Human Cdc7-related kinase complex: In vitro phosphorylation of MCM by concerted actions of Cdks and Cdc7 and that of a critical threonine residue of Cdc7 by Cdks

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Running title: Interplay between Cdc7 and Cdks for initiation of S phase
Summary

huCdc7 encodes a catalytic subunit for *Saccharomyces cerevisiae* Cdc7-related kinase complex of human. ASK, whose expression is cell cycle-regulated, binds and activates huCdc7 kinase in a cell cycle-dependent manner [Kumagai et al. Mol. Cell. Biol. Vol. 19, 5083-5095. (1999)]. We have expressed huCdc7 complexed with ASK regulatory subunit using the insect cell expression system. To facilitate purification of the kinase complex, glutathione-S-transferase (GST) was fused to huCdc7 and GST-huCdc7-ASK complex was purified. GST-huCdc7 protein is inert as a kinase on its own, and phosphorylation absolutely depends on the presence of the ASK subunit. It autophosphorylates both subunits in vitro and phosphorylates a number of replication proteins to different extents. Among them, MCM2 protein, either in a free form or in a MCM2-4-6-7 complex, serves as an excellent substrate for huCdc7-ASK kinase complex in vitro. MCM4 and MCM6 are also phosphorylated by huCdc7 albeit to less extent. MCM2 and 4 in the MCM2-4-6-7 complex are phosphorylated by Cdks as well, and prior phosphorylation of the MCM2-4-6-7 complex by Cdks facilitates phosphorylation of MCM2 by huCdc7, suggesting collaboration between Cdks and Cdc7 in phosphorylation of MCM for initiation of S phase. huCdc7 and ASK proteins can also be phosphorylated by Cdks in vitro. Among four possible Cdk phosphorylation sites of huCdc7, replacement of T376, corresponding to the activating threonine of Cdk, with alanine (T376A mutant) dramatically reduces kinase activity, indicative of kinase activation by phosphorylation of this residue. In vitro, Cdk2-CyclinE, Cdk2-CyclinA, and Cdc2-CyclinB, but not Cdk4-CyclinD1, phosphorylates the T376 residue of huCdc7, suggesting possible regulation of huCdc7 by Cdks.
**Introduction**

*Saccharomyces cerevisiae* Cdc7 encodes a serine/threonine kinase essential for G1/S transition (1-2). DBF4, originally isolated as a temperature-sensitive mutant defective in initiation of DNA replication (3) was later identified as a multicopy suppressor of *cdc7*(ts) (4). Furthermore, Dbf4 was shown to associate with Cdc7 and to activate its kinase activity (5). Kinases related to Cdc7 are widely conserved in eukaryotes, suggesting conserved important functions of this class of kinase in initiation of S phase (6-10). Recently, functional homologues of Dbf4 were identified in fission yeast and human (11-17). Genetic studies in fission yeast clearly demonstrate essential role of Hsk1-Dfp1/Him1 kinase complex (fission yeast homologue of Cdc7-Dbf4) in S phase initiation (11,14).

In human, cellular Cdc7 kinase activity appears to be largely determined by the level of ASK protein, the human homologue of Dbf4, which fluctuates during the cell cycle. ASK protein as well as huCdc7 kinase activity are kept high during S phase and decreases by the next G1 phase. Microinjection of anti-ASK antibody into human primary fibroblast inhibited progression into S phase, indicating essential roles of ASK in mammalian DNA replication (13). Cdk2-CyclinE is also essential for G1-S transition in mammalian cells, although its crucial target for S phase initiation has not been elucidated. It is not known if Cdc7 and Cdk2-CyclinE function in a parallel pathway or in the same pathway for S phase initiation. Results in budding yeast have suggested that Cdc7 may be regulated by phosphorylation of the conserved threonine present near the APE motif (T281), although the kinase responsible for this phosphorylation has not been known (18,19,19a).

Similar to prokaryotic DNA replication, initiation of DNA replication in eukaryotes requires assembly of a series of replication proteins at the origins (20). These include ORC (origin recognition complex), Cdc6, MCM (minichromosomal maintenance), Cdc45, DNA polymerases, and single-stranded DNA binding protein (RFA). One hybrid assays in yeast indicated that Dbf4 protein is tethered at the origins (21), strongly suggesting that Cdc7 kinase complex is also present on chromatin in association with
replication complexes at the origin. Biochemical studies have indicated that MCM complex may be an important target of Cdc7 kinase (7,12,14). Furthermore, genetic studies have shown that MCM2 may be a physiologically important substrate of Cdc7 (22; Takeda et al., submitted).

In order to characterize mammalian Cdc7 kinase complex in more detail, we have overexpressed and purified active human Cdc7-ASK kinase complex. Using a purified Cdc7 kinase preparation, we have examined phosphorylation of MCM complex and other various replication proteins by huCdc7-ASK kinase complex in vitro. We have shown that MCM2, either by itself or in a complex, serves as an efficient substrate of Cdc7, and that other replication proteins including SV40 Tag are also phosphorylated by huCdc7-ASK complex to different degrees. We have shown that Cdk-mediated phosphorylation of a MCM complex facilitates phosphorylation of MCM2 by huCdc7. We have also shown that Cdks including Cdk2-CyclinE can phosphorylate huCdc7 and ASK proteins in vitro and identified a possible phosphorylation site mutation (T376A) of huCdc7 which significantly affects the kinase activity of huCdc7. We have shown that the critical T376 residue is phosphorylated by Cdk2-CyclinE, Cdk2-CyclinA and Cdc2-CyclinB in vitro, providing the possible functional links between Cdks and Cdc7.

Experimental Procedures

Construction of insect cell expression vectors for huCdc7 and ASK
NotI-linearized pKU3-HA-short huCdc7 (7) was partially digested with XbaI and the 2.3 kb fragment containing the HA-tagged 562 amino acid (residues 13-574) huCdc7 coding frame was subcloned at NotI-XbaI sites of pVL1392, resulting in pVL1392-huCdc7(WT). The kinase negative mutants, KE and KR, were similarly subcloned from pKU3-HAuCdc7 into pVL1392. For expression of GST-fused huCdc7 proteins in insect cells, the GST fragment, amplified by a set of primers (5’ AT A AGA ATG CGG CCG CAT ATG TCC CCT AT A CT A GGT T AT 3’ and 5’ AT A AGA ATG CGG CCG CCA CGA CCT TCG ATC AGA TCC 3’) on pGEX-5X-3, was digested with NotI and was inserted at the NotI site of
pVL1392-huCdc7 (wild-type or mutants), resulting in in-frame fusion of GST and huCdc7 coding frames. For expression of ASK, pME18S-mycASK (13) was digested with StuI (present in the vector at 40 bp upstream of the myc tag) and NotI and subcloned at SmaI-NotI sites of pVL1393, resulting in myc-tagged ASK protein (containing 63 extra amino acids derived from the 5’ non-coding region).

