Cleavage of Claspin by Caspase-7 during Apoptosis Inhibits the Chk1 Pathway*

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Claspin is required for the phosphorylation and activation of the Chk1 protein kinase by ATR during DNA replication and in response to DNA damage. This checkpoint pathway plays a critical role in the resistance of cells to genotoxic stress. Here, we show that human Claspin is cleaved by caspase-7 during the initiation of apoptosis. In cells, induction of DNA damage by etoposide at first produced rapid phosphorylation of Chk1 at a site targeted by ATR. Subsequently, etoposide caused activation of caspase-7, cleavage of Claspin, and dephosphorylation of Chk1. In apoptotic cell extracts, Claspin was cleaved by caspase-7 at a single aspartate residue into a large N-terminal fragment and a smaller C-terminal fragment that contain different functional domains. The large N-terminal fragment was heavily phosphorylated in a human cell-free system in response to double-stranded DNA oligonucleotides, and this fragment retained Chk1 binding activity. In contrast, the smaller C-terminal fragment did not bind Chk1, but did associate with DNA and inhibited the DNA-dependent phosphorylation of Chk1 associated with its activation. These results indicate that cleavage of Claspin by caspase-7 inactivates the Chk1 signaling pathway. This mechanism may regulate the balance between cell cycle arrest and induction of apoptosis during the response to genotoxic stress.

Eukaryotic cells maintain genomic integrity by monitoring DNA for damage or incomplete replication. In the event of aberrant structures being detected, checkpoint mechanisms are activated that delay cell cycle progression and that allow the damage to be repaired or replication to be completed (1). DNA damage can also initiate pathways leading to apoptosis and the removal of a damaged cell from a tissue (2). The balance between cell cycle arrest and damage repair on one hand and the initiation of apoptosis on the other determines the survival of individual cells and the maintenance of genomic stability. Defects in the balance between these responses may lead to hypersensitivity to DNA damage or, conversely, to resistance to apoptosis and maintenance of genomic defects, which characterize cancer cells. Furthermore, the response of cells to anticancer treatments that damage DNA may determine therapeutic outcomes (3, 4).

The Chk1 protein kinase is a central component of a conserved checkpoint pathway activated by DNA damage or replication stress (5). Once activated, Chk1 inhibits Cdc25 phosphatases, which control inhibitory phosphorylation sites on cyclin-dependent protein kinases, critical regulators of cell cycle transitions (3, 6). In vertebrates, activation of Chk1 in response to DNA damage or replication arrest induced by UV light or hydroxyurea involves the ATR (ATM- and Rad3-related) kinase. ATR directly phosphorylates Chk1 at serines 317 and 345 in vitro, and phosphorylation of these sites in human cells in response to UV light and hydroxyurea is ATR-dependent (7, 8). Chk1 also undergoes autophosphorylation associated with its full activation (9). Defects in the ATR/Chk1 pathway have been implicated in the loss of genomic stability and in the development of cancer (3, 7, 10). Conversely, genetic ablation of ATR (11, 12) or Chk1 (7, 10, 13, 14) or their inhibition by kinase inhibitors (15–17) sensitizes cells to DNA damage and induces cell death, showing that this checkpoint pathway plays a critical role in determining cell survival following genotoxic stress.

Phosphorylation and activation of Chk1 by ATR require Claspin, a protein first identified by co-purification with Chk1 from Xenopus egg extracts (18) and subsequently shown to be involved in Chk1 activation in human cells (19, 20). Mrcl, an apparent homolog in yeast, is instead involved in activation of the Chk2 kinase homologs, Rad53 (Saccharomyces cerevisiae) and Cds1 (Schizosaccharomyces pombe), in response to DNA replication stress (21, 22). In vertebrates, Claspin may act as a scaffolding protein that brings together ATR and Chk1 (18, 23). Claspin interacts with chromatin during S phase, indicating that it may also act as a sensor of DNA replication (24, 25). Although Claspin has a DNA-binding motif in its N-terminal half (26), similar to Mrcl (27), it is not clear if this direct interaction with DNA occurs in cells. The association of Claspin with chromatin during S phase involves the pre-replication complex and Cdc45 (24). Claspin has also been reported to associate with ATR and the Rad9-Rad1-Hus1 complex (19) and with BRCA1 (20) in human cells.

