Repeated Phosphopeptide Motifs in Human Claspin Are Phosphorylated by Chk1 and Mediate Claspin Function*

Claudia C. S. Chini and Junjie Chen†

From the Department of Oncology Research, Mayo Clinic College of Medicine, Rochester, Minnesota 55905

Claspin is a checkpoint protein involved in ATR (ataxia telangiectasia mutated- and Rad3-related)-dependent Chk1 activation in Xenopus and human cells. In Xenopus, Claspin interacts with Chk1 after DNA damage through a region containing two highly conserved repeats, which becomes phosphorylated during the checkpoint response. Because this region is also conserved in human Claspin, we investigated the regulation and function of these potential phosphorylation sites in human Claspin. We found that Claspin is phosphorylated in vivo at Thr-916 in response to replication stress and UV damage. Mutation of these phosphorylation sites on Claspin inhibited Claspin-Chk1 interaction in vivo, impaired Chk1 activation, and induced premature chromatin condensation in cells, indicating a defect in replication checkpoint. In addition, we found that Thr-916 on Claspin is phosphorylated by Chk1, suggesting that Chk1 regulates Claspin during checkpoint response. These results together indicate that phosphorylation of Claspin repeats in human Claspin is important for Claspin function and the regulation of Claspin-Chk1 interaction in human cells.

Genomic integrity of DNA is maintained by checkpoint mechanisms that monitor DNA damage or incomplete replication. These cell cycle checkpoint mechanisms, once activated, delay the cell cycle transitions to allow damage to be repaired or replication to be completed. Key components of the checkpoint pathway are ATM and ATR, members of the phosphoinositide 3-kinase-related protein kinase family (1, 2). These kinases phosphorylate the effector kinases Chk1 and Chk2. Although the ATM-Chk2 pathway is primarily activated by ionizing radiation (IR), the ATR-Chk1 pathway is typically activated by UV damage and stalled replication forks. Chk1 is an essential protein, and its activation is involved in regulation of S phase and mitotic entry after DNA damage (3). Activation of Chk1 requires not only ATR but also several additional checkpoint proteins, including Claspin (4), Rad9, Rad17, TopBP1, and BRCA1 (3). The mechanism by which these proteins work together to regulate Chk1 function remains to be elucidated.

To understand how Chk1 is regulated, it was important to determine the function and regulation of these Chk1 activators. In both Xenopus and human cells Claspin has been shown to be a key regulator of Chk1 activation (5–7). Claspin is a cell cycle-regulated protein (6) that associates with chromatin in a regulated manner during S phase (8). Because Claspin binds with high affinity to DNA, it has been proposed that Claspin may be a component of the replication ensemble (9). After DNA damage and replication stress, Claspin is phosphorylated and interacts with Chk1 (5, 6). This Claspin-Chk1 interaction is required for ATR-dependent Chk1 activation (10).

The mechanism by which Claspin and Chk1 interact appears to be complex and has been explored in more detail in Xenopus. XClaspin interacts with Chk1 through the Chk1-binding domain, a region of 57 amino acids that contains two highly conserved repeats of ~10 amino acids with the consensus sequence EXXLCS/T/GXF (11). These motifs are also conserved in hClaspin. While XClaspin has two of these motifs, hClaspin has three. Mutation of serines in these repeats abolishes the Claspin-Chk1 interaction as well as Chk1 activation in Xenopus egg extracts (11). However, these motifs are not necessary for the binding of XClaspin to chromatin (12), indicating that retention of XClaspin on the chromatin is not necessary for Chk1 activation. These repeats also appear to be involved in hClaspin-Chk1 interaction. In an in vitro human cell-free system, mutation of two of these putative phosphorylation sites in the Chk1-binding domain of human Claspin inhibited Claspin-Chk1 interaction (13). However it is still not clear how these phosphorylation events are regulated and how they contribute to Claspin function in vivo.