Purification of GST-huCdc7-ASK complex
Three days after infection of Sf9 cells with recombinant virus solutions of GST-huCdc7 and myc-ASK (m.o.i. 2 of each), cells were scraped off from the plates and harvested. 10⁹ cells were resuspended in 100 ml of IP buffer (40 mM Hepes•KOH [pH7.6], 40 mM potassium glutamate, 10 % glycerol, 1 mM EDTA, 1 mM DTT, 0.1 % NP40 and protease inhibitors). Cells were lysed in a glass hand homogenizer, and supernatant was obtained by centrifugation at 8000 krpm at 4°C. After addition of glutathione Sepharose 4B resin, the extract was incubated for 1 hour with gentle rotation. The resin was thoroughly washed with the IP buffer, and washed resin could be directly used for in vitro kinase assays. To obtain purified soluble preparations of huCdc7 kinase complex, bound proteins were eluted from the resin with IP buffer containing 10 mM glutathione.

Purification of MCM2, MCM complexes and SV40 Tag and p27
Baculoviruses for preparation of mouse MCM2-4-6-7 complex were constructed as reported (23). For preparation of the MCM4-6-7 complex, Sf9 cells were coinfected with baculoviruses containing MCM7, cloned onto pVL1392, and those containing his-tagged MCM4 and MCM6. Baculoviruses containing MCM3, cloned onto pVL1392, and those containing his-tagged MCM5, cloned onto pAcHLT-A (Phamingen), were used for coinfection to prepare MCM3-5 complex. Mouse MCM2 gene was cloned into pAcHLT-A. Free his-tagged MCM2 and MCM complexes (MCM2-4-6-7, MCM4-6-7 and MCM3-5) were purified as described above except that cells expressing his-tagged MCM2 were resuspended in IP buffer containing 0.5 M NaCl. Nickel resin was added to the extracts, was extensively washed with IP buffer and proteins were eluted from the resin by IP buffer
containing 10 mM imidazole. Proteins were further purified by monoQ column on SMART system (Amersham-Pharmacia). The peak fractions containing the MCM2-4-6-7 complex, which eluted at 0.3M NaCl, were dialyzed against IP buffer and were used for in vitro kinase assays. SV40 T antigen was purified from insect cells by using anti-Tag monoclonal antibody column, as previously described (23a). Histidine-tagged mouse p27 protein, expressed in insect cells (gift from Dr. Junya Kato), was purified by nickel column, as described above for the MCM2-4-6-7 complex.

In vitro kinase assays for huCdc7-ASK kinase complex
The standard reaction mixtures (25 µl) for huCdc7 in vitro kinase assays contained 40 mM Hepes/KOH (pH 7.6), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM β-glycerophosphate, 1 mM NaF, 2 mM DTT, 10 mM magnesium acetate, 80 µg/ml BSA, 0.1 mM ATP, 1 µCi of [γ-32P]ATP, 0.1-0.5 µg of MCM2 (or MCM2-4-6-7 complex) unless otherwise stated, and 50 ng of huCdc7-ASK kinase complex or immunoprecipitates. The reaction mixtures were normally incubated at 30°C for 30 min. After addition of SDS-PAGE sample buffer, the reaction mixtures were incubated at 75°C for 3 min and applied on 8% SDS-PAGE.

Expression and purification of Cdk-Cyclin complexes in insect cells
Insect cell vectors for expression of mouse Cdk2, mouse Cdk4, human Cdc2, mouse CyclinD1, human CyclinE, and human CyclinA were generously provided by Dr. Hitoshi Matsushime (24), and were used for coexpression with huCdc7 and ASK. Expression vectors for purification of mouse Cdk-Cyclin complexes, Cdk4, Cdk2, Cdc2, GST-CyclinD1, GST-CyclinE, GST-CyclinA and GST-CyclinB were generous gifts from Dr. Hideyo Yasuda, and Cdk-Cyclins were purified with glutathione Sepharose 4B column, as described for purification of GST-huCdc7 kinase complexes.

Generation of dephosphorylated MCM proteins
Affinity-purified preparations of free MCM2 or MCM2-4-6-7 complex were treated with λ phosphatase (New England BioLabs, Inc; 4000 units per 1 ml of affinity-purified
preparation), and dephosphorylation of MCM subunits was confirmed on SDS-PAGE. Then, it was further purified on monoQ column to separate MCM from the phosphatase.

Two-step kinase reaction
In the first stage, dephosphorylated or untreated MCM2-4-6-7 was incubated in the standard kinase reaction mixtures (lacking radioactive ATP) with Cdk5 for 20 min at 30°C, and p27 (0.5 microgram) was added and was further incubated at 30°C for 5 min. The second stage reaction was initiated by adding huCdc7-ASK and [γ-32p]ATP (1 µCi) and aliquots were mixed with SDS-PAGE sample buffer at the times indicated. In control, p27 protein was present in the first stage reaction mixture.

Mutagenesis of huCdc7
In order to generate mutant huCdc7 harboring an alanine substitution at four individual threonine or serine residue in TP or SP sequence, PCR-directed mutagenesis was conducted. Four sets of primers (5’ ATG GCT TTT gCT CCC CAG CGT G 3’ and 5’ CAC GCT GGG GAG cAA AAG CCA T 3’; 5’ ATT TCA CAT GAG gcC CCT GCA GTG AAA 3’ and 5’ TTT CAC TGC AGG Gge CTC ATG TGA AAT 3’; 5’ TAG GGC AGG TgC ACC AGG ATT CA 3’ and 5’ TGA ATC CTG GTG cAC CTG CCC TA 3’; 5’ GGA TTC TAG CgC TCC CAA GTT AA 3’ and 5’ TTA ACT TGG GAG cGC TAG AAT CC 3’) were used to introduce alanine substitution at S16, S302, T376 and T472, respectively. The first and the second of the pair was combined, respectively, with 5’ CGG ATT TCC TTG AAG AGA GTG 3’ and 5’ CGG ATT TCC TTG AAG AGA GTG 3’ and 5’ CCT ATA AAT ATT CCG GAT TAT TC 3’ (vector primers), for PCR amplification of C-terminal and N-terminal halves of the coding region. The PCR fragments were recovered from polyacrylamide gel and were mixed with the vector primers for amplification of the entire coding region with an expected mutation. The fragment was digested with NotI + SmaI, and inserted at NotI-SmaI site of pVL1392.
Cyanogen bromide (CNBr) digestion of phosphorylated huCdc7 protein
After in vitro phosphorylation in the presence of 10-fold excess of labeled nucleotide, the products were run on 8% SDS-PAGE and were transferred to a nitrocellulose filter. The transferred protein band was excised, and the filter was incubated in 100mg/ml CNBr in formic acid for 90 min at room temperature. The solution containing digested polypeptides was recovered and evaporated. After addition of 500 µl of water, it was further lyophilized and resuspended in SDS-PAGE sample buffer. The polypeptides were analyzed on a 24% low-bis Tricine gel.

