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第３次対がん総合戦略研究事業

ヒトパピローマウイルスを標的とする発がん予防の研究

平成２２年度～２５年度　総合研究報告書

Ⅲ　研究成果の刊行物・別冊
その１

研究代表者　温川　恭至

平成２６（２０１４）年　５月

Ⅲ　研究成果の刊行物・別冊

その１

平成２２年度

CDC6 interaction with ATR regulates activation of a replication checkpoint in higher eukaryotic cells

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Summary

CDC6, a replication licensing protein, is partially exported to the cytoplasm in human cells through phosphorylation by Cdk during S phase, but a significant proportion remains in the nucleus. We report here that human CDC6 physically interacts with ATR, a crucial checkpoint kinase, in a manner that is stimulated by phosphorylation by Cdk. CDC6 silencing by siRNAs affected ATR-dependent inhibition of mitotic entry elicited by modest replication stress. Whereas a Cdk-phosphorylation-mimicking CDC6 mutant could rescue the checkpoint defect by CDC6 silencing, a phosphorylation-deficient mutant could not. Furthermore, we found that the CDC6-ATR interaction is conserved in *Xenopus*. We show that the presence of *Xenopus* CDC6 during S phase is essential for *Xenopus* ATR to bind to chromatin in response to replication inhibition. In addition, when human CDC6 amino acid fragment 180-220, which can bind to both human and *Xenopus* ATR, was added to *Xenopus* egg extracts after assembly of the pre-replication complex, *Xenopus* Chk1 phosphorylation was significantly reduced without lowering replication, probably through a sequestration of CDC6-mediated ATR-chromatin interaction. Thus, CDC6 might regulate replication-checkpoint activation through the interaction with ATR in higher eukaryotic cells.

Key words: CDC6, ATR, Cdk phosphorylation, Replication checkpoint, Higher eukaryotic cells

Introduction

In eukaryotic cells, chromosomal DNA replication is tightly regulated during the cell cycle and closely coordinated with other cell-cycle events, such as mitosis and DNA-damage response. The periodic assembly and disassembly of pre-replication complexes (pre-RCs) at replication origins is a central mechanism that ensures that there is only one DNA replication per single cell cycle (Bell and Dutta, 2002; Diffley, 2004; Fujita, 2006). During the period of low cyclin-dependent kinase (Cdk) from late mitosis through G1 phase, the origin recognition complex (ORC), CDC6 and Cdt1 load a presumptive replicative helicase, the MCM2-7 complex, onto chromatin to assemble the pre-RC. During the S, G2 and M phases, inappropriate reassembly of the pre-RC and subsequent re-replication are strictly prohibited by redundant suppression mechanisms against multiple MCM loaders. In human cells, Cdt1 activity is very tightly restricted by multiple pathways (Fujita, 2006). Human ORC1 is degraded through ubiquitylation by SCF^{Skp2} (Méndez et al., 2002). CDC6 is a target of Cdk and, although this leads to degradation in yeast (Drury et al., 1997; Jallepalli et al., 1997; Jallepalli et al., 1998; Kominami and Tada, 1997), the outcome in human cells is nuclear export (Fujita et al., 1999; Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998). Whereas phosphorylation of human CDC6 by Cdk negatively regulates its function in pre-RC formation (Sugimoto et al., 2009), it is noteworthy that a significant proportion of CDC6 proteins remains in the nuclei during S phase (Fujita et al., 1999), suggesting a potential CDC6 function(s) other than pre-RC assembly.

When chromosomal DNA is damaged, for example by DNA double-strand breaks (DSBs) and replication stress, cells activate DNA-damage response cascades to halt the cell cycle, repair the damage and, if necessary, induce apoptosis (Bartek and Lukas, 2003; Burrows and Elledge, 2008; Kastan and Bartek, 2004; Nyberg et al., 2002). Ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) kinases are phosphatidylinositol-3-kinase-related kinases and are major upstream kinases that signal several downstream pathways by phosphorylating many effector molecules, including the Chk1 and Chk2 kinases. The activated Chk1 and Chk2 eventually delay cell-cycle progression to facilitate DNA repair, mainly through inhibiting CDC25 phosphatases required for Cdk activation (Bartek and Lukas, 2003). ATM primarily responds to DSBs, and mainly phosphorylates and activates Chk2. Several other checkpoint factors co-function with ATM. For example, The Mre11-Rad50-Nbs1 complex is a sensor for DSBs, substrate for ATM and regulator of ATM activity (Lee and Paull, 2005).