The interaction of Claspin with Chk1 requires two phosphorylation sites in a tandem motif that lies within the Chk1-binding domain (28, 29), which interacts with the kinase domain of Chk1 (30). Phosphorylation of these two sites is ATR-dependent, but may not be directly catalyzed by ATR (20, 28, 29). The interaction of Claspin with chromatin in Xenopus egg extracts is inhibited by phosphorylation by the Xenopus Polo-like kinase Plx1, which inactivates the checkpoint as part of an adaptive response (31). In cultured human cells, depletion of Claspin by small interfering RNA increases sensitivity to genotoxic stress and promotes cell death (19, 20). Claspin may therefore be pivotal in determining the balance between cell cycle control and induction of cell death.

Here, we report that human Claspin is cleaved into two functional domains during the initiation of apoptosis by caspase-7 both in cell-free systems and in intact cells. Using a human cell-free system in which the checkpoint pathway is activated by double-stranded oligonucleotides, we show that the larger N-terminal fragment retains Chk1 binding activity, whereas the smaller C-terminal fragment associates with DNA. This smaller fragment has a dominant inhibitory effect on Chk1 phos-
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phorylation associated with its activation. In human cells, cleavage of Claspin by caspase-7 following genotoxic stress and the subsequent loss of Chk1 phosphorylation may switch the cellular response from cell cycle arrest to apoptosis.

MATERIALS AND METHODS

Claspin Proteins—Human full-length Claspin and Claspin-(679–1332) were produced as described previously (29). Other Claspin fragments were generated in pcDNA3.2-DEST (Invitrogen) by site-directed mutagenesis to create stop codons or restriction digestion at convenient restriction sites and subcloning into a pET vector (Novagen). Claspin mutagenesis to create stop codons or restriction digestion at convenient restriction sites and subcloning into a pET vector (Novagen). Claspin mutants D841A, D1031A, D1072A, D1085A, and D1158A were generated by site-directed mutagenesis. His$_6$-tagged Claspin proteins were expressed from pET vectors in reticulocyte lysate using the TNT Quick coupled transcription/translation system (Promega) and purified on nickel-nitrilotriacetic acid beads (Qiagen Inc.).

Cell Extracts—A mixture of equal volumes of HeLa nuclear and cytosolic S100 extracts was incubated for 30 min at 30 °C in the presence of 50 ng/μl (dA)$_{70}$(dT)$_{70}$ (synthesized by MWG Biotech and annealed as described previously (36)), 10 μM okadaic acid (BIOMOL Research Labs Inc.), 1 mM ATP, 10 μM creatine kinase, 5 μM creatine phosphate, and 0.02 μl of in vitro translation product/μl of extract. For each sample, 75 μg of protein was run on a 12% polyacrylamide gel and analyzed by Western blotting or autoradiography. 3′-Biotinylated (dA)$_{70}$ and 5′-biotinylated (dT)$_{70}$ (MWG Biotech) were annealed with untagged (dA)$_{70}$ and (dT)$_{70}$, respectively, as described (36) to generate 3′-biotinylated (dA)$_{70}$(dT)$_{70}$ and 5′-biotinylated (dA)$_{70}$(dT)$_{70}$. The beads were collected using the Dynal magnetic particle concentrator. Proteins were boiled off the beads into SDS loading buffer and analyzed by Western blotting with mouse monoclonal anti-Chk1 antibody or by autoradiography.

Immunodepletion of Chk1—Chk1 was phosphorylated in 600 μg of extract as described above with the addition of 2 μl of in vitro translated Claspin and then immunoprecipitated for 1 h at 4 °C using sheep anti-Chk1 or control sheep anti-Xenopus Mog1 antibody prebound in the presence of 2% (w/v) bovine serum albumin to protein G-Dynabeads (Dynal Biotech). Beads were isolated using a Dynal magnetic particle concentrator. Proteins were boiled off the beads into SDS loading buffer and analyzed by Western blotting with mouse monoclonal anti-Chk1 antibody or by autoradiography.