Development of phosphorylated T376-specific antibody
A synthetic oligopeptide, CVAPRAGT(PO₃H₂)PGFRA (residues 370-381 of huCdc7), was synthesized and used as antigen to develop rabbit anti-sera. Antibody was affinity-purified using the non-phosphorylated peptide and the antigen peptide.

Results
Expression of active HA-tagged huCdc7-myc-tagged ASK kinase complex in insect cells
The huCdc7 coding frame, either the wild-type or kinase negative, lacking the first 12 amino acids and tagged with the 7 amino acid haemaglutinin epitope tag (7), was cloned under the Polyhedrin promoter on the insect cell expression vector pVL1393. Similarly, the ASK coding frame containing extra 63 amino acids derived from the 5' non-coding region and fused to the myc epitope tag was cloned on pVL1393 (13). Both plasmids were transfected into insect cell Sf9, and recombinant viruses were recovered, which were coinfected into Sf9 cells. The huCdc7 and ASK formed a complex in insect cells, as indicated by coimmunoprecipitation of huCdc7 protein with anti-myc antibody (Figure 1, lanes 1-3, lower panel). The immunoprecipitate containing wild-type huCdc7 and ASK exhibited two smeared phosphorylated bands on SDS-PAGE, which were identified as ASK, as previously shown with mammalian cell extracts overexpressing the both subunits.
(13, Figure 1, lane 1). This phosphorylation was not detected with KE or KR mutant huCdc7, in which the conserved lysine residue at position 90 was replaced with glutamic acid or arginine, respectively, although they associated with ASK (Figure 1, lanes 2 and 3). Autophosphorylation of huCdc7 was not detected, when huCdc7 alone was expressed (Figure 1, lane 4). Gel filtration analyses of the extract expressing both huCdc7 and ASK indicated that both proteins migrated at around 150 kDa, consistent with formation of a heterodimer (data not shown).

**Purification and characterization of GST-fused huCdc7 protein complexed with myc-ASK**

In order to facilitate purification of an active huCdc7 kinase complex, we have fused GST tag at the N-terminus of huCdc7 and expressed the GST-huCdc7 fusion protein in Sf9. In separate experiments, we have fused the GST tag to the N-terminus of Hsk1 (6), the fission yeast homologue of Cdc7, and showed the resulting GST-Hsk1 can complement the growth defect of the hsk1 null mutant (our unpublished data), strongly suggesting that the presence of the GST-tag at the N-terminus of Cdc7-related kinases may not affect their functions. Sf9 cells, coinfected with recombinant viruses encoding GST-huCdc7 (wild-type or KE mutant) and myc-ASK, were harvested three days later and extracts were prepared. GST-huCdc7-myc-ASK complex was affinity-purified onto glutathione Sepharose 4B resin, and these preparations attached to the resin exhibited strong autophosphorylation activity in vitro (data not shown). The kinase complex could be eluted from the resin with buffer containing 10 mM glutathione. Since ASK is extensively phosphorylated in insect cells when coexpressed with huCdc7 and appears as smeared bands on SDS-PAGE (Figure 2A, lane 2), the preparations containing the complex was pretreated with λ phosphatase prior to loading onto gel (Figure 2A, lane 1). A major band corresponding to GST-huCdc7 and a cluster of several bands corresponding to myc-ASK were detected after silver staining in addition to several other contaminating bands (Figure 2A, lanes 1 and 3). Only the huCdc7 band was detected in the preparations from the extract singly-infected by WT GST-huCdc7 or KE GST-huCdc7 (Figure 2A, lanes 4 and 5). These preparations were used for in vitro
kinase assays using GST-MCM2N containing the N-terminal 206 amino acids of human MCM2 protein generated in E. coli. Autophosphorylation of GST-huCdc7 and myc-ASK as well as phosphorylation of GST-huMCM2N was detected with the wild-type huCdc7 complex, but not with the kinase-negative mutant huCdc7 nor with free GST-huCdc7 proteins (Figure 2B, lanes 1-4), consistent with requirement of ASK protein for expression of huCdc7 kinase activity. The purified protein in solution was more efficient in phosphorylating an exogenous substrate than the resin-bound form or the immunoprecipitates (data not shown; see Figure 7B for comparison), probably due to absence of steric hindrance caused by the resin or associated antibody which may inhibit the access of the substrate to the kinase.

The amount of the incorporation could be quantified by spotting an aliquot on P81 paper, which was extensively washed in 75 mM phosphoric acid. At 30°C, the reaction continued linearly for 10 min, and leveled off after 60 min (Figure 3A). With 5 pmole of the full-length MCM2 protein, expressed in and purified from insect cells, as a substrate, the incorporation increased linearly up to 0.7 pmole of huCdc7 kinase complex and reached 20 pmole of incorporation of phosphate (Figure 3B), indicating that the kinase functions in a catalytic manner. The presence of salt was generally inhibitory for kinase activity. The level of incorporation was inhibited by 50% at 100 mM KCl (data not shown). The kinase reaction required the presence of a divalent cation, and the reaction reached plateau at over 4 mM Mg(OAc) and stayed at the similar level at up to 20 mM. Manganese could support only less than 20% of the incorporation of that attained with magnesium (Figure 3C). The reaction was not significantly affected by pH between the range of 6.0 to 8.5, and slightly decreased at pH 9.0 (data not shown). The reaction was most efficient at 37°C and was inhibited by 30 % at 48°C. Incubation of the purified protein at 55°C for 5 min resulted in loss of 50% activity. Titration of ATP concentration indicated the apparent Km for ATP in phosphorylation of MCM2 (in the MCM2-4-6-7 complex purified similarly from insect cells, see below) by huCdc7 is 2-4 μM, indicating high affinity of huCdc7 kinase complex for ATP. GTP, CTP and UTP at 1mM did not support any detectable level of phosphorylation (Figure 3D).
Phosphorylation of MCM2 complex and T-antigen by huCdc7 kinase complex in vitro

Previous biochemical and genetic evidence indicated that MCM complex may be the physiological substrate of Cdc7-related kinases (7,12,22). Among the MCM components, MCM2 protein appears to be a conserved target of Cdc7 in various eukaryotes examined so far. We have purified various subassemblies of mouse MCM complex expressed in insect cells, and used them as substrates for in vitro kinase assays.

Uncomplexed full-length MCM2 served as an excellent substrate for huCdc7-ASK (Figure 4A, lane 3) and quantification of $^{32}$P incorporated into MCM2 indicated 2-2.5 molecules of phosphates were transferred to one molecule of MCM2 (Figure 4B). MCM2-4-6-7 also served as an excellent substrate, and MCM2 was efficiently phosphorylated (Figure 4A, lane 5). MCM4 and 6 also were phosphorylated by huCdc7 albeit with much less efficiency (Figure 4A, lane 5). MCM4 and MCM6 in the MCM4-6-7 complexes were also phosphorylated to a similar extent (Figure 4A, lane 7). Phosphorylation of the MCM3-5 complex by huCdc7 was not evident due to the presence of insect cell-derived unknown kinases in the preparation (Figure 4A, lanes 9 and 10).