ATR primarily responds to replication stress and mainly phosphorylates and activates Chk1 (Burrows and Elledge, 2008). A current model for ATR recruitment and activation at damaged sites is as follows: ATR forms a stable complex with the ATRIP (ATR-interacting protein; Cortez et al., 2001). When DNA replication is impeded, extensive single-stranded DNA (ssDNA) is generated through discordance between DNA polymerases and the MCM helicase, and then coated by replication protein A (RPA), which in turn recruits ATR through ATRIP binding (Namiki and Zou, 2006; Zou and Elledge, 2003). However, the need for the final step has been challenged by the recent identification of ATRIP mutants that are deficient in RPA binding and, thus, do not support stable ATR retention at damaged sites but still promote Chk1 phosphorylation (Ball et al., 2005; Kim et al., 2005). Also in this pathway, several crucial cofactors are required for optimal activation of ATR-Chk1. The Rad9-Rad1-Hus1 complex is a PCNA-like clamp loaded by Rad17-RFC onto damaged sites (Burrows and Elledge, 2008). TopBP1 promotes ATR kinase activity (Hashimoto et al., 2006; Kumagai et al., 2006), through interactions with Rad9-Rad1-Hus1 and ATR-ATRIP (Mordes et al., 2008). Claspin is a mediator that facilitates Chk1 phosphorylation by ATR (Lee et al., 2003).

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However, the mechanisms for ATR recruitment and activation at damaged sites have yet to be completely elucidated.

Several studies in higher eukaryotic cells have implicated the CDC6 proteins in replication-checkpoint activation. In human cells, overexpression of CDC6 in G2 cells blocks mitotic entry by activating Chk1 (Clay-Farrace et al., 2003). In addition, Lau et al. showed that, despite CDC6 depletion in S-phase cells slowing replication, it does not activate Chk1 and leads to cells that prematurely enter mitosis with DNA synthesis (Lau et al., 2006). In an in vitro DNA-replication system with *Xenopus* egg extracts, *Xenopus* CDC6 (XCDC6) was shown to be required for XChk1 activation induced by replication inhibition (Oehlmann et al., 2004). Nevertheless, the molecular mechanism by which CDC6 contributes to replication-checkpoint activation remains unknown. Here, we show that human CDC6 physically interacts with ATR in a Cdk-phosphorylation-stimulated manner and that this interaction is required for proper activation of the replication checkpoint induced by modest replication stress. Further experiments suggested that a similar mechanism might also operate in *Xenopus* cells. In fission yeast, Cdc18 (CDC6) physically interacts with Rad3-Rad26 (ATR-ATRIP) via Rad26 to sustain the replication-checkpoint activation (Hermand and Nurse, 2007). Thus, involvement of CDC6/Cdc18 interaction with ATR-ATRIP in a replication checkpoint might be a conserved feature throughout eukaryotes.

Results

Human CDC6 physically interacts with ATR in a Cdk-phosphorylation-stimulated manner

We investigated whether human CDC6 physical interacts with certain checkpoint protein(s) by GST-CDC6 pulldown assays with HeLa cell nuclear extracts followed by immunoblotting. ATM and ATR, but not Chk1, Chk2, TopBP1, claspin, Nbs1 or Mre11, were pulled down specifically with GST-CDC6 (Fig. 1AII). Rad9-Hus1-Rad1 proteins also did not bind to GST-CDC6 (data not shown). In these assays, cyclin A and Cdh1, proteins that bind to CDC6 (Mailand and Diffley, 2005; Petersen et al., 1999), served as controls. To confirm whether endogenous proteins are associated with CDC6 in vivo, HeLa cell nuclear extracts were immunoprecipitated with an anti-CDC6 antibody. As shown in Fig. 1B, ATR co-precipitated with CDC6. Thus far, we have been unable to detect co-immunoprecipitation between endogenous CDC6 and ATM. Therefore, the CDC6-ATR interaction is considered to be physiologically important.