Although it has been demonstrated that Claspin is phosphorylated in response to DNA damage and replication stress, it is not known which protein kinases phosphorylate Claspin. Phosphorylation of the EXXLCS/T/GXF motifs in XClaspin (11) and human Claspin (14) is ATR-dependent, but these sites are not consensus sites for ATR/ATM kinases, suggesting that an ATR-dependent kinase is directly responsible for Claspin phosphorylation at these residues. In the present study we investigated whether the conserved repeats on hClaspin are required for hClaspin-Chk1 interaction and Claspin function in the cell. We confirmed that these sites are phosphorylated in vivo in response to replication stress and UV damage. Phosphorylation of these residues is important for Claspin function and for the regulation of hClaspin-Chk1 interaction in vivo. More importantly, we found that these sites are phosphorylated by Chk1.

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† Recipient of U. S. Department of Defense Breast Cancer Career Development Award DAMD 17-02-1-0472. To whom correspondence should be addressed: Dept. of Therapeutic Radiology, Hunter Bldg., Rm. 213C, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06520, Tel.: 203-785-2967; Fax: 203-785-7482; E-mail: Junjie.Chen@yale.edu.
2 The abbreviations used are: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia mutated- and Rad3-related; hClaspin, human Claspin; PCC, premature chromatin condensation; siRNA, small interfering RNA; HU, hydroxyurea; UV, ultraviolet light; GST, glutathione S-transferase.
indicating that Claspin and Chk1 may regulate each other to ensure optimal checkpoint activation following DNA damage.

MATERIALS AND METHODS

Cell Lines and Antibodies—Human cell lines HeLa, U2OS, and 293T were grown in RPMI 1640 supplemented with 10% fetal bovine serum. Mouse monoclonal and polyclonal anti-Claspin antibodies have been described previously (6). Phospho-antibody against Thr-916 was raised against the peptide DENAMDANMDELLDLC(p)TGKFTSQAE. Anti-FLAG M2 antibody was purchased from Sigma. Anti-Chk1 antibody was form Santa Cruz Biotechnology (Santa Cruz, CA), and anti-phospho-Chk1 (Ser-317) antibody was from Cell Signaling Technology (Beverly, MA). UCN01 was a gift of J. N. Sarkaria (15).

Expression and Purification of Recombinant Proteins—We generated wild-type Claspin, Claspin deletion mutants, Chk1, and Chk2 in the baculovirus/insect cell expression system. Claspin, Chk1, and Chk2 were cloned in gateway vector pDEST20, and Myc epitope-tagged Chk1 was cloned into pDEST8. Baculoviruses were generated using the Bac-to-Bac system (Invitrogen). FLAG-Claspin baculovirus was a gift from A. Sancar (9). Sf9 insect cells (Invitrogen) were infected with the appropriate baculoviruses and harvested 48 h after infection. Cells were washed with 1× phosphate-buffered saline and then lysed in NETN buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing protease and phosphatase inhibitors. After incubation on ice for 20 min, cell lysates were cleared by centrifugation for 10 min at 14,000 × g. Supernatants were incubated with anti-FLAG-agarose (Sigma) or glutathione-agarose beads for 30 min at 4 °C. Cell lysates were centrifuged for 10 min at 4 °C. Proteins were eluted from FLAG beads with lysis buffer containing 0.2 mg/ml FLAG peptide (Sigma) for 30 min at 4 °C. Cell lysates were centrifuged for 10 min at 4 °C and 293T were grown in RPMI 1640 supplemented with 10% fetal bovine serum. Mouse monoclonal and polyclonal anti-Claspin antibodies have been described previously (6). Phospho-antibody against Thr-916 was raised against the peptide DENAMDANMDELLDLC(p)TGKFTSQAE. Anti-FLAG M2 antibody was purchased from Sigma. Anti-Chk1 antibody was form Santa Cruz Biotechnology (Santa Cruz, CA), and anti-phospho-Chk1 (Ser-317) antibody was from Cell Signaling Technology (Beverly, MA). UCN01 was a gift of J. N. Sarkaria (15).

Immunoprecipitation and Western Blotting—Cells were lysed in NETN containing protease and phosphatase inhibitors for 20 min at 4 °C. Cell lysates were centrifuged for 10 min at 4 °C. Immunoprecipitation was performed for 1 h at 4 °C with specific antibodies. Samples were subjected to SDS-PAGE followed by immunoblotting.

siRNA Transfection and Preparation of Mitotic Spreads—siRNA duplexes were 21 base pairs including a 2-deoxynucleotide overhang. The target cDNA sequence for the Claspin and control siRNA were described previously (16). For siRNA transfection, U2OS cells were plated in 6-well plates and transfected at 40% confluence with the siRNA duplex and Oligofectamine. Transfection was repeated 24 h later, and cells were analyzed 72 h after the first transfection. Mitotic spreads were performed as described previously (6).