DNA Precipitation Assay—Chk1 was phosphorylated in 600 μg of extract as described above using either non-biotinylated or biotinylated (dA)$_{70}$(dT)$_{70}$ with the addition of 3 μl of in vitro translated Claspin (or truncated). Particulate material was removed by centrifugation for 1 min in a bench-top centrifuge, and the supernatant was incubated with 30 μl of M-280 streptavidin-Dynabeads (Dynal Biotech) for 2 h at 4 °C. Beads were collected through a 1 ml sucrose cushion (0.5 ml) by centrifugation at 6000 × g for 5 min and washed twice with 10 mM Hepes (pH 7.6), 80 mM NaCl, 0.1% Nonidet P-40, 2.5 mM EGTA, and 20 mM β-glycerophosphate. The beads were collected using the Dynal magnetic particle concentrator. Proteins were boiled off the beads into SDS loading buffer and analyzed by autoradiography.

Immunodepletion of Caspases—Approximately 1 mg of cell extract was subjected to three cycles of depletion (1 h each at 4 °C) with ~1.5 μg of specific antibody prebound to beads coated with protein A (rabbit antibodies) or protein G (goat polyclonal or mouse monoclonal antibodies). Samples were used to confirm depletions by Western blotting with antibodies raised in different species from the immunoprecipitatinig antibodies.
Cleavage of Claspin by Caspase-7

FIGURE 1. Human Claspin is cleaved by a caspase during apoptosis. A, cleavage of Claspin in a cell-free system. Caspase activation was induced in a system made from equal volumes of HeLa cytosolic (S100) and nuclear extracts by incubation with cytochrome c. B, cleavage of Claspin in a cell-free system is blocked by caspase inhibitors. HeLa extracts were incubated with cytochrome c (cycloheximide (CHX) together with Ac-DEVD-CHO as shown, D, caspase-dependent cleavage of Claspin in Jurkat cells. Cells were treated with etoposide with or without 10 μM Z-VAD-fmk for the times shown. In each experiment, samples were removed after the times shown and were analyzed by Western blotting with antibody against Claspin, PARP, caspase-7, or Chk1. C, caspase-dependent cleavage of Claspin in HeLa cells. Cells were treated with tumor necrosis factor-α (TNFα) and cycloheximide (CHX) together with Ac-DEVD-CHO as shown. D, caspase-dependent cleavage of Claspin in Jurkat cells. Cells were treated with etoposide with or without 10 μM Z-VAD-fmk for the times shown.

RESULTS

Claspin Is Cleaved by a Caspase during Apoptosis—Human Claspin has a predicted molecular mass of 151 kDa, but migrates anomalously on SDS-polyacrylamide gel with an apparent molecular mass of ~250 kDa as noted previously (26). In a human cell-free system made from a mixture of nuclear and cytoplasmic extracts of HeLa cells (29) in which caspases are activated by the addition of cytochrome c (35), Claspin was modified from the apparent 250-kDa form to a doublet with an apparent molecular mass of ~220 kDa, suggesting that it was being proteolytically cleaved (Fig. 1A). Claspin cleavage followed processing of caspase-7 (Fig. 1A), which is associated with activation of this aspartyl protease and cleavage of PARP, a substrate for caspase-7 and caspase-3 (37). Cleavage of Claspin, PARP, and caspase-7 in this system was completely blocked by the tetrapeptide caspase inhibitors Ac-DEVD-CHO and Z-VAD-fmk (Fig. 1B), confirming that cleavage of Claspin, like PARP, was due to caspase activity.

In HeLa cells in which apoptosis was induced by a combination of tumor necrosis factor-α and cycloheximide, Claspin was similarly processed from the 250-kDa form to the ~220-kDa form after 6 h of incubation, when PARP cleavage and caspase-7 activation were also apparent (Fig. 1C). An antibody raised against a peptide derived from the C-terminal region of Claspin detected a polypeptide that migrated at ~30 kDa, consistent with the generation of a smaller C-terminal fragment. As in the cell-free system, processing of Claspin, PARP, and caspase-7 was inhibited by Ac-DEVD-CHO. In addition to apoptotic cleavage, the total amount of Claspin declined substantially following treatment with tumor necrosis factor-α and cycloheximide, and this loss was not prevented by Ac-DEVD-CHO. This indicates that there is also a caspase-independent pathway for the degradation of Claspin in cells.