It should be noted that phosphorylation of free MCM2 causes upward mobility shift on SDS-PAGE (Figure 4A, lane 3), while MCM2 in the MCM2-4-6-7 complex shifts downward upon phosphorylation (Figure 4A, lane 5). This indicates the presence of specific phosphorylation site(s) on MCM2 which become available for phosphorylation by huCdc7-ASK only in the complex.

Among other replication proteins examined, p180, p68 and p54 subunits of DNA polymerase α (in collaboration with Drs. Takeshi Mizuno and Fumio Hanaoka), ORC4 protein (25; in collaboration with Dr. Anyndia Dutta), Geminin (26; in collaboration with Dr. Tom McGarry) were phosphorylated by huCdc7 in vitro (data not shown). Viral initiators, SV40 Tag and Bovine papilloma virus E1 proteins (in collaboration with Dr. Jim Borowiec) were also phosphorylated by huCdc7-ASK (Figure 4A, lane 11 and data not shown). SV40 Tag was phosphorylated to the level comparable to that of MCM2. The functional significance of this phosphorylation is being investigated.
Cdk and huCdc7 may cooperate for phosphorylation of MCM2

The recombinant MCM proteins purified from Sf9 have undergone phosphorylation at the time of harvest presumably by a variety of kinases present in the insect cells, which is estimated from the presence of mobility-shifts on SDS-PAGE. This phosphorylation may affect its efficacy as a substrate for Cdc7 kinase. Sugino et al. have shown that phosphorylation of MCM2 with budding yeast Cdc7-Dbf4 in vitro depends on the presence of “prephosphorylation” of MCM2 by some other kinases (manuscript submitted). Therefore, we have generated “dephosphorylated” substrates by treating them with a phosphatase and further purifying them on a column, and compared Cdc7-mediated phosphorylation of these “dephosphorylated” substrates with that of untreated ones. Treatment of the MCM complex with λ phosphatase resulted in disappearance of mobility-shifted bands on MCM2 and MCM4 (Figure 5A, compare the silver staining pattern of the rightmost lanes of left and middle panels).

Interestingly, the efficiency of phosphorylation of dephosphorylated MCM complex, as detected by the appearance of the fully mobility-shifted form, was lower than that of untreated substrate (Figure 5A and B). MCM2 in the dephosphorylated MCM complex can be converted to this mobility-shifted form with 100% efficiency, if sufficient amount of huCdc7 is added, indicating that prephosphorylation of MCM2 only contributes to the efficiency of phosphorylation but not to the phosphorylation pattern by huCdc7. This may indicate that the presence of “prephosphorylated” residues on MCM2 may be required for efficient phosphorylation by huCdc7.

Some MCM proteins are known to be phosphorylated by Cdk. We have examined whether known Cdk can phosphorylate the MCM complex in vitro. Among Cdk examined, Cdk2-CyclinE, Cdk2-CyclinA and Cdc2-CyclinB, but not Cdk4-CyclinD1, phosphorylated MCM2 and MCM4 to a similar extent (Figure 6A, lanes 5-7). Biochemical and genetic evidence which showed Cdc2-mediated phosphorylation of MCM4 was previously reported (27,28). Ishimi et al. recently reported that Cdk2-CyclinA phosphorylates MCM42 and that this phosphorylation inhibits DNA helicase activity of
MCM4-6-7 complex (28a). Phosphorylation of MCM2 by Cdks did not cause any additional mobility-shift on SDS-PAGE (Figure 6A, compare lane 1 and lanes 5-7), whereas huCdc7 caused downward mobility-shift (Figure 6A, lane 2), indicating that huCdc7 phosphorylates sites on MCM2 distinct from those by Cdks. MCM4 is also mobility-shifted by phosphorylation with huCdc7-ASK but not with Cdks (Figure 6, compare lane 2 and lanes 5-7), indicative of phosphorylation of distinct sites on MCM4 by huCdc7-ASK.

Therefore, it would be interesting to examine whether phosphorylation of MCM by Cdks affects efficiency of phosphorylation by hyCdc7. We have conducted kinase assays on dephosphorylated MCM2-4-6-7 complex in the presence of both ahuCdc7-ASK and a Cdk. The reaction mixtures contained suboptimal concentration of huCdc7 kinase, and less than half of MCM2 in dephosphorylated MCM2-4-6-7 complex was converted to the mobility-shifted form, whereas nearly all the MCM2 protein in the untreated MCM2-4-6-7 complex was converted to the shifted form (Figure 6B, lanes 1 and 15). Under this condition, the presence of Cdk2-CyclinA significantly stimulated huCdc7-mediated phosphorylation, as judged by the amount of the downward mobility-shifted form generated (Figure 6B, compare lanes 1 and 10). Stimulation was observed also with Cdk2-CyclinE and Cdc2-CyclinB (Figure 6B, lanes 7 and 13). Autophosphorylation of huCdc7 and ASK does not appear to be significantly affected by the presence of Cdk, suggesting that effect of Cdk is not due to modulation of Cdc7 activity per se. These results indicate that phosphorylation of MCM2 by huCdc7 may be stimulated by cophosphorylation by Cdks.

In order to show more directly that the effect of Cdks is mediated by their actions on MCM, not on huCdc7-ASK, we have conducted the reactions in two steps by taking advantage of the fact that p27 protein inhibits Cdk2-CyclinE and Cdk2-CyclinA, but has no effect on huCdc7 kinase activity (Figure 6C). We first incubated the phosphatase-treated MCM2-4-6-7 with Cdk2-CyclinE or Cdk2-CyclinA, and then added p27 protein to inactivate Cdks. huCdc7-ASK, together with radioactive [$\gamma^{32}$P]-ATP, was then added and phosphorylation of MCM2 was analyzed on SDS-PAGE. Both Cdk2-CyclinE and Cdk2-
CyclinA significantly increased the rate of MCM2 phosphorylation and the amount of the mobility-shifted form (Figure 6D, lanes 6-15). When p27 was present in the first stage, no effect of the Cdks was observed (Figure 6D, lanes 19 and 21). With the untreated MCM2-4-6-7 complex, which has presumably been phosphorylated by insect cell-derived Cdks, efficient phosphorylation of MCM2 was observed, resulting in the appearance of mobility-shifted form after as early as 5 min incubation (Figure 6D, lanes 22-26). The results clearly indicate that Cdks affect the efficacy of MCM2 phosphorylation by huCdc7 through phosphorylation of the MCM complex.

