The hCds1 (Chk2)-FHA Domain Is Essential for a Chain of Phosphorylation Events on hCds1 That Is Induced by Ionizing Radiation*

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hCds1 (Chk2) (1–4) is an evolutionarily conserved kinase that functions in DNA damage response and cell cycle checkpoint. The Cds1 family of kinases are activated by a family of large phosphatidylinositol 3-kinase-like kinases. In humans, ataxia telangiectasia-mutated (ATM) (5) and ataxia-telangiectasia and Rad3-related (6) kinases activate hCds1 by phosphorylating Thr69 (7–9). hCds1 and Cds1-related kinases contain the FHA (forkhead-associated) domain (10), which appears to be important for integrating the DNA damage signal. It is not known how ATM phosphorylation activates hCds1 function and whether the phosphorylation is linked to the FHA. Here, we demonstrate that the hCds1-FHA domain is essential for Thr69 phosphorylation. Thr69 phosphorylation, in turn, is required for ionizing radiation-induced autophosphorylation of two amino acid residues in hCds1, Thr386 and Thr387. These two amino acid residues, located in the activation loop of hCds1, are conserved in hCds1-related kinases and are essential for hCds1 activity. Thus, the hCds1-FHA domain mediates a chain of phosphorylation events on hCds1, which includes phosphorylation by ATM and hCds1 autophosphorylation, in response to DNA damage.

DNA damage activates a signal cascade that causes a cell cycle arrest to allow cells to repair damage before progressing further in the cell cycle. In eukaryotes, disruption of this signal cascade can lead to genomic instability, which in mammals can lead to cancer (11). Therefore, the DNA damage signal cascade and the proteins participating in this signal cascade are immensely important to understanding cancer and to finding effective treatments for cancer. Two groups of kinases play a key role in the DNA damage signal cascade: phosphatidylinositol 3-kinase-like kinases ataxia telangiectasia-mutated (ATM) (5) and ataxia-telangiectasia and Rad3-related (ATR) (6) kinases and their substrates, hCds1(1–4) and Chk1 (12). hCds1 phosphorylates and regulates tumor suppressors BRCA1 (13) and p53 (14–16). Chk1 phosphorylates Cdc25C (12, 17), which inhibits the ability of Cdc25C to activate Cdc2.

In addition to being a regulator of tumor suppressors, hCds1 may be a tumor suppressor itself. hCds1 is mutated in a variant form of Li-Fraumeni syndrome, a highly penetrant familial cancer syndrome that is more commonly associated with inherited mutations in the p53 gene (18). Being linked with a syndrome classically associated with p53 mutation may be an indication that hCds1 is a tumor suppressor that functions in the same pathway as p53. This notion is supported by the observation that hCds1 phosphorylates Ser392 of TP53 and stabilizes it in response to DNA damage (14–16). If hCds1 is indeed a tumor suppressor, it is surprising that the hCds1 mutation is so rare in cancers (one heterozygotic mutation in 49 cancers studied) (18). One explanation may be that the high frequency of the p53 mutation or destabilization may obviate the need for the hCds1 mutation.

Activation of hCds1 in response to ionizing radiation (IR) requires phosphorylation of Thr69 of hCds1 by ATM (7–9). But, activation of hCds1 and phosphorylation of Thr69 in response to replication block does not require ATM (3, 4, 9). This suggests that ATR, which responds to replication block (19, 20), may phosphorylate Thr69 in response to replication block. It is not known how hCds1 is activated by phosphorylation of its Thr69.

hCds1 and hCds1-related kinases contain a domain called the FHA (forkhead-associated) domain. The FHA domain is a phospho-specific protein-protein interaction motif (21, 22). In Rad53, the Cds1-homologue in budding yeast, the FHA domains integrate the DNA damage signal by coupling with phosphorylated Rad9. The FHA domain is found in proteins of both prokaryotes and eukaryotes (10). Most of FHA-containing proteins in eukaryotes are nuclear, and many have been linked to the regulation of DNA damage repair, cell cycle, and transcription. In addition to hCds1 and hCds1-related kinases, other well known FHA-containing proteins include the DNA repair protein Nbs1, which is mutated in the ataxia-telangiectasia-like syndrome called the Nijmegan breakage syndrome (23) and Chfr1 (24), a mitotic checkpoint protein that is mutated in many cancers.

At least in Rad53, it is clear that the FHA domain plays a crucial role in responding to the DNA damage signal, but it is not known how the signal transmitted through the FHA domain activates Rad53 or other FHA domain-containing proteins. It is also not known how the phosphorylation of Thr69 by ATM or ATR activates hCds1 and whether Thr69 phosphorylation is linked to the FHA domain. In this report, we investigated the mechanistic role of the FHA domain and Thr69 phosphorylation in transmitting the DNA damage signal to hCds1. Our results indicate that the hCds1-FHA domain is essential for initiating a chain of phosphorylation events on hCds1 that activate hCds1 in response to DNA...
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The FHA domain is essential for Thr<sup>68</sup> phosphorylation in response to DNA damage. Thr<sup>68</sup> phosphorylation, in turn, is necessary for autophosphorylation of two amino acid residues in the activation loop of hCds1 that are essential for its activity, Thr<sup>383</sup> and Thr<sup>387</sup>.