RESULTS

Human Claspin Is Phosphorylated at the Thr-916 Site in Vivo in Response Replication Stress and UV Radiation—Previous studies in Xenopus and a human cell-free system show that binding of Chk1 to Claspin requires two conserved repeats on Claspin that contain phosphorylation sites (11, 13). However, the importance of these phosphorylation sites in vivo for human Claspin function and its interaction with Chk1 has not been addressed. To study the regulation and function of these phosphorylation sites in human cells, we prepared antibodies specifically recognizing a peptide containing phosphorylated Thr-916, one of the phosphorylation sites within this repeat region. We first tested this phospho-specific antibody in 293T cells before and after treatment with hydroxyurea or UV damage. Exposure of cells to either of these treatments induced phosphorylation of hClaspin at the Thr-916 site, and phosphatase treatment abolished the immunoreactivity of this phospho-specific antibody (Fig. 1A). These results confirm that human Claspin is indeed phosphorylated at this conserved repeat region following DNA damage.

Although the phosphorylation sites like the Thr-916 site within these conserved repeats are not consensus ATM and/or ATR phosphorylation sites, phosphorylation of these repeats is ATR-dependent (11, 14), suggesting that an ATR-dependent kinase is directly involved in the phosphorylation of these residues in vivo. To address this question, we decided to investigate whether the major ATR downstream kinase Chk1 is required for phosphorylation of these residues in human cells. To investigate the potential role of Chk1 in Claspin phosphorylation, we treated cells with UCN01, an inhibitor of Chk1 (15). As shown in Fig. 1B, pretreatment with UCN01 blocked HU-induced Claspin phosphorylation, suggesting that Chk1 is required for Claspin phosphorylation following replication stress. This HU-induced phosphorylation of human Claspin at Thr-916 site is also inhibited by caffeine, an inhibitor of ATM/ATR (17) (Fig. 1B), which is in agreement with data from Xenopus (11). Similar results were obtained using 293T cells, in which pretreatment with UCN01 reduced HU- or UV-induced Thr 916 phosphorylation of Claspin (Fig. 1C). We also performed experiments using Chk1 siRNA or prolonged treatment with UCN01. How-
Claspin is phosphorylated in vivo at the Thr-916 site. A, 293T cells were treated with 10 mM HU or 40 J/m² UV. After 2 h, cells were collected and lysed, and Claspin was immunoprecipitated with anti-Claspin antibodies. Western blots were performed using anti-Claspin and anti-Claspin-P-Thr-916 antibodies. B, HeLa cells were mock treated or pretreated with 300 nM UCN01 or 3 mM caffeine for 30 min before the addition of 10 mM HU. Claspin was immunoprecipitated with anti-Claspin antibody and immunoblotted with anti-Claspin or anti-Claspin-P-Thr-916 antibodies. C, 293T cells were mock treated or treated with 300 nM UCN01 for 30 min before treatment with 10 mM HU or 40 J/m² UV. Immunoprecipitation and immunoblotting were performed in a manner similar to that described in B.

However, Claspin levels were reduced dramatically following these treatments, because Chk1 also regulates Claspin stability (16). In conclusion, these results suggest that Claspin is phosphorylated in vivo on these conserved repeats upon DNA damage and that Chk1 may be the kinase responsible for these phosphorylation events.

Claspin Is Phosphorylated by Chk1—To confirm a role for Chk1 in Claspin phosphorylation and further characterize the interaction between Claspin and Chk1, we prepared baculoviruses for expression of these proteins in insect cells. When insect cells were infected with baculoviruses expressing FLAG-tagged Claspin with GST-Chk1, we observed an interaction between Claspin and Chk1 by either pulldown with glutathione beads (Fig. 2A) or immunoprecipitation using anti-FLAG beads (Fig. 2B). Claspin only interacted with Chk1, but not with Chk2 (Fig. 2A and B), suggesting that the interaction was specific. The Claspin-Chk1 interaction was also observed when GST-Claspin was co-expressed with Myc-tagged Chk1 (Fig. 3, C and D).