**Threonine 376, corresponding to activation threonine of Cdk, is required for full kinase activity of huCdc7**

Cdc7-ASK mainly functions in G1/S transition and S phase presumably downstream of G1 Cdks, Cdk4-CyclinD1 and Cdk2-CyclinE. However, functional interactions, if any, between Cdc7 and Cdks are not known. There are four SP or TP sequences (16S, 302S, 376T and 472T), possible Cdk target sites, on huCdc7 (Figure 7A). We have replaced each of them with alanine, and expressed the resulting mutant huCdc7 in insect cells. We then coexpressed the mutant huCdc7-ASK complexes, which were coimmunoprecipitated with huCdc7 antibody for kinase assays. The mutations did not affect the complex formation between Cdc7 and ASK, as far as immunoprecipitation could measure (Figure 7B, middle and lower panels). All the mutants except for T376A displayed kinase activity comparable to the wild-type (Figure 7B, upper panel). T376A mutant huCdc7 exhibited significantly reduced phosphorylation activity. The extent of mobility shift of ASK and huCdc7 was significantly reduced in T376A mutant (data not shown), indicating that only subset of autophosphorylation sites on huCdc7 and ASK are phosphorylated with the T376A mutant.

We have purified GST-fused huCdc7 T376A mutant complexed with myc-tagged ASK, and examined its kinase activity. The T376A supported only 10 % level of phosphorylation of MCM2 protein compared to the wild-type (Figure 7C, lower panel; compare lanes 2 and 4, 6 and 8). Whereas the upward mobility-shift in free form of MCM2 and downward mobility-shift of MCM2 in the MCM2-4-6-7 complex are obvious after
phosphorylation with the wild-type huCdc7-ASK (Figure 7C, upper panel; lanes 2 and 6), they were barely detected with T376A mutant (Figure 7C, upper panel; lanes 4 and 8). These results clearly show that phosphorylation activity is impaired in the T376A mutant. T376 corresponds to T160 of Cdk, whose phosphorylation by CAK is required for activation of Cdk (28a). Therefore, huCdc7 may also be activated by phosphorylation of T376 by some unknown kinase.

**Phosphorylation of T376 of huCdc7 by Cdks**

In order to explore possible interplay between Cdks and Cdc7, we examined whether Cdks can phosphorylate huCdc7-ASK kinase complex. When purified Cdks were incubated with purified GST-huCdc7 in vitro, Cdk2-CyclinE, Cdk2-CyclinA and Cdc2-CyclinB phosphorylated huCdc7 (Figure 8A, upper panel, lanes 6-8). No significant phosphorylation of huCdc7 was detected with Cdk4-CyclinD1 (Figure 8A, lane 5). The T376A mutant was also phosphorylated by the three Cdks (Figure 8A, upper panel, lanes 10-12). However, the level of phosphorylation by Cdks on the T376A mutant was slightly lower than that on wild-type. Both huCdc7 and ASK subunits in the huCdc7-ASK complex appear to be phosphorylated by the three Cdks (Figure 8A, upper panle, lanes 18-20). Phosphorylation of the T376 residue of huCdc7 by Cdk2-CyclinE was also indicated by comparing the peptide mapping pattern of wild-type and T376A huCdc7 phosphorylated by Cdk2-CyclinE in vitro. The polypeptide band indicated by an arrow in Figure 8B is seen only in the sample from wild-type huCdc7 (Figure 8B, lane 2).

To further confirm phosphorylation of the T376 residue by Cdks, we have developed an antibody specific for the phosphorylated T376 residue. The GST-huCdc7 protein phosphorylated by Cdk2-CyclinE, Cdk2-CyclinA and Cdc2-CyclinB reacted with the phosphopeptide antibody (Figure 8A, middle panel, lanes 6-8), whereas T376A GST-huCdc7 protein did not react with the antibody under the same condition (Figure 8A, middle panel, lanes 10-12). The KR mutant of GST-huCdc7, either free form or in a complex with myc-ASK also reacted with the phosphopeptide antibody after phosphorylation by Cdks (Figure 8A, middle panel, lanes 14-16 and 18-20). Cdk2-CyclinE,
Cdk2-CyclinA, and Cdc2-CyclinB could phosphorylate the T376 residue to similar levels, although extra mobility-shift was observed only with Cdk2-CyclinE (Figure 8A, middle panel, lanes 14 and 17). These results indicate that the critical T376 residue of huCdc7 could be a target of phosphorylation by Cdks.

Discussion

Cdc7 kinase plays a crucial role in activation of preRC at each origin (29,30). Genetic and biochemical evidence indicates that the MCM complex is an important target of Cdc7 kinase. Among the six components of MCM, MCM2 appears to be the conserved target of Cdc7-related kinases (7,12,22). Cdc7 and its related kinases require a regulatory subunit for expression of its kinase activity (16). Dbf4, Him1/ Dfp1 and ASK form complexes with Cdc7, Hsk1 and huCdc7, respectively, and activate or stimulate the kinase activity of the latter subunits. In this report, we have purified the huCdc7-ASK kinase complex and characterized its biochemical properties and phosphorylation reactions. We attempt to explore the functional interactions among MCM, Cdc7 and Cdks, using in vitro kinase assays with purified proteins.

ASK subunit is absolutely required for kinase activity of huCdc7

We previously reported that coexpression of ASK is required for kinase activity of huCdc7 in transient transfection assays in mammalian cells (13). We have confirmed this using purified protein preparations. The purified GST-huCdc7 protein was completely inert as a kinase. The complex of GST-huCdc7 and ASK was active and autophosphorylated both subunits in vitro. S. cerevisiae Cdc7 kinase also totally depends on association with its regulatory subunit Dbf4 protein for expression of its kinase activity (Masai, H., unpublished results). In contrast, fission yeast Hsk1 kinase possesses intrinsic kinase activity on its own, which is highly stimulated by the presence of Him1/ Dfp1 protein (12,14). Whereas purified Him1/ Dfp1 protein is capable of activating purified Hsk1 protein in vitro (our unpublished result; 12), our attempt to reconstitute the active huCdc7-
ASK kinase complex from purified proteins failed. An assembly factor may be required for formation of the active huCdc7-ASK kinase complex, as was reported for assembly of Cdk7-CyclinH complex (31). Alternatively, prior modification of either or both subunits may be required for stable association of the two proteins.

**Substrates of huCdc7-ASK kinase complex in vitro**

We previously reported that MCM subunits could be phosphorylated by huCdc7 in vitro (7). Subsequently, MCM2 was reported to be phosphorylated by Cdc7-related kinases in yeasts both in vivo and in vitro (12,22; Takeda et al., submitted). We therefore tested whether the purified huCdc7 kinase complex could phosphorylate MCM proteins in vitro. We purified various subassemblies of MCM protein complexes and used them in in vitro phosphorylation assays. Free MCM2 as well as MCM2 in the MCM2-4-6-7 complex served as an excellent substrate for huCdc7. Quantification of in vitro phosphorylated MCM2 protein indicated the presence of multiple phosphorylation sites on MCM2 by huCdc7. The phosphorylation sites on MCM2 in the complex may not be identical with those on free MCM2, as indicated by differential mobility shift on SDS-PAGE as well as by two-dimensional peptide mapping (Cho et al.; unpublished data).

