A Second Human Dbf4/ASK-related Protein, Drf1/ASKL1, Is Required for Efficient Progression of S and M Phases*\[S\]

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Cdc7-Dbf4 kinase is conserved through evolution and regulates initiation and progression of DNA replication. In human, ASK/hsDbf4 binds and activates huCdc7 during S phase and this kinase complex is essential for DNA replication and cell proliferation. Drf1/ASKL1, a second human Dbf4/ASK-related protein, shares three conserved Dbf4 motifs previously identified on all of the Dbf4-related molecules. Drf1/ASKL1 can bind and activate huCdc7, and Cdc7-ASKL1 complex phosphorylates MCM2. ASKL1 transcription and protein levels oscillate during cell cycle and increase at late S to G2/M phases. The protein is detected predominantly in the nuclear-soluble fraction but not in the chromatin-bound fraction. Inhibition of Drf1/ASKL1 expression by siRNA results in attenuation of cell growth and in the increase of late S and G2/M phase population. siRNA treatment on synchronized cell population revealed that S phase progression is delayed when ASKL1 protein level is decreased. S phase delay may be linked to replication fork block, because increased levels of γH2AX and activated form of Chk2 are detected with ASKL1 siRNA in the absence of any additional DNA damages. Furthermore, mitotic progression is retarded in ASKL1 or Cdc7 siRNA-treated cells. Our results suggest that ASKL1 in a complex with Cdc7 may play a role in normal progression of both S and M phases.

Cell cycle progression of eukaryotic cells is strictly regulated by a series of phosphorylation events. Among them, multiple cyclin-dependent kinases play crucial roles to facilitate the progression of cell cycle at various stages. Multiple cyclins and cyclin-dependent kinases have been identified in various eukaryotes, each acting at specific stages of the cell cycles (1–7). Conserved cyclin-box sequences have been identified on the genome of budding yeast. Spo4 and Spo6 were shown to play essential roles during meiosis but not during the mitotic cell cycle, in contrast to Hsk1-Dfp1/Him1, which is essential for cell viability. The presence of more than one Cdc7-Dbf4-related kinase in a single eukaryotic species suggests a possibility that they also constitute a novel kinase family, each member of which may play distinct roles in cell cycle progression.

We have identified a novel human cDNA, designated ASKL1,1 which shares significant homology with Dbf4 protein family. ASKL1 is identical to Drf1 reported recently by Montagnoli et al. (26). It was reported that Drf1 binds to huCdc7 and activates its kinase activity and that its transcription and protein levels increase during late S to G2/M. The protein exists in nuclei, and its role in S phase progression is speculated (26). However, the functional analyses of Drf1 have not been conducted and its precise roles during cell cycle remained elusive. Therefore, we have undertaken studies aimed at clarifying the roles of the Cdc7-ASKL1 complex during cell cycle regulation of human cells.

We have found that Drf1/ASKL1 is primarily recovered in nuclear-soluble fractions but is not associated with Triton-insoluble chromatin fractions. To elucidate biological functions of the Cdc7-ASKL1 complex, we examined the effects of down-regulation of Drf1/ASKL1 expression by siRNA on cell cycle progression. Inhibition of Drf1/ASKL1 expression resulted in retardation of both S phase and M phase progression. We discuss possible mechanisms of how Drf1/ASKL1 may regulate the cell cycle through association with huCdc7 kinase subunit.