Claspin Cleavage Coincides with Inactivation of Checkpoint Signaling—When Jurkat cells were treated with the topoisomerase II poison etoposide, which induces DNA damage through generation of double-strand breaks, there was an initial strong activation of the Chk1 pathway. This activation was evident from the strong induction of phosphorylation of Chk1 at Ser345 (pChk1), or ATR as indicated. The migration positions of Claspin forms with apparent molecular masses of 250 kDa (p250), 220 kDa (p220), and 30 kDa (p30) are indicated by arrows. Nonspecific bands reacting with antibody are labeled with an asterisk.

Claspin Is Cleaved by a Caspase at a Single Site—Because cleavage of Claspin produced a large fragment with an apparent molecular mass of 220 kDa and a smaller C-terminal fragment of 30 kDa, a major site of cleavage is likely to reside within the C-terminal half of the protein.
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FIGURE 2. Human Claspin is cleaved by a caspase at a single site, Asp1072. A, cleavage of Claspin in apoptotic cell extracts. Claspin-(679–1332) was incubated with HeLa cytosolic extract (S100) or Xenopus egg extract (XEE) in which caspases were activated by cytochrome c. The caspase inhibitor Ac-DEVD-CHO was added as indicated. B, comparison of caspase-3/7 cleavage sites in some substrates and putative sites in Claspin-(679–1332). PKC, protein kinase C. C, cleavage of Claspin requires Asp1072. Claspin-(679–1332) proteins with single Asp residues changed to Ala were incubated in apoptotic HeLa cytosolic extract. D, Claspin is cleaved at a single site, Asp1072. Full-length Claspin or truncated proteins containing the amino acids indicated were incubated in apoptotic HeLa cytosolic extract with or without Ac-DEVD-CHO. E, comparison of amino acid sequences of Claspin from different species with amino acids 1063–1085 of human Claspin. Conserved amino acids are shaded; the putative caspase recognition motif is underlined; and the caspase cleavage site in human Claspin is indicated by an arrow. In the experiments shown in A, C, and D, in vitro translated 35S-labeled Claspin proteins were incubated in extracts at 30°C for 9 h with the addition of cytochrome c to activate caspases. Samples were analyzed by SDS-PAGE and autoradiography. The migration positions of molecular mass markers (in kilodaltons) are shown on the left.

Indeed, in vitro transcribed and translated human Claspin-(679–1332), which migrated on SDS-polyacrylamide gel at 120 kDa, was cleaved to produce one fragment with an apparent molecular mass of 75 kDa and a second of 30 kDa in apoptotic extracts of HeLa cells (S100) and Xenopus eggs. In both extracts, Claspin-(679–1332) cleavage was completely inhibited by Ac-DEVD-CHO (Fig. 2A). Examination of the amino acid sequence of Claspin-(679–1332) revealed five DXXD motifs that might be recognized by the effectors caspase-3 and caspase-7, which are inhibited by Ac-DEVD-CHO. Similar motifs are present in other substrates for these caspases (Fig. 2B). In these substrates, cleavage occurs after the second Asp residue, which is essential for recognition by the caspase. We therefore mutated the five Asp residues in Claspin-(679–1332) at which cleavage might occur to Ala and found that mutation of only Asp1072 prevented caspase cleavage (Fig. 2C). Comparison of Claspin-(679–1332) with full-length Claspin and Claspin-(1–1072) demonstrated that Asp1072 was the only site at which cleavage occurred. Indeed, the fragments predicted to be generated by caspase cleavage of the full-length protein, Claspin-(1–1072) and Claspin-(1073–1332), were stable against caspase activity in apoptotic cell extracts (Fig. 2D).

The doublet formed during cleavage of the endogenous protein in cell extracts (Fig. 1A) may therefore be due to another post-translational modification of a fraction of Claspin molecules. Comparison of the amino acid sequences of Claspin from different species indicated that the site of cleavage in human Claspin is conserved in mouse, but not in Xenopus, although a number of Asp residues are present within this region of the Xenopus protein. In Drosophila Claspin, there is a DEYD motif within this region of the protein that is a potential caspase cleavage site (Fig. 2E).

