論文内容の要旨
Dissertation Abstract

論文題目 Title :

Analyses of Expression and Functions of Human Cdc7 Kinase in Cell Cycle Regulation

(ヒト Cdc7 キナーゼの発現と細胞周期制御における機能の解析)

氏 名 Name ： トウ　ゲック　ティング

背景と目的

The processes of DNA replication are tightly regulated so that it occurs only once in each cell cycle. Defective regulation in DNA replication and cell division often leads to genome instability and cancer development. Cdc7 (cell division cycle 7), an evolutionary conserved serine/threonine kinase, is an important DNA replication regulatory protein overexpressed in many human tumors and neoplastic cells. Studies demonstrating cell death in Cdc7-depleted cancer cells but not that in the normal fibroblast highlighted Cdc7 as a useful candidate target for cancer therapy. Nevertheless, a limited knowledge on the function of human Cdc7 which mostly focuses on the S phase of the cell cycle so far may pose a potential limitation to the significance and potential development of Cdc7 inhibitor as an anti-cancer drug. Hence, the present study was undertaken to unravel the biological functions of Cdc7 during mitotic (M) phase of the cell cycle in human cells.

実験結果

Cdc7 is a cell-cycle regulated protein abundantly expressed in mitotic phase.

By using HeLa cells released from double-thymidine block (dTB), I show that human Cdc7 is a cell-cycle regulated protein, accumulating from G1/S phase transition and peaking at M phase (Fig. 1A). Consistent with the endogenous data, time-lapse imaging of HeLa cells expressing monomeric-Azami Green (mAG)-tagged Cdc7 shows similar oscillation during cell cycle with predominant nuclear localization during S phase and cytoplasmic localization during M phase (Fig. 1B). Examination of fluorescent-tagged human Cdc7 regulatory subunit, Dbf4/ASK or Drf1/ASKL1, in HeLa cells also show oscillation and nuclear-cytoplasmic localization similar to that of the mAG-tagged Cdc7-fusion.

Regulation of Cdc7 protein level during cell cycle.

I then examined the mechanism that regulates Cdc7 protein in human cells. Cycloheximide (CHX) at 75 μg/mL, a translation inhibitor, reduces Cdc7 protein level at as soon as 0.5 hr post-treatment, indicating that Cdc7 is an unstable protein (Fig. 1C). In contrast, MG132, a proteasome inhibitor, results in accumulation of Cdc7 after 24 hr-treatment at 5 μM, suggesting regulation of Cdc7 protein by the proteasomal degradation pathway (Fig. 1D). *In silico* analysis reveals two putative Destruction (D)-box motifs and a D-box Activating Domain (DAD)-box in the Cdc7 sequence. Among these mutants, mAG-tagged Cdc7 R^{373}xxT>AxxA mutant shows slight stabilization during the cell cycle, suggesting that R^{373}xxT motif may be a potential D-box targeting Cdc7 for APC/C^{Cdh1}-dependent proteolysis in early G1 phase. However, this mutant does not cause any significant cell cycle effect possibly due to the absence of sufficient Dbf4/ASK for Cdc7 kinase activation.

Potential roles of Cdc7 during transition from M to G1 phase.

Chromatin binding and band shift of Cdc7 protein in Nocodazole-treated HeLa cells suggest phosphorylation and potential functionality of Cdc7 during M phase (Fig 1E). Here, I show that Cdc7 interacts with both N-term kinase domain (1-330 amino acids) and C-term Polo-box domain (331-603 residues) of human Plk1, an important mitotic kinase and cell cycle regulator (Fig. 2A, B). Cdc7 also interacts with human Plk2 and Plk3 after coexpression albeit with reduced affinity (Fig. 2A). Noteworthy, coexpression of Cdc7 and Dbf4/ASK rescued the G2/M phase arrest caused by Plk1 overexpression, although this rescue is independent of Cdc7 kinase activity since coexpression of Cdc7 kinase-dead form also leads to similar effect (Fig. 2C). Kinase activity of Plk1 is not affected but its interaction with endogenous Cdt1, an important DNA licensing factor, is weakened by Cdc7-ASK overexpression (Fig. 2D, E). Further examination shows that Cdt1 interacts with the N-term 331-603 residues of Plk1, suggesting that Cdc7 and Cdt1 may compete for binding to the N-terminus of Plk1. Based on these observation, I propose that Plk1 overexpression causes cell cycle arrest by inhibiting Cdt1 function through direct interaction, and Cdc7 may counteract the arrest by competing with this interaction.