Prior phosphorylation of MCM2 in insect cells may affect the efficacy of Cdc7-mediated phosphorylation. In fact, prior dephosphorylation of the MCM complex reduced the efficiency of huCdc7-mediated phosphorylation by about 50%, although the identical mobility-shift was observed on both untreated and dephosphorylated MCM2 proteins. Cophosphorylation of the MCM2-4-6-7 complex with Cdk2-CyclinA/Cdk2-CyclinE/Cdc2-CyclinB and huCdc7-ASK resulted in enhanced phosphorylation of MCM2 by huCdc7 kinase. Most notably, the appearance of the mobility-shifted form of MCM2 was significantly stimulated by prior phosphorylation of the MCM complex by Cdks (Figure 6D), suggesting the presence of a specific phosphorylation site(s) on MCM2, the recognition of which by huCdc7 is highly facilitated by Cdk-mediated phosphorylation of the complex. This phosphorylation appears to be specific to MCM2 in the complex, since stimulation of Cdc7-mediated phosphorylation of MCM2 by Cdks occurs only with
MCM2-4-6-7 complex, not with free MCM2 protein (data not shown). Cdk2-CyclinE and Cdk2-CyclinA can phosphorylate both MCM2 and MCM4 in the MCM2-4-6-7 complex. These phosphorylation events may induce conformational change of the MCM2-4-6-7 complex, facilitating the phosphorylation of MCM2 by huCdc7 kinase. Our results suggest the importance of concerted actions of Cdks and Cdc7 on MCM in regulation of S phase.

Although ample genetic and biochemical evidence demonstrates that MCM is essential for initiation of DNA replication, the precise role of MCM complex in origin activation is still not clear. Recent demonstration that DNA helicase activity is associated with the MCM4-6-7 complex (23,32) may suggest that MCM plays essential roles in duplex melting at the origin and/ or duplex unwinding at the replication forks. The possibility that phosphorylation of MCM by huCdc7 modulates DNA helicase activity of MCM complex is currently being explored. It is intriguing that SV40 T antigen, which is known to act as DNA helicase at the forks (33), can be efficiently phosphorylated by huCdc7-ASK in vitro (Figure 4A). BPV-encoded E1 protein, a viral helicase, can also be phosphorylated by huCdc7 in vitro (34 and data not shown). DNA helicase activity of SV40 T antigen is known to be activated through phosphorylation of specific residues by multiple kinases (35), and the possibility that huCdc7 kinase is capable of activating T antigen is currently being examined.

A number of other replication proteins were phosphorylated in vitro by purified huCdc7-ASK protein, including p180, p68 and p54 subunits of DNA polymerase α (36) and ORC4 (25, 37). The physiological significance of these phosphorylation events in vivo needs to be examined with genetic characterization in yeasts.

**huCdc7 may be a target of Cdks**

Although huCdc7 kinase activity appears to be largely determined by the level of ASK subunit, which increases during S phase, posttranslational regulation of its kinase activity may also operate. The timing of kinase activation places this kinase downstream of G1 and G1/S Cdk. There are four SP/ TP sites on huCdc7 which are potential phosphorylation sites by Cdks. Among the four serine/ threonine-to-alanine mutants of huCdc7, only
T376A mutant displayed significantly reduced kinase activity in vitro. This T residue corresponds to T160 of Cdk, an activation threonine, whose phosphorylation by CAK is essential for the kinase activity (38). Alanine substitution of the corresponding threonine of budding yeast Cdc7 resulted in a mutant with attenuated activity both in vivo and in vitro (18, 19; Masai, H., unpublished data).

We have shown that huCdc7 protein can be phosphorylated in vitro by Cdk2-CyclinA, Cdk2-CyclinE, and Cdc2-CyclinB, whereas Cdk4-CyclinD1 did not phosphorylate it to any significant level. We have developed a specific antibody which recognizes the phosphorylated T376 residue, and have presented evidence that Cdk2-CyclinE, Cdk2-CyclinA and Cdc2-CyclinB are able to phosphorylate T376 in vitro. It is especially an intriguing possibility that Cdk2-CyclinE targets huCdc7 for activation by phosphorylating the T376 residue. However, as seen in Figure 6B, the presence of Cdks did not stimulate the kinase activity of huCdc7-ASK complex in vitro as judged by the level of autophosphorylation. This could be because the T376 residue of huCdc7 within the complex may have already been phosphorylated by insect cell-derived kinase(s). In fact, significant level of phosphorylation of T376 in GST-huCdc7(WT)-myc-ASK preparation is detected by the phospho-T376-specific antibody (data not shown). Therefore, fully dephosphorylated huCdc7-ASK complex needs to be prepared in order to examine the effect of T376 phosphorylation on its kinase activity. Furthermore, analyses of in vivo phosphorylation of huCdc7 kinase during cell cycle would be needed to clarify the physiological role of this phosphorylation.

Although other three TA/SA mutations did not affect the intrinsic kinase activity of huCdc7, it is possible that phosphorylation of these residues by Cdks may regulate interaction of huCdc7-ASK complex with chromatin or other replication proteins. Multiple mobility-shifted bands of huCdc7 after phosphorylation by Cdks indicate the presence of more than one phosphorylation sites on huCdc7. In yeasts, Cdc7-Dbf4 was reported to be associated with chromatin only during S phase (39). The possibility that cell cycle-dependent association and dissociation of Cdc7 kinase complex with chromatin may be regulated by phosphorylation by Cdks is now being examined.
Acknowledgement

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References


16. Johnston, L. H., Masai, H., and Sugino, A. First the CDKs, now the DDKs. (1999)

Legends to figures

Figure 1  **In vitro** phosphorylation assays with insect cell-expressed huCdc7-myc-ASK complex
The wild-type, KE or KR mutant of huCdc7 was coexpressed with myc-tagged ASK (lanes 1-3) or only the wild-type huCdc7 was expressed (lane 4) in insect cells and extracts were prepared. Proteins were immunoprecipitated by anti-myc antibody (lanes 1-3) or with anti-huCdc7 antibody (lane 4), and used for *in vitro* kinase assays as described in “Experimental Procedures”. Products were analyzed on 10% SDS-PAGE and were autoradiographed (upper panel) or blotted with anti-huCdc7 antibody (lower panel). Lane 1, wild-type huCdc7 and myc-ASK; lane 2, KE huCdc7 and myc-ASK; lane 3, KR huCdc7 and myc-ASK; and lane 4, wild-type huCdc7 alone.