We then mapped the interaction domain(s) of CDC6 with ATR (Fig. 1C,D). GST-CDC6 and the truncated derivatives studied were mixed with HeLa cell nuclear extracts and bound proteins were analyzed. ATR was found to bind to CDC6 fragments (amino acids) 89-268 and 180-268, but the binding was significantly weaker with fragment 1-180 (Fig. 1D). Binding studies to further narrow the ATR-binding domain found that CDC6 180-220, which contains a Walker A motif of the AAA ATPase family (Fig. 1C) (Williams et al., 1997; Herbig et al., 1999), is sufficient for ATR interaction (Fig. 1D). As shown below, this fragment was also able to bind to *Xenopus* ATR. In these assays, cyclin A, which binds to CDC6 via the Cy motif, was retained with CDC6 1-180 and 89-268, but not with CDC6 180-268 and 180-220, as expected. ATR forms a complex with ATRIP (Cortez et al., 2001; Zou and Elledge, 2003). In the studies reported here, it was difficult to determine whether ATRIP is retained with full-length GST-CDC6 because our antibody against ATRIP (86 kDa) cross-reacted with GST-CDC6 (Fig. 1E). However, using truncated GST-CDC6 89-268 and 180-220, which could bind to ATR, we detected specific retention of ATRIP (Fig. 1E). Similar to ATR, there was only weak binding between ATRIP and GST-CDC6 1-180.

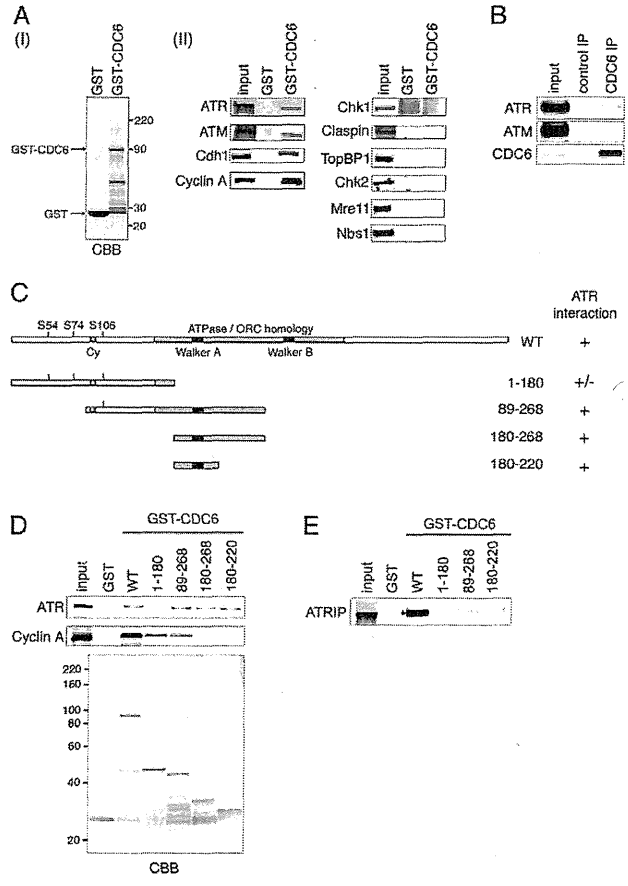

Fig. 1. Interaction of human CDC6 and ATR. (**A**) GST-CDC6 was incubated with HeLa cell nuclear extracts and bound proteins were analyzed by Coomassie Brilliant Blue (CBB) staining (I; left panel) or immunoblotting with the indicated antibodies (II; right panels). Fifteen percent of the input was also loaded. (**B**) HeLa cell nuclear extracts were immunoprecipitated with anti-CDC6 antibody or control rabbit IgG. The immunoprecipitates (IP) were subjected to immunoblotting. One percent of the input was also analyzed. (**C**) Schematic of wild-type human CDC6 and its truncated mutants used in D. The Cdk-phosphorylation sites (S54, S74, S106), cyclin-binding motif (Cy; R94 R95 L96) and ATPase/ORC-homology-domain containing Walker-A and -B motifs are marked. The ability of each mutant to interact with ATR, analyzed in D, is shown on the right. (**D**) Full-length GST-CDC6 (WT), its truncated mutants (amino acids 1-180, 89-268, 180-268 and 180-220) or GST were incubated with HeLa cell nuclear extracts and bound proteins were analyzed by CBB staining (lower panel) or immunoblotting. (**E**) Full-length GST-CDC6 (WT), its truncated mutants (1-180, 89-268 and 180-220) and GST were each incubated with HeLa cell nuclear extracts and bound proteins were immunoblotted with anti-ATRIP antibodies. The asterisk marks a non-specific band, with a molecular weight similar to ATRIP, due to cross-reaction of the anti-ATRIP antibody with GST-CDC6 WT.