Claspin Is Cleaved Specifically by Caspase-7—Cleavage of human Claspin after a DEYD motif suggests that it is a substrate for caspase-3 or caspase-7, both of which show a strong preference for sites with aspartate at the P4 position and glutamate at the P3 position (39). To determine whether one or the other of these caspases is required for Claspin cleavage, we depleted them from HeLa cell extracts using specific antibodies (Fig. 3A). Depletion of caspase-3 only slightly reduced cleavage of Claspin-(679–1332) in response to cytochrome c, whereas depletion of caspase-7 strongly inhibited cleavage. Depletion of caspase-9, which is required for activation of caspase-3 and caspase-7 in response to cytochrome c (40), completely inhibited Claspin-(679–1332) cleavage. In contrast, depletion of caspase-8 had no effect on Claspin-(679–1332) cleavage. This indicates that Claspin is cleaved at Asp1072 predominantly by caspase-7 downstream of caspase-9. Claspin-(679–1332) was indeed cleaved into two fragments by active caspase-7 (Fig. 3B), and this processing was completely inhibited by mutation of Asp1072 to Ala (D1072A). Active caspase-3 was also capable of cleaving Claspin directly at this site (data not shown), although caspase-3 was clearly less important than caspase-7 in cell extracts (Fig. 3A).

Cleavage of Claspin Does Not Prevent Its Phosphorylation in a Human Cell-free System—Claspin is phosphorylated in response to DNA damage or replication arrest, and this controls the interaction of Claspin...
with other proteins involved in the checkpoint signaling pathway. Phosphorylation of two sites in human Claspin, Thr^{916} and Ser^{945}, forms a Chk1-binding motif that is conserved with respect to *Xenopus* Claspin (28, 29). Cleavage of Claspin by caspase-7 could potentially regulate the phosphorylation of Claspin and thereby affect its function. We therefore examined the phosphorylation of Claspin-(679–1332) and the fragments generated by caspase-7 cleavage, Claspin-(679–1072) and Claspin-(1073–1332), in response to activation of the checkpoint pathway in a human cell-free system (29) in which the checkpoint pathway is activated by an oligonucleotide duplex made of annealed (dA)_{70} and (dT)_{70} together with the protein-serine/threonine phosphatase inhibitor okadaic acid. Like Claspin-(679–1332), Claspin-(679–1072) was phosphorylated at multiple sites in the presence of okadaic acid, and phosphorylation was enhanced by (dA)_{70}(dT)_{70} showing that the majority of phosphorylation sites within the C-terminal region of Claspin reside in fragment 679–1072 and that this phosphorylation does not appear to be affected by removal of the C-terminal 260 amino acids. Phosphorylation of Claspin-(679–1072) and Claspin-(679–1332) was partially inhibited by caffeine, which inhibits ATR (15, 41), and was strongly but not completely blocked by staurosporine and Ro 31-8220, which inhibit several protein kinases (42). Claspin-(1073–1332) was only partially up-shifted to a single band in response to okadaic acid/(dA)_{70}(dT)_{70} but this result nevertheless indicates that at least one previously unidentified phosphorylation site exists with the C-terminal 260 amino acids.

**Cleavage of Claspin Separates Its Chk1-binding Domain from a DNA-interacting Domain**—To test whether Claspin cleavage at Asp^{1072} affects the interaction with Chk1, we determined the interaction between Chk1 and fragments of Claspin in human cell extracts. As found previously (29), the non-phosphorylated form of Claspin is a sticky protein that precipitates nonspecifically with beads, and Claspin-(679–1332) and Claspin-(679–1072) showed similar characteristics (Fig. 4B). However, in the presence of (dA)_{70}(dT)_{70} and okadaic acid, phosphorylated Claspin-(679–1332) and Claspin-(679–1072) precipi-
tated specifically with Chk1, whereas Claspin-(1073–1332) did not. This result is consistent with separation by caspase-7 cleavage of the C-terminal 260 amino acids from the Chk1-binding motif in residues 679–1072, which contain phosphorylation sites at Thr916 and Ser945.

To examine the possible interaction between Claspin and the double-stranded oligonucleotide, we used (dA)70/(dT)70 duplexes labeled with a biotin group at either the 5'-end of the (dT)70 strand or the 3'-end of the (dA)70 strand. We found that the 3'-biotinylated duplex was less effective than either the 5'-biotinylated or non-biotinylated duplex in inducing phosphorylation of Claspin-(679–1332) in cell extracts. Full-length Claspin was precipitated from extracts when the 5'-biotinylated duplex was retrieved on streptavidin-coupled beads, but was not precipitated from extracts incubated with the non-biotinylated duplex (Fig. 5A). Removal of residues 1073–1332 completely abolished the interaction of Claspin with Chk1.