**MATERIALS AND METHODS**

**Plasmid Constructs**—The mammalian expression vector for hCds1 (V5-hCds1) was made by inserting hCds1 cDNA EcoRI/PvuI fragment (nucleotides −27 to +1689) into EcoRI/NsiI sites of pcDNA6A (Invitrogen). The kinase-dead version of hCds1 construct was made by substituting the hCds1 cDNA fragment (−27 to +1441) with a fragment containing the K249R mutation. Site-specific mutagenesis of the other mutations was performed using the QuickChange mutagenesis kit (Stratagene). The oligonucleotides used have the following sequences with the lowercase letters indicating mutations, 5′-CATACGCAAGAAACTTTTGGGATGAGCAAGACCTACTTGG-3′ (CL61) for T383A, 5′-GAGACCGATGTGGAGCtCCCACCTACTTGG-3′ (CL78) for R145W, 5′-CATACAGCAAGAAACCCTTTTGG-3′ (CL76) for T68A, and 5′-CATACAGCAAGAAACTTTTGG-3′ (CL78) for R145W, 5′-CATACAGCAAGAAACCCTTTTGG-3′ (CL76) for T68A. The mammalian expression vector for hCds1 (V5-hCds1) was made by inserting hCds1 cDNA into pcDNA6A (Invitrogen) with the anti-V5 antibody. The kinase activity of recombinant GST-hCds1 purified from S. pombe was tested using GST-Cdc25C as substrate. The total amount of GST-Cdc25C substrate is shown in the bottom panel.

**Immunoprecipitation Kinase Assay**—293T cells were transfected with hCds1 expression constructs using the Effectene transfection reagent (Qiagen). The cell lysates were prepared 1–2 days after transfection as described previously (3). V5-tagged hCds1 was immunoprecipitated with 0.2 μl of anti-V5 antibody (Invitrogen) and 5 μl of protein A-agarose (Life Technologies, Inc.) in 150 μl of total volume for 1 h at 4 °C after preclearing the lysate with 5 μl of protein A-agarose. After washing beads with the lysis buffer (3) four times and with the kinase buffer (10 mM HEPES, pH 7.5, 75 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 1 mM dithiothreitol, 10 μM ATP, and 1 μCi of [γ-<sup>32</sup>P]ATP (Amersham Pharmacia Biotech; AA0068) with 2 μg of GST-Cdc25C (200–256) (25) as substrate.

**Phosphopeptide-specific Antibody and Immunoblotting—Phosphopeptides (Thr(P))<sup>68</sup>: CGTLSSLETVSpTQELY; Thr(P)<sup>383</sup>: CGETSLM-...
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**RESULTS AND DISCUSSION**

The FHA Domain Is Required for hCds1 Phosphorylation by ATM—We examined the role of the hCds1-FHA domain on hCds1 activity by investigating naturally occurring FHA domain mutations (Fig. 1). Two hCds1-FHA mutations have been described: Arg^{145} → Trp (R145W) in colon cancer cell line HCT15 and Ile^{157} → Thr (I157T) in the Li-Fraumeni syndrome-variant MGH005 (18). As shown in Fig. 2A, the R145W mutation abolished the IR-induced activity of transiently expressed hCds1. The I157T mutation did not significantly affect hCds1 activity (data not shown). The R145W mutation may inhibit hCds1 activity either by directly decreasing the kinase activity of hCds1 or by disrupting the transmission of the activating signal to hCds1. We found that the R145W mutation decreased the hCds1 activity either by directly decreasing the kinase activity of hCds1 by less than 50%, suggesting that the FHA domain is more likely to function in responding to ATM and ATR (Fig. 3).

**Entrez accession numbers of these proteins are as follows:** hCds1, NP009125; SpCds1, Q09170; ScRad53, NP015172; Cd2, NP001789; Camk1, NP003647; MAPK, P28482; protein kinase A, NP002721; Chk1, NP001265; and Ck1, NP001883.
FHA domain (amino acids 114–195) is important for Thr68 phosphorylation by ATM, we examined Thr68 phosphorylation after IR in the R145W mutant. Consistent with previous observations (8, 9), Thr68 phosphorylation was important for hCds1 activity (Fig. 2C). Interestingly, Thr68 phosphorylation was abolished in the R145W mutant (Fig. 2D), suggesting that the FHA domain is essential for ATM phosphorylation of hCds1 in response to IR. Deletions (21 and 42 amino acids) of the FHA domain that span Arg145 had similar effects as the R145W mutation (data not shown). Because a stable interaction between ATM and hCds1 was not seen, we have not been able to determine whether the FHA domain mediates Thr68 phosphorylation by directly recruiting ATM to hCds1.