Functional interactions between Cdc7 and mitotic kinases.

Cdc7 kinase activity appears to be downregulated by Plk1 since the Cdc7 autophosphorylation level was slightly reduced upon coexpression (Fig. 2E). I have identified the T68 residue as a potential Plk1-mediated phosphorylation site on Cdc7. *In vitro* experiments show that Plk1 reduces the Cdc7-mediated phosphorylation of the MCM complex, and that Cdc7 can stimulate kinase activity of Aurora B. Taken together, these results suggest that Cdc7 may regulate M phase progression through diverse functional interactions with mitotic kinases.

結論

Cdc7 is a cell cycle regulated protein abundantly expressed in M phase and degraded at G1 phase potentially through APC/C^{Cdh1} dependent pathway. Interaction between Cdc7 and Plk1 in M phase may be crucial to regulate the timing for DNA origin licensing by releasing Cdt1 from Plk1 (Fig 3). Potential significance of functional interactions between Cdc7 and mitotic kinases for progression of mitosis is also indicated. Thus, findings from this study will provide new insights into the roles of Cdc7 during M phase and facilitate the understanding of the molecular pathways for maintenance of genomic stability.
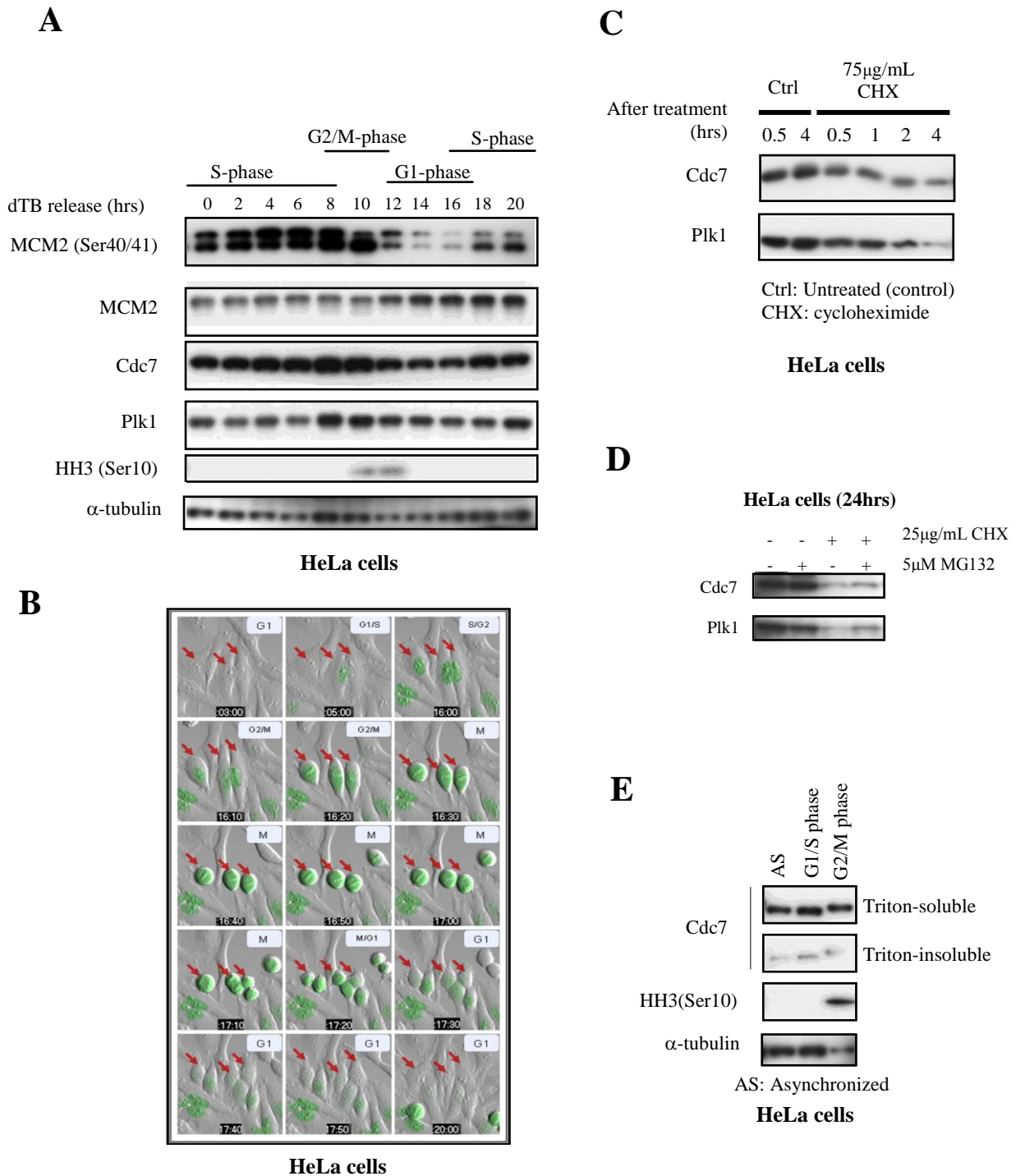
Figure 1. Dynamics and protein stability of human Cdc7 protein in HeLa cells. (A) Cdc7 protein level during cell cycle. HeLa cells were arrested at G1/S phase by double thymidine block (dTB). Cells were harvested at every 2-hr interval after release and whole cell extract (WCE) was separated by SDS-PAGE and immunoblotted with the antibodies against the proteins indicated. (B) Time-lapse imaging of mAG-tagged Cdc7-fusion (Green) in HeLa cells during cell cycle. (C, D) Levels of Cdc7 after Cycloheximide (CHX) and/or MG132 treatment, as indicated in the figure. Plk1 was run as a control since it is known to be degraded via APC-C dependent pathway. (E) Subcellular localization of Cdc7. HeLa cells were synchronized at G1/S boundary or G2/M phase by using dTB or Nocodazole block, respectively. Triton-soluble and –insoluble extracts were prepared by using CSK buffer containing 0.5% triton-X.
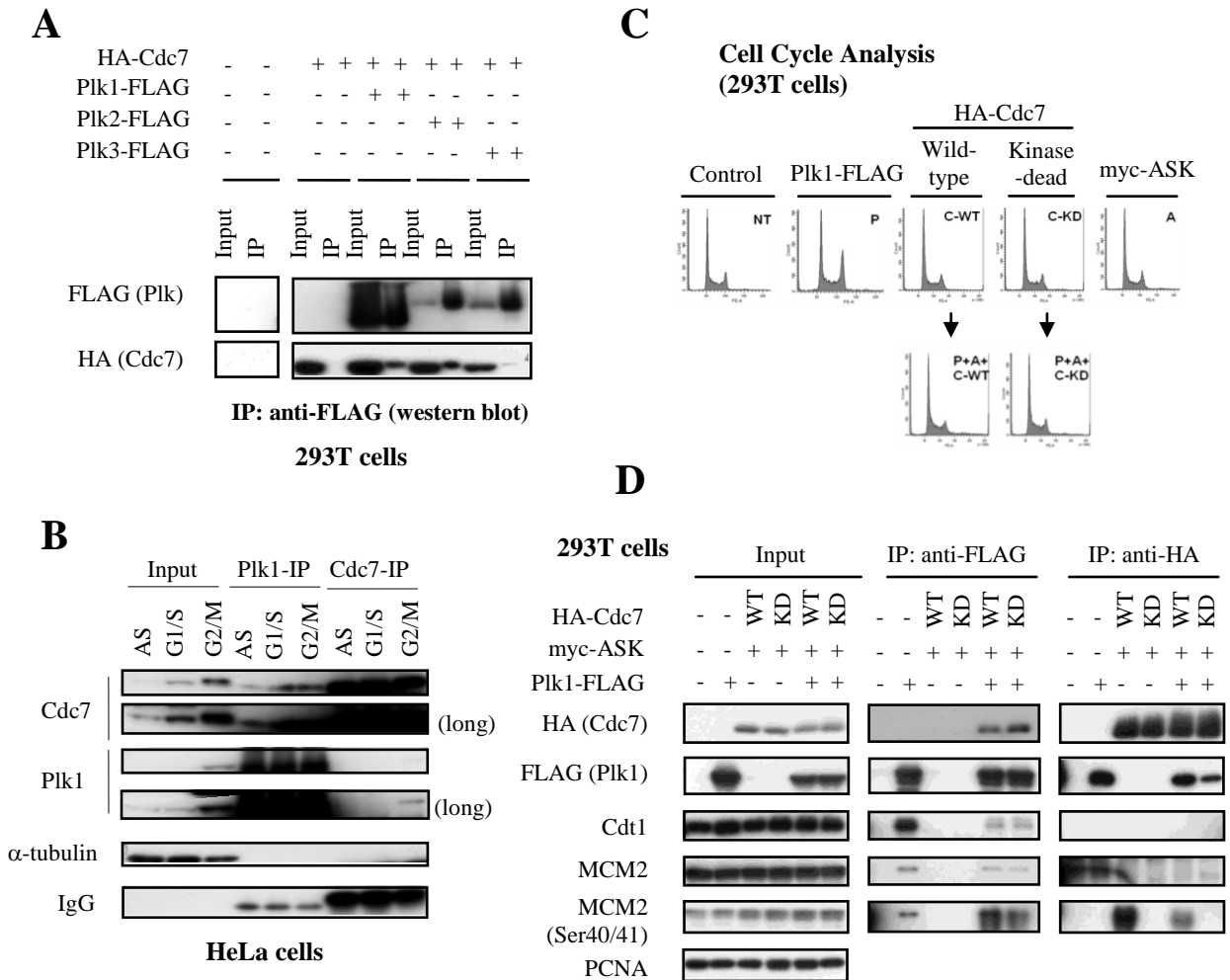
A