FIGURE 4. Phosphorylation of Claspin and interaction with Chk1. A, phosphorylation of Claspin fragments in a human cell-free system. In vitro translated [35S-labeled Claspin-(679–1332), Claspin-(679–1072), and Claspin-(1073–1332) (C-terminal fragment (CTF)) were incubated for 30 min at 30 °C in a mixture of HeLa cytosolic (S100) and nuclear extracts supplemented with 50 ng/μl (dA)70/(dT)70 (poly(dA/dT)70), 10 μM okadaic acid (OA), 5 mM caffeine (caff), 5 μM staurosporine (stau), or 20 μM Ro 31-8220 (Ro) as indicated. Samples were analyzed by SDS-PAGE and autoradiography. B, interaction of Chk1 with phosphorylated Claspin. In vitro translated [35S-labeled Claspin-(679–1332), Claspin-(679–1072), and Claspin-(1073–1332) were incubated for 30 min at 30 °C in a mixture of HeLa cytosolic (S100) and nuclear extracts supplemented with 50 ng/μl (dA)70/(dT)70 and 10 μM okadaic acid as indicated. Immunoprecipitations with anti-Chk1 (Chk1 IP) or control sheep (Control IP) antibody were carried out, and bound proteins were analyzed by blotting for Chk1 with mouse monoclonal anti-Chk1 antibody or by autoradiography for Claspin proteins.
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Claspin with the 5′-biotinylated duplex, whereas Claspin-(1073–1332) did interact (Fig. 5B). This demonstrates that the interaction of Claspin with (dA)₇₀(dT)₇₀ requires the C-terminal 260 amino acids that are separated from the Chk1-binding domain through cleavage by caspase-7. To test whether this C-terminal DNA-interacting domain might have a dominant effect on downstream signaling to Chk1, we added recombinant Claspin-(1073–1332) to extracts and found that there was a dose-dependent inhibition of Chk1 phosphorylation detected by mobility on SDS-polyacrylamide gel and specifically at Ser⁴⁴⁵, a site targeted by ATR, using a site-specific antibody (Fig. 5C). Thus, cleavage of Claspin by caspase-7 not only separates the Chk1- and DNA-binding domains of Claspin, but also generates a competitive inhibitor of Chk1 phosphorylation.

DISCUSSION

Claspin plays a critical role in activation of the Chk1 protein kinase in response to incomplete DNA replication and DNA damage. In this study, we have shown that, during apoptosis, Claspin is targeted for cleavage by caspase-7 after a single residue, Asp¹⁰⁷². Using a human cell-free system in which the Chk1 pathway is activated by double-stranded oligonucleotides, we have shown that this cleavage separates the Chk1-binding motif in Claspin from a C-terminal domain that is required for interaction with DNA (Fig. 6A). The released C-terminal fragment prevents the phosphorylation of Chk1 associated with its activation and thereby blocks checkpoint signaling through the Chk1 pathway.

Inactivation of Claspin by caspase-7-mediated cleavage may play an important role in switching the response of a cell from cell cycle arrest, an initial response that provides an opportunity for damage repair and cell survival, to induction of cell death following prolonged arrest of DNA replication or the persistence of DNA damage. In this model (Fig. 6B), activation of ATR and Chk1 is an acute response to DNA damage or ongoing replication that provides a protective mechanism for the cell by arresting cell cycle progression and by permitting DNA damage to be repaired or DNA replication to be completed. A survival role of the ATR/Claspin/Chk1 pathway is supported by the sensitization of cells lacking one of these proteins to DNA damage (11, 12, 14, 20). Although this role might be an indirect one, due to functions in restraining cell cycle progression and perhaps initiating damage repair, it remains possible that the ATR/Claspin/Chk1 pathway also controls apoptosis more directly. With prolonged arrest, additional responses, in part through transcription controlled by activation of p53 (43), induce apoptotic pathways that cause activation of caspase-7, resulting in cleavage of Claspin and inactivation of the checkpoint. This may relieve a direct inhibitory effect on apoptosis or cause unrestrained progression through a critical cell cycle transition and indirect activation of apoptosis through mechanisms that detect the presence of genomic damage during such transitions or in a subsequent cell cycle phase.