Next we examined whether Claspin would be phosphorylated by Chk1 in these cells. When FLAG-Claspin was expressed alone, we could not detect phosphorylation of Claspin at Thr-916 site using our anti-Claspin phospho-specific antibody (Fig. 2B). However, co-expression of FLAG-Claspin with active GST-Chk1 induced Claspin phosphorylation at the Thr-916 site (Fig. 2A and B). Although GST-Chk1 kinase dead mutant (KD) also interacted with Claspin, it did not promote Claspin phosphorylation. As a control, when Claspin was expressed with GST-Chk2, we did not observe Claspin phosphorylation or an interaction between Claspin and Chk2 (Fig. 2A and B), suggesting that Claspin specifically interacts with and is phosphorylated by Chk1.

To confirm this phosphorylation of Claspin by Chk1, we isolated both proteins separately and performed in vitro kinase assays. When Claspin was incubated with active GST-Chk1 it became phosphorylated at Thr-916. This residue was not phosphorylated when Claspin was incubated with GST-Chk1 kinase dead (data not shown) or GST-Chk2. Immunoprecipitation was performed with anti-P-Claspin and with anti-GST antibodies to detect the associated GST-Chk1 in these samples. In vitro kinase assays were carried out using GST-Chk1 or GST-Chk2 as kinase source and wild-type (WT) or mutant (3A) GST-Claspin as substrates. Immunoblotting was performed with anti-P-Claspin, anti-Claspin, and anti-GST antibodies.
Regulation and Function of Claspin Phosphorylation

Phosphorylation of Claspin repeats is not essential for its interaction with Chk1 in vitro. A, upper panel, phospho- and nonphosphorylated Claspin peptides were incubated with streptavidin beads, and whole cell lysates (WCL) were prepared from 239T cells or 293T cells treated with 10 gray of IR or 10 mM HU for 1 h. Associated proteins were detected by Western blots using anti-Chk1 antibody. Lower panel, phosphorylated and nonphosphorylated biotinylated Claspin peptides were incubated with GST-Chk1 and streptavidin beads. Western blots were performed with anti-GST antibody. B, WT, phosphorylation of Claspin repeats is not essential; WT, phosphorylation of Claspin repeats is not essential for Claspin-Chk1 interaction. C, WT, phosphorylation of Claspin repeats is not essential; WT, phosphorylation of Claspin repeats is not essential for Claspin-Chk1 interaction. D, WT, phosphorylation of Claspin repeats is not essential; WT, phosphorylation of Claspin repeats is not essential for Claspin-Chk1 interaction. E, WT, phosphorylation of Claspin repeats is not essential; WT, phosphorylation of Claspin repeats is not essential for Claspin-Chk1 interaction.
Regulation and Function of Claspin Phosphorylation

In this study, we have shown that hClaspin is phosphorylated in vivo at the repeat region and that phosphorylation of these motifs after replication stress is important for Claspin function. Surprisingly, our results also demonstrate that Chk1 itself may be directly involved in the phosphorylation of the Claspin repeats. Previous studies (5, 10, 11) proposed a model in which Claspin is phosphorylated after DNA damage and then interacts with Chk1, with this damage-induced Claspin-Chk1 interaction being essential for the activation of Chk1 by ATR. Because these phospho-repeats on Claspin mediate the interaction between Claspin and Chk1 in *Xenopus*, it was believed that the repeats had to be phosphorylated before Claspin/Chk1 interacted and Chk1 was activated (11). However, our results indicate that phosphorylation of these repeats by Chk1 may occur after both proteins have already associated and the phosphorylation-dependent interaction between these two proteins may regulate the stability or the affinity of the interaction.