Figure 2  **In vitro** kinase assays with purified GST-fused huCdc7-myc-ASK complexes.
Either wild-type or KE mutant GST-fused huCdc7 was coexpressed with myc-ASK in insect cells and the complex was purified by glutathione Sepharose 4B column. A. Purified proteins were analyzed on 8% SDS-PAGE and were stained with silver. Lanes 1 and 2,
wild-type huCdc7 and myc-ASK; lane 3, KE-huCdc7 and myc-ASK; lane 4, wild-type huCdc7 alone; and lane 5, KEhuCdc7 alone. In lane 1, purified protein fraction was treated with λ phosphatase prior to electrophoresis to eliminate phosphorylation which causes mobility-shift on the gel. The smear appearing between the 80 and 110 kDa markers in lane 2 represents autophosphorylated forms of myc-ASK protein. B. Kinase assays were conducted with the purified proteins in the presence of GST-huMCM2N protein as a substrate and products were analyzed on 8% SDS-PAGE, followed by autoradiograph. Lane 1, wild-type huCdc7 and myc-ASK; lane 2, KE-huCdc7 and myc-ASK; lane 3, wild-type huCdc7 alone; and lane 4, KEhuCdc7 alone.

Figure 3 Characterization of phosphorylation reaction by purified Cdc7 kinase complex.
A. Five X reaction mixture (125 µl) of the standard in vitro kinase assay for huCdc7 was incubated at 30°C. B. Standard in vitro phosphorylation reactions were conducted in the presence of various amount of purified huCdc7 kinase complex as indicated. C. In vitro phosphorylation assays were conducted in the presence of various concentrations of magnesium acetate (open circles) and manganese chloride (closed circles). In A and B, open and closed squares indicate the reaction with wild-type GST-huCdc7+myc-ASK and KE GST-huCdc7+myc-ASK, respectively. In A, B and C, full-length MCM2 protein (5 pmole per 25µl reaction mixture) was used as a substrate and ten µl was withdrawn at each time point or from each reaction mixture and incorporation of 32P into proteins were measured by spotting onto P81 filter paper followed by extensive washing in 75 mM phosphoric acid. The values represent those for 25µl reaction. D. In vitro phosphorylation assays were conducted in the presence of various concentrations of ATP or 1 mM of GTP, CTP or UTP with purified MCM2-4-6-7 complex as a substrate. The MCM2-4-6-7 complex was affinity-purified and used without prior phosphatase treatment and purification with monoQ. The reactions did not contain labeled nucleotides and the products were analyzed on 8% SDS-PAGE, followed by silver staining of the gel. The phosphorylation of MCM2 and MCM4 was monitored by the intensities of the mobility-
shifted bands on SDS-PAGE, which are phosphorylated forms. The ratio of the mobility-shifted forms (P-MCM2 or P-MCM4) to the unphosphorylated form (MCM2 or MCM4) was calculated and plotted for titration of ATP.

**Figure 4** Phosphorylation of various substrates by purified huCdc7 kinase complex in vitro.

A. Standard reaction mixtures for in vitro phosphorylation by huCdc7-ASK kinase complex were incubated in the presence of various substrates shown for 30 min at 30°C, and products were analyzed on 8% SDS-PAGE. The gel was stained with silver (upper panel) and dried for autoradiogram (lower panel). Lanes 1 and 2, no substrate; lanes 3 and 4, uncomplexed MCM2; lanes 5 and 6, MCM2-4-6-7 complex; lanes 7 and 8, MCM4-6-7 complex; and lanes 9 and 10, MCM3-5 complex; and lanes 11 and 12, SV40 T-antigen. 

B. Quantification of ³²P incorporation into MCM2. In vitro phosphorylation assays were conducted with various amounts of the full length MCM2 protein as substrate. After the products were run on 8% SDS-PAGE and transferred to 3MM paper, the phosphorylated MCM2 bands were excised and radioactivity was measured by scintillation counter. The lower panel is the autoradiogram of the SDS-PAGE on which products were run. The upper panel shows MCM2 protein in each reaction mixture visualized by silver staining on a separate SDS-PAGE. The specific activity of [γ-³²P] ATP in the reaction mixtures were 860 cpm per pmole of ATP. Below the panels, the amounts of MCM2 protein and incorporation of ³²P into MCM2 in each reaction are indicated. The values for incorporation have been corrected by subtraction of background incorporation on the lane 1 (without MCM2 protein), which represents portion of the autophosphorylation of myc-tagged ASK protein.

**Figure 5** Effect of prior dephosphorylation of MCM complex on efficacy of phosphorylation by huCdc7 kinase.

Affinity-purified MCM2 and MCM2-4-6-7 were further purified by monoQ column. One portion of MCM2-4-6-7 was treated with phosphatase prior to purification with monoQ. A. In vitro phosphorylation assays were conducted with increasing amount of each substrate in
the reaction mixtures, and the products were analyzed on 8% SDS-PAGE, which was silver-stained (upper panels), dried and autoradiographed (lower panels). The numbers indicate the positions of each subunit of MCM. B. The levels of MCM2 phosphorylation on the autoradiogram in A were quantified by Fuji image analyzer. The amount of MCM2 protein in each reaction mix was also measured by scanning the intensities of silver stained bands. The both measurements were expressed as relative values and they were plotted. Open squares, untreated MCM2-4-6-7; closed squares, phosphatase-treated MCM2-4-6-7; open circles, untreated full-length MCM2.

Figure 6 Phosphorylation of MCM by various Cdks in vitro and its effect on phosphorylation by huCdc7 kinase
A. Affinity-purified MCM2-4-6-7 was phosphorylated in vitro by huCdc7-ASK or by various Cdks (lanes 1-7). Lanes 8-12 do not contain any MCM. Kinases used are; lane 1, none; lanes 2 and 12, wild-type huCdc7-myc-ASK; lane 3, KEhuCdc7-myc-ASK; lanes 4 and 8, Cdk4-CyclinD1; lanes 5 and 9, Cdk2-CyclinE; lanes 6 and 10, Cdk2-CyclinA; lanes 7 and 11, Cdc2-CyclinB. Upper and lower panels show respectively, the silver staining and autoradiogram of the 8% SDS-PAGE on which products were analyzed. B. Effect of Cdk on rate of MCM2 phosphorylation by huCdc7. Lanes 1-14, dephosphorylated MCM2-4-6-7 complex as substrate; lanes 15-16, untreated MCM2-4-6-7 complex as substrate. Lanes 1 and 2, no Cdk; lanes 3-5 ; Cdk4-CyclinD1; lanes 6-8; Cdk2-CyclinE; lanes 9-11, Cdk2-CyclinA; lanes 12-14, Cdc2-CyclinB. Lanes 1, 4, 7, 10, 13, and 15, wild-type GST-huCdc7 + myc-ASK; lanes 2, 5, 8, 11, 14 and 16, KE GST-huCdc7 + myc-ASK. The lower panel is the short exposure of the same gel, in which only the protein bands of MCM2 are visible. C. p27 protein was incubated in the standard kinase assays for Cdks and huCdc7 and incorporation was measured by spotting an aliquot on P81 paper. HistoneH1 (200 pmole) and MCM2-4-6-7 complex (3 pmole) were used as substrates for Cdks and huCdc7, respectively. One hundred % incorporation in the absence of p27 was 81 pmole, 56 pmole and 11 pmole for Cdk2-CyclinE, Cdk1-CyclinA and huCdc7-ASK, respectively. D. Two-step kinase reactions were conducted as described in “Experimental Procedures”.