Phosphorylation by ATM Is Required for Phosphorylation of hCds1 Activation Loop—Some kinases are activated by phosphorylation of residues in the activation loop of the kinase, and hCds1 may be one of these. According to structural analyses, kinases that are activated by phosphorylation of the activation loop have Thr/Tyr in the position corresponding to Thr383 of hCds1 (reviewed in Ref. 27). To determine whether Thr383 and Thr387 of hCds1 are phosphorylated in response to IR, we performed experiments with GST-hCds1 (wild type, kinase-dead, T383A, and T387A) using GST-Cdc25C as substrate. The total level of GST-hCds1 was measured by immunoblotting with V5 antibody (right panel). B, Thr383 and Thr387 are phosphorylated in bacteria. The phosphorylation status of Thr383 and Thr387 in bacterially expressed GST-hCds1 (wild type, kinase-dead, T383A, and T387A) was determined by immunoblotting with the respective phosphospecific antibody. The total level of GST-hCds1 as determined by immunoblotting with anti-hCds1 antibody is shown in the bottom panels. C, Thr383 and Thr387 are essential for the intrinsic kinase activity of hCds1. In vitro kinase reactions were performed with GST-hCds1 (wild type, kinase-dead, T383A, and T387A) using GST-Cdc25C as substrate. The level of 32P incorporation is shown in the left panel, and the Coomassie staining of the same gel is shown in the right panel. The bands for GST-hCds1 and GST-Cdc25C are marked with arrows. D, schematic diagram of the order of phosphorylation events on hCds1.

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Thr383 mutation also abolished the ability of hCds1 to trigger G1 checkpoint. Unfortunately, we were not able to perform this study for hCds1 with the R145W mutation because it was expressed very poorly in U2OS cells.

To determine whether the FHA domain and ATM phosphorylation of Thr68 are important for phosphorylation of Thr383 and Thr387, we examined the phosphorylation status of Thr383 and Thr387 in R145W and T68A mutant proteins. The FHA domain and Thr68 were found to be important for phosphorylation of Thr383 and Thr387 because Thr383 and Thr387 were not phosphorylated in the R145W or the T68A mutant after IR (Fig. 5A). Consistent with this, phosphorylation of Thr383 and Thr387 after IR did not occur in ATM-mutated cells (Fig. 5B). On the other hand, phosphorylation of Thr387 did not require Thr383 (Fig. 5C) or Thr387 (data not shown). This suggests that ATM phosphorylation of Thr68 is required for the subsequent phosphorylation of Thr383 and Thr387 in vivo.

The Activation Loop of hCds1 Kinase Is Autophosphorylated—Because phosphatidylinositol 3-kinase-like kinases such as ATM and ATR phosphorylate Ser/Thr followed by a Gln residue (31, 32), Thr383 and Thr387 of hCds1 are most likely not phosphorylated by ATM or ATR. Another possibility is that they are autophosphorylated. Many kinases, including protein kinase A, are activated by autophosphorylation in the activation loop (reviewed in Ref. 27). To determine whether Thr383 and Thr387 of hCds1 are autophosphorylated, we examined the phosphorylation status of these two residues in transiently expressed kinase-dead hCds1 after IR. As shown in Fig. 6A, Thr383 and Thr387 were not phosphorylated in kinase-dead hCds1. Furthermore, Thr383 and Thr387 were found to be phosphorylated in bacterially expressed hCds1 but not kinase-dead hCds1 or mutant hCds1 (T383A or T387A) (Fig. 6B), suggesting that hCds1 autophosphorylates the activation loop residues. To determine whether Thr383 and Thr387 were essential for the intrinsic kinase activity of hCds1, we compared the kinase activities of recombinant wild type hCds1 and T383A and T387A mutant proteins. As shown in Fig. 6C, there was no detectable kinase activity in T383A and T387A mutants, suggesting that autophosphorylation of these two threonine residues may be essential for the kinase activity of hCds1.

The work described in this report indicates that the FHA domain plays a central role in initiating a chain of phosphorylation events.
phosphorylation step that is dependent on the FHA domain occurs at Thr68 by ATM kinase, which is activated by double-stranded DNA breaks. The exact role that the FHA domain plays in ATM phosphorylation of hCds1 in response to DNA damage (Fig. 6). Phosphorylation of hCds1 is not known. Phosphorylation of Thr68 causes autophosphorylation of hCds1 in vivo, which appears to be necessary for its activity. How Thr68 phosphorylation causes autophosphorylation of hCds1 is currently under investigation. Based on the findings presented here, we speculate that the tumors that carry an FHA domain mutation (e.g. HCT15) may have risen partly as a result of the failure to initiate the chain of hCds1 phosphorylation events described here. As a consequence, the hCds1-dependent processes such as DNA damage response, checkpoint, and apoptosis may have been compromised during tumor formation.

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Note Added in Proof—While this manuscript was under review, Wu et al. (33) also reported that the R145W mutation of the FHA domain inhibited Thr68 phosphorylation.

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