The specific role for caspase-7 in the cleavage of Claspin is unusual. Caspase-7 and the closely related enzyme caspase-3 have very similar primary substrate recognition requirements, both targeting DXD motifs (39). Caspase-3 is the predominant activity in most cells toward substrates containing such motifs (44), although caspase-7 can compensate for the loss of caspase-3 to some extent, e.g. in MCF-7 cells (45) and in caspase-3⁻/⁻ mouse cells (46, 47). A distinct biological role for caspase-7 is indicated by the apparent lethality of gene knockout in mice (48). Caspase-7 has been reported to cleave PARP (37), but cleavage of caspase-3 can also perform this function (49). In the case of Claspin, although purified caspase-7 and caspase-3 can both cleave the protein at the DEYD¹⁰⁷² motif (Fig. 3 and data not shown), when cleavage is analyzed under near physiological conditions in concentrated cell extracts, caspase-7 is clearly the predominant activity.

A distinct, early role for caspase-7 in DNA damage-induced apoptosis has been indicated by experiments in which deletion of caspase-7 in chicken DT40 cells delayed activation of caspase-3 and caspase-6 after treatment with etoposide (49). Interestingly, active caspase-7 has been reported to be present in the nucleus (37, 50), where it could act on...
nuclear substrates such as Claspin. Furthermore, experiments in mouse cells lacking the caspase-9 activator Apaf-1 have suggested that caspase-7 can be activated by a mechanism distinct from the mitochondrial Apaf-1/caspase-9/caspase-3 pathway. Such a mechanism activating caspase-7 in the nucleus in response to DNA damage or DNA replication arrest might be particularly important in the initial induction of Claspin cleavage and inactivation of the Chk1 pathway. With further, prolonged DNA damage or DNA replication arrest, activation of caspase-7 results in proteolytic cleavage of Claspin, dephosphorylation of Chk1, inactivation of the checkpoint signal, and promotion of apoptosis. Activated forms of Chk1 and ATR are indicated by asterisks.

Our results indicate that cleavage of Claspin by caspase-7 effectively inactivates the protein by separating the Chk1-binding domain from a C-terminal domain that is required for interaction with DNA and the phosphorylation and activation of Chk1 by ATR. A number of other proteins involved in responses to DNA damage, including PARP, ATM, and DNA-dependent protein kinase, are also inactivated by caspase cleavage during apoptosis by separation of functional domains. Previous work has identified a putative DNA-binding motif in the N-terminal half of Claspin that is conserved in yeast Mrc1, although another, weaker, direct interaction with DNA through the C-terminal region was also found. Our results indicate that a region contained within the C-terminal 260 amino acids of human Claspin is essential for interaction with DNA in a cell-free system in which the checkpoint pathway to Chk1 is functional. This interaction may well involve interaction with other proteins rather than direct binding to DNA. Surprisingly, such a stable interaction with double-stranded oligonucleotides was not observed in Xenopus egg extracts by Kumagai et al. (23), possibly because of a more dynamic interaction of Claspin with DNA molecules in that system. The separation of the C-terminal domain from the Chk1-binding domain by caspase-7 creates a dominant inhibitor of the pathway that prevents Chk1 phosphorylation associated with its activation, probably due to the inability to recruit Chk1 to ATR. However, phosphorylation of Claspin itself does not appear to be affected by the inability to interact stably with DNA, suggesting that the relevant kinases do not require assembly of Claspin into a DNA-interacting complex. Our results also indicate that the C-terminal domain contains at least one phosphorylation site that could regulate the interaction of this domain with DNA or other proteins.

In summary, we have identified human Claspin as a substrate for caspase-7, supporting a distinct role for this caspase in the control of early events during genotoxic responses. Inactivation of Claspin by caspase-7 may play a pivotal role in the switch from cell cycle arrest and restraint of apoptosis to inactivation of the checkpoint and induction of apoptosis in response to genotoxic damage. The control of Claspin function through caspase-7-mediated cleavage may play a role in determining the response of cancer cells to genotoxic therapies.