HA-Cdc7	-	-	+	+	+	+	+	+	+
Plk1-FLAG	-	-	-	-	+	+	-	-	-
Plk2-FLAG	-	-	-	-	-	-	+	+	-
Plk3-FLAG	-	-	-	-	-	-	-	-	+

IP: anti-FLAG (western blot)

293T cells

B

HeLa cells

C

Cell Cycle Analysis (293T cells)

D

293T cells

E

***In-vitro* kinase assay**

293T cells

Figure 2. Physical and functional interactions between Cdc7 and Plk1. (A) Interaction between overexpressed Cdc7 and Plk1, Plk2 and Plk3. 293T cells were transfected using polyetherimide (PEI) with the indicated plasmids for 48 hrs. Triton-soluble extracts were prepared and immunoprecipitates by anti-FLAG (Plks) antibody were separated by SDS-PAGE and immunobloted with antibodies indicated. (B) Interaction between endogenous Cdc7 and Plk1. HeLa cells were synchronized as described in (1E). Triton-soluble extracts were prepared and immunoprecipitation was conducted by using anti-Plk1 or anti-Cdc7 antibody. (C) Effects of Plk1, Cdc7 (WT: wildt-ype; KD: kinase-dead) and/or ASK transfection on the cell cycle of 293T cells. Transfection was performed as described in (A) prior to preparation for FACS analysis. (D) Triton-soluble extracts were prepared. The immunoprecipitates by anti-HA (Cdc7) or anti-FLAG (Plk1) antibody were examined by immunoblotting using antibodies against the replication factors indicated. Interaction between Plk1 and Cdt1 as well as that between Plk1 and MCM2 Ser40/41 were affected upon Cdc7-ASK coexpression. (E) Immunoprecipitates by anti-HA (Cdc7) or anti-FLAG (Plk1) antibody were prepared and used for *in vitro* kinase assays.
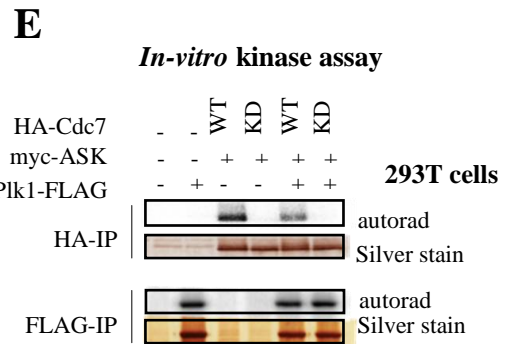
Figure 3. A proposed model for regulation of DNA origin licensing by Plk1 and Cdc7. Plk1 and Cdt1 interaction may prevent chromatin loading of Cdt1, thereby reduce recruitment of MCM complex to replication origin to form pre-Replication Complex (pre-RC). Cdc7-ASK may facilitate release of Cdt1 from Plk1 through competitive interaction with Plk1 and thus allow origin licensing.