27
protein was included in the second stage (lanes 1-15) or in the first stage (lanes 16-26). Lanes 1-21, dephosphorylated MCM2-4-6-7 as substrate; lanes 22-26, phosphorylated MCM2-4-6-7 as substrate. Cdk2-CyclinE (lanes 6-10, 18 and 19), Cdk2-CyclinA (lanes 11-15, 21 and 21) or none (lanes 1-5, 16, 17, 22-26) was added to the first stage reaction. Aliquots were withdrawn at t=0 min (at the time of addition of huCdc7-ASK and radioactive ATP; lanes 1, 6, 11, 16, 18, 20 and 22), 5 min (lanes 2, 7, 12 and 23), 10 min (lanes 3, 8, 13 and 24), 20 min (lanes 4, 9, 14 and 25), 30 min (lanes 5, 10, 15, 17, 19, 21 and 26) for analysis on 8% SDS-PAGE. The levels of phosphate incorporation into HistoneH1 (400 pmole) by Cdk2-CyclinE, Cdk2-CyclinA and Cdc2-CyclinB used in A, B and D above were 280 pmole, 190 pmole, and 210 pmole, respectively. The levels of phosphate incorporation into Rb protein (residue 769-921 of mouse Rb protein fused to GST, 150 pmole; purchased from Santa Cruz) by Cdk4-CyclinD1 and Cdk2-CyclinE were 61 pmole and 93 pmole, respectively.

**Figure 7** Kinase activities of huCdc7 mutants lacking possible Cdk phosphorylation sites

A. Schematic representation of the positions of possible Cdk phosphorylation sites and that of the conserved lysine residue mutated in KE and KR mutants. Roman letters indicate the conserved kinase motifs proposed by Hank et al. (40). B. Wild-type or mutant huCdc7 was coexpressed with myc-tagged ASK in insect cells and extracts were prepared. Proteins were immunoprecipitated by anti-huCdc7 antibody and were used for in vitro phosphorylation assays in the presence of GST-huMCM2N protein as a substrate. The huCdc7 used in the assays were: lane 1, wild-type; lane 2, KE; lane 3, S16A; lane 4, S302A, lane 5, T376A, and lane 6, T472A. Upper panel, autoradiogram of products of kinase assays run on 8% SDS-PAGE; middle panel, huCdc7 protein in the immunoprecipitates detected by western blotting with anti-huCdc7 antibody; lower panel, myc-ASK protein in the immunoprecipitates detected by western blotting with anti-myc antibody. Note that, with wild-type, S16A, S302A and T472A, myc-ASK is autophosphorylated and appears as a smear as a result of mobility shift. C. Phosphorylation of MCM2 by the purified T376A
GST-huCdc7-myc-ASK kinase complex. Upper panel, silver staining of the gel; lower panel, autoradiogram of the same gel. Kinases used were: lanes 1 and 5, no kinase; lanes 2 and 6, wild-type huCdc7 + myc-ASK; lanes 3 and 7, KE huCdc7 + myc-ASK; and lanes 4 and 8, T376A huCdc7 + myc-ASK. Substrates used were: lanes 1-4, free full-length MCM2; and lanes 5-8, MCM2-4-6-7 complex. M, molecular weight marker. The affinity-purified substrates were further purified with monoQ after λ phosphatase treatment.

**Figure 8** Phosphorylation of huCdc7 and ASK by Cdks.

A. *In vitro* phosphorylation of huCdc7-ASK with Cdks. Purified huCdc7 proteins were phosphorylated by various Cdks *in vitro* in the standard kinase reaction. Reaction was run on 8% SDS-PAGE in duplicate and one was dried and autoradiographed (Upper panel), while other was blotted by anti-phosphoT376 peptide antibody (middle panel). The latter was deprobed and reprobed by the huCdc7 monoclonal antibody 4A8 (lower panel). Lanes 1-4, no substrate; lanes 5-8, wild-type GST-huCdc7; lanes 9-12, T376A GST-huCdc7; lane 13-16, KR GST-huCdc7; lanes 17-20, KR GST-huCdc7 complexed with myc-ASK. Lanes 1, 5, 9, 13 and 17, no Cdk added; lanes 2, 6, 10, 14 and 18, Cdk2-CyclinE; lanes 3, 7, 11, 15 and 19, Cdk2-CyclinA; lanes 4, 8, 12, 16 and 20, Cdc2-CyclinB. The levels of Cdk activities used in this experiment are similar to those used in Figure 6. B. The *in vitro* phosphorylated T376A (lane 1) and wild-type (lane 2) GST-huCdc7 proteins were digested with CNBr and analyzed on a 24% Tricine gel. huCdc7 preparations used in the above experiments (A and B) were pretreated with λ phosphatase prior to affinity purification.
Figure 1

IP anti-myc anti-huCdc7

1 2 3 4 (kDa)

myc-ASK

huCdc7

33 50 80 106 (kDa)
Figure 2
Figure 3
**Figure 4**

A

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Silver staining

 Autoradiogram

- MCM2
- MCM6
- MCM4
- MCM7
- phosphorylated ASK
- MCM3
- MCM5

B

- 1
- 2
- 3
- 4
- 5

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Figure 5

(A) Silver staining and autoradiogram images showing the comparison of MCM2-4-6-7 untreated, MCM2-4-6-7 dephosphorylated, and MCM2 untreated samples.

(B) Graphical representation of relative phosphorylation vs. substrate (relative value) for MCM2-4-6-7; -PPase, MCM2-4-6-7; +PPase, and Free MCM2.
Figure 6

A

Silver staining

 Autoradiogram

 MCM2

 MCM6

 MCM4

 MCM7

 BSA

 Cdk4-D1

 Cdk2-E

 Cdk2-A

 Cdc2-B

 huCdc7/myc-ASK

 1 2 3 4 5 6 7 8 9 10 11 12

(kDa)

 B

 Dephosphorylated MCM2-4-6-7

 untreated MCM2-4-6-7

 Cdk4-D1  Cdk2-E  Cdk2-A  Cdc2-B

 WT KE  WT KE  WT KE  WT KE  WT KE  WT KE

 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

 MCM2

 MCM4

 huCdc7

 autoradiogram

 C

 Relative Phosphorylation (per cent)

 p27 Protein (microgram)

 D

 p27 in the second stage

 None  Cdk2-CyclinE  Cdk2-CyclinA

 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

 non-shifted MCM2

 mobility-shifted MCM2

 p27 in the first stage

 None  K2E  K2A

 (untreated substrate)

 16 17 18 19 20 21 22 23 24 25 26

 non-shifted MCM2

 mobility-shifted MCM2
Human Cdc7-related kinase complex: In vitro phosphorylation of MCM by concerted actions of Cdk5 and Cdc7 and that of a critical threonine residue of Cdc7 by Cdk5

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