Bartek J. (2004). *Cell Cycle* **3**: 941–945.
Tomlinson GE, Chen TT, Stastny VA, Virmani AK, Spillman MA, Tonk V *et al*. (1998). *Cancer Res* **58**: 3237–3242.
Wang X, Zou L, Zheng H, Wei Q, Elledge SJ, Li L. (2003). *Genes Dev* **17**: 965–970.
Ward IM, Wu X, Chen J. (2001). *J Biol Chem* **276**: 47755–47758.
Yarden RI, Pardo-Reoyo S, Sgagias MM, Cowan KH, Brody LC. (2002). *Nature Gen* **30**: 285–289.
Yu X, Chini CCS, He M, Mer G, Chen J. (2003). *Science* **302**: 639–642.
Zhao H, Watkins JL, Piwnica-Worms H. (2002). *Proc Natl Acad Sci* **99**: 14795–14800.