Exactly how Claspin interacts with Chk1 is complex. In this study, we have shown that Claspin can interact with Chk1 in both phosphorylation-dependent and -independent manners. When we overexpressed both Claspin and Chk1 in insect cells, it appeared that Claspin could interact with Chk1 in a phosphorylation-independent manner, because...
the phaso-mutant of Claspin had reduced Chk1 binding but nevertheless still interacted with Chk1 (Fig. 3D). The phosphorylation-dependent interaction between Claspin and Chk1 was also observed in our study. First we observed a reduction of Claspin-Chk1 interaction when we used the 3A mutant of Claspin. Second, we showed that only a peptide containing phosphorylated Thr-916 of Claspin, but not the unphosphorylated control peptide, could interact with Chk1 (Fig. 3A). Third, this phosphorylation-dependent interaction appeared to be more important for Claspin-Chk1 interaction in human cells, because in vivo Chk1 only interacted with the wild type, but not the phoso-mutant of Claspin, in response to replication stress (Fig. 4B). This latter finding is consistent with the data from a recent study using an in vitro human cell-free system (13). In that study, mutation of two phosphorylation sites (Thr-916 and Ser-945) in the C-terminal half of Claspin inhibited its association with Chk1. Indeed, the importance of these phosphorylation events is further supported by our observations that these phosphorylation sites on Claspin are required for optimal Chk1 activation and Claspin function following replication stress. The unanswered question is why there is a phosphorylation-in-phosphorylation-independent interaction between Claspin and Chk1. One explanation is that the phosphorylation-independent interaction is just an in vitro artifact due to the overexpression of these two proteins in insect cells. Alternatively, the phosphorylation-independent interaction of these proteins may have some role in the activation of checkpoint response following DNA damage. We speculate that the later explanation may have some merit. First, even in insect cells, the phosphorylation-independent interaction between Claspin and Chk1 is quite specific, because Chk2 does not bind to Claspin in this system (Fig. 2A). Second, we have shown that the repeated motifs on Claspin are phosphorylated by Chk1, suggesting that Chk1 may at least transiently associate with and phosphorylate Claspin even before the phosphorylation-dependent Claspin-Chk1 interaction become possible. Based on these observations, we believe that Claspin does not merely act upstream of Chk1. Claspin and Chk1 may interact transiently in a phosphorylation-independent manner in normal cells. Following DNA damage and/or replication stress, this phosphorylation-independent interaction between Claspin and Chk1 may allow the initial ATR-dependent phosphorylation of Chk1. This initial phosphorylation of Chk1 increase Chk1 activity and leads to the Chk1-dependent phosphorylation of Claspin at these conserved repeated motifs. The phosphorylation of these motifs further increases the Claspin-Chk1 interaction and results in full activation of Chk1 by ATR. This model of dynamic interaction between Claspin and Chk1 can explain the seemingly paradoxical results presented here.

The phosphorylation and regulation of Claspin are highly complex, more than we imagined several years ago. A recent study in Xenopus suggests that ATR-dependent phosphorylation of S/TQ sites on Claspin regulates Chk1 activation in response to double strand breaks but not in response to aphidicolin (19). These observations imply that ATR may be directly involved in regulating Claspin phosphorylation and that Claspin may be differently regulated in response to various types of DNA damage. In Xenopus and human cells, Claspin can also be phosphorylated by Polo-like kinase 1 (Plk1) (20–23). This Plk1-dependent phosphorylation of Claspin in Xenopus is involved in the regulation of adaptation following DNA damage. In human cells, Claspin phosphorylation by Plk1 also controls the ability of β-TrCP to recognize and degrade Claspin before mitosis (21–23). We have shown previously that Chk1 is required to maintain Claspin stability in S phase cells (16). Although the exact mechanism responsible for this regulation remains to be determined, it may involve phosphorylation of Claspin by Chk1. In the present study, we have demonstrated that the repeat region in Claspin is phosphorylated by Chk1. However, these may not be the only sites that are phosphorylated by Chk1, because Chk1 can still phosphorlyate the Ser-3/Thr-to-Ala mutant of Claspin in vitro.3 To understand the complex interaction between Claspin and Chk1 and Claspin regulation, it will be important in the future to determine the identity and function of these additional Chk1-dependent phosphorylation sites on Claspin.

Besides protein kinases, other proteins such as TopBP1 and Rad17 have also been implicated in the regulation of Claspin-Chk1 complexes and Claspin phosphorylation (24, 25). Uncovering how these proteins regulate human Claspin phosphorylation after replication stress will also contribute to our understanding of the mechanisms that control the ATR-Claspin-Chk1 pathway following DNA damage.

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