When CDC6 is overexpressed in G2 cells, it blocks mitotic entry through Chk1 activation (Clay-Farrace et al., 2003). CDC6 has three Cdk-phosphorylation sites (S54, S74 and S106) (Jiang et al., 1999; Petersen et al., 1999) and Cdk phosphorylation is required for CDC6 activity to induce G2 arrest (Clay-Farrace et al., 2003). Therefore,

we investigated whether CDC6-ATR interaction might be regulated by Cdk phosphorylation. Wild-type CDC6, a phosphorylation-deficient CDC6 mutant (S54A, S74A, S106A) and a phosphorylation-mimic mutant (S54E, S74E, S106E) (Jiang et al., 1999) were tagged with tandem hemagglutinin (HA) at the N-terminus (2HA-CDC6 WT, AAA and EEE, respectively), and the vectors expressing CDC6 were transiently transfected into 293T cells. The nuclear extracts were then immunoprecipitated with anti-HA antibodies. ATR co-precipitated with 2HA-CDC6 WT and EEE, but little co-precipitated with the AAA mutant (Fig. 2A). However, the steady-state levels of 2HA-CDC6 AAA proteins and, therefore, the levels of immunoprecipitated AAA proteins were lower than 2HA-CDC6 WT and EEE upon transient expression (Fig. 2A), as expected from the fact that Cdk phosphorylation attenuates APC/C^{Cdh1}-mediated CDC6 degradation (Mailand and Diffley, 2005). Therefore, the amount of co-precipitated ATR was normalized to that of immunoprecipitated CDC6. These results demonstrated that the efficacy of ATR co-precipitation with 2HA-CDC6 AAA is reduced to ~40% compared with 2HA-CDC6 WT and EEE (Fig. 2A, lower panel). To demonstrate reciprocal co-immunoprecipitation, 293T cells were transfected with a vector expressing FLAG-ATR along with 2HA-CDC6 WT, AAA or EEE and immunoprecipitated with anti-FLAG antibodies (Fig. 2B). 2HA-CDC6 WT was co-precipitated specifically with FLAG-ATR. Immunoblotting with anti-phospho-S54-CDC6 antibodies revealed that CDC6 proteins phosphorylated by Cdk were co-precipitated. Although FLAG-ATR was precipitated with comparable efficacy in all experiments, the efficacy of 2HA-CDC6 AAA co-precipitation relative to the input was decreased to one-third that of the WT and, conversely, that of 2HA-CDC6 EEE increased threefold.

We studied this result further with a different system. 293T cells transiently expressing 2HA-CDC6 were treated with 10 mM purvalanol A, a specific inhibitor of Cdk1 and Cdk2 (Gray et al., 1998; Villerbu et al., 2002), or left untreated, and then immunoprecipitated with anti-HA antibodies. Also, in these experiments, the steady-state levels of 2HA-CDC6 were reduced by Cdk inhibition (Fig. 2C). Therefore, the amount of co-precipitated ATR was normalized to that of immunoprecipitated 2HA-CDC6. The efficiency of ATR co-precipitation was found to be reduced to ~25% by purvalanol-A treatment (Fig. 2C, lower panel). Cells were also treated simultaneously with purvalanol A and MG132, an inhibitor of 26S proteasomes, and immunoprecipitated with anti-HA antibodies. Simultaneous MG132 treatment decreased the reduction in 2HA-CDC6 by Cdk inhibition (Fig. 2C). Even under

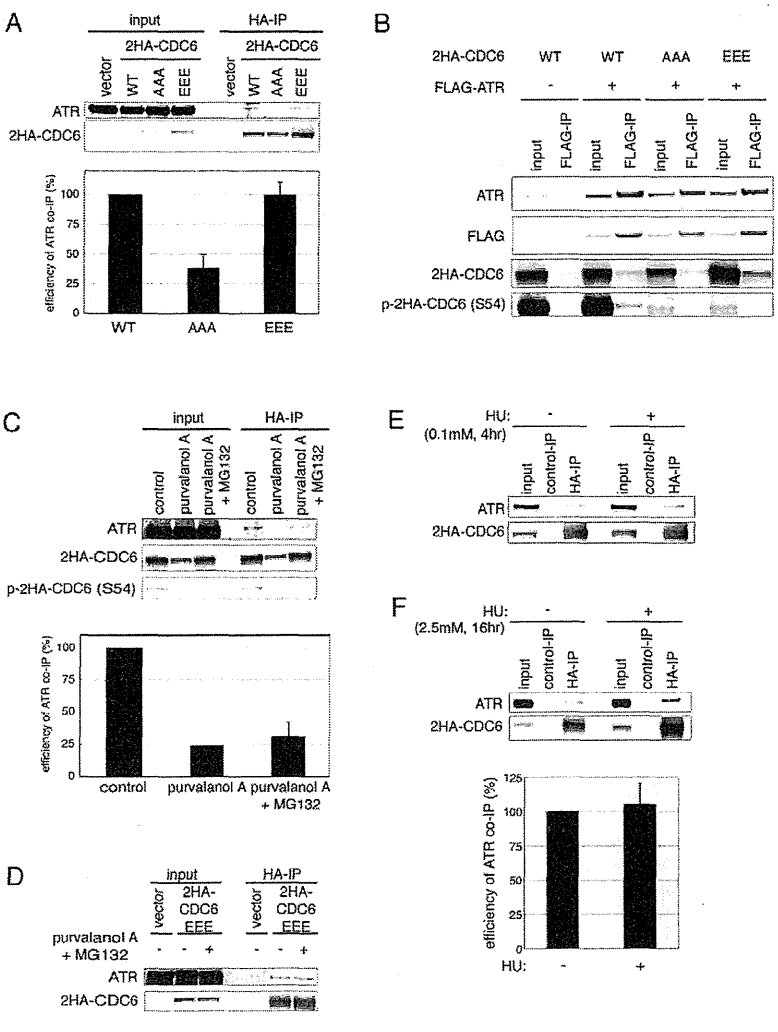

Fig. 2. Stimulation of CDC6-ATR interaction by Cdk phosphorylation. (**A**)293T cells were transfected with expression vectors for 2HA-CDC6 wild type (WT), the AAA mutant (S54A, S74A, S106A), the phospho-mimic EEE mutant (S54E, S74E, S106E) or with a control vector and then immunoprecipitated with anti-HA antibody at 48 hours post-transfection. The immunoprecipitates (IP) and 1% of the inputs were immunoblotted with anti-CDC6 and anti-ATR antibodies. The signal intensities of the bands were quantitated and the efficiency of ATR co-immunoprecipitation (ATR co-IP) was calculated as the intensity of the co-precipitated ATR relative to that of the precipitated CDC6. The mean ± s.d. from three independent experiments are given with the WT value set at 100% (lower panel). (**B**) 293T cells were transfected with expression vectors for 2HA-CDC6 WT, AAA or EEE, along with an expression vector for FLAG-ATR or a control vector and then immunoprecipitated with anti-FLAG antibodies at 48 hours post-transfection. The immunoprecipitates (IP) and 4% of the inputs were immunoblotted with the indicated antibodies. (**C**)2HA-CDC6 (WT) was transiently expressed in 293T cells and immunoprecipitated 48 hours post-transfection. As indicated, 10 μM purvalanol A, an inhibitor of Cdk1 and Cdk2, was added to the medium, with or without 20 μM MG132, at 3 hours before harvest. The precipitates and 1% of the inputs for ATR or 20% of the input for 2HA-CDC6 were immunoblotted. The efficiency of ATR co-IP was calculated as described in A and the mean ± s.d. from two independent experiments are given with the control value set at 100% (lower panel). (**D**)The same experiments as in C,were carried out with 2HA-CDC6 EEE. (**E**,**F**)HU treatment at 0.1 mM for 4 hours (E) or 2.5 mM for 16 hours (F) does not alter the CDC6-ATR interaction. 293T cells were transfected with the vector for 2HA-CDC6 WT and then treated with HU as above before harvest or left untreated. At 48 hours post-transfection, cells were subjected to immunoprecipitation with anti-HA antibodies as described in A. For the experiments shown in F, incubation with 2.5 mM HU for 16 hours arrested most cells in S phase, leading to CDC6 accumulation. Therefore, the amount of co-precipitated ATR was normalized to that of immunoprecipitated 2HA-CDC6, as in A, and the mean ± s.d. from two independent experiments are given with the value of untreated cells set at 100% (lower panel).