EXPERIMENTAL PROCEDURES

Development of Antibody against ASKL1 Protein—DNA encoding a polypeptide of ASKL1 (residues 211–355; minimum ASKL1) was amplified by PCR (5'-CGGGGATCCCCGGAACATGTCCAGCAGCA-3' and 5'-CGGAATTCACCTGGGGAG GCCAGCCTG-3') and cloned into the BamHI-EcoRI site of pGEX-5X-3 to generate a GST-ASKL1[minimum] fusion protein. The protein was insoluble after over-expression and purified from inclusion bodies by extraction into SDS-PAGE running buffer followed by dialysis against PBS containing 0.1% SDS. The antibody was developed in rabbit, and the serum was run through a GST-affinity column to remove GST-reactive antibody and was further affinity-purified by adsorption to polyvinylidene difluoride membrane to which purified His-tagged ASKL1c protein (448 amino acids, also see Supplemental Fig. S2B) was transferred. The ASKL1c DNA was amplified by 5'-GGGATCCCATATGAGCGAACCGG-
GAAGGGGAGC-3' and 5'-CGAATTTCTAGTGATGGTGATGATGATGGAGAGAAGATGCTCCGGGGCGGGA-3' to be subcloned into a T7 promoter vector, pT7-7. The antibody was eluted from the membrane by the 0.2 M glycine, pH 2.8, and 1 mM EGTA, and the pH of the eluate was adjusted by addition of 0.1 volume of 1 M Tris base.

Other Antibodies—Antibodies against huCdc7, anti-huCdc781, were described previously (13). Anti-H2AX S139P antibody was a gift from Katsuyuki Tamai. Anti-GST, anti-Myc, anti-Ieβ, anti-lamin B2, anti-Chk1, and anti-Chk2 antibodies were from Santa Cruz Biotechnology. Anti-α-tubulin and anti-FLAG M2 monoclonal antibodies were from Sigma. Anti-Chk1 S37P and anti-Chk2 T68P antibodies were from Cell Signaling Technology. Anti-RPA p34 antibody was from NeoMarkers. In some experiments, anti-Cdc7 mouse monoclonal antibody from MBL was used.

Establishment of Stable Cell Lines Expressing GST-ASKL1—A NotI-fragment containing GST-ASKL1(minimum) or GST coding region was cloned at the NotI site of pGEM vector, and the resulting plasmid DNA was transfected into HeLa cells with Lipofectamine Plus reagent (Invitrogen). After 2 days of incubation, cells were grown in a medium containing 100 μg/ml G418 for 10 days and the surviving colonies were selected. They were further cloned and amplified to establish HeLa cells constitutively expressing GST-ASKL1 or GST protein.

Synchronization of Cell Cycle and Cell Fractionation—Cell cycle of HeLa cells was synchronized either by release from G1/S arrest or that from mitotic arrest. For G1/S arrest, the cells were treated with 2.5 mM thymidine for 14–16 h twice with a 9-h interval of growth without the drug. For mitotic arrest, the cells were first treated with 2.5 mM thymidine for 16 h and then treated with 50 μg/ml nocodazole for 8 or 10 h. The cells then were incubated in the absence of the drug for indicated times and harvested. Half of the cells were resuspended in 70% ethanol for 16 h and then treated with 50 ng/ml nocodazole for 8 or 10 h. The medium containing three short stretches of amino acids conserved in Dbf4-related molecules and named them Dbf4 motif-N, -M, and -C (27). Further characterization of these motifs revealed that the motif-M and -C polypeptides are capable of binding to the catalytic subunit and that the bipartite binding of these two motifs is essential and sufficient for activation of Cdc7 kinase (28, 29).
and hemagglutinin-tagged huCdc7 in bacterial cells and purified the complex. The wild-type complex was active in phosphorylating MCM2 in the MCM2-MCM4-MCM6-MCM7 (data not shown). These results indicate that Drf1/ASK1 can form active kinase complexes with huCdc7 and that the amino acid segment containing motif-M and motif-C is required and sufficient for the generation of an active kinase.

We also examined whether endogenous huCdc7 associates with Drf1/ASK1. We have established a stable HeLa cell line expressing GST-ASK1 fusion protein containing the “minimum” ASKL1 region (Dbf4 motif-M and -C) under a constitutive active promoter. In control, a stable cell line expressing GST alone was also established. The GST-ASK1[minimum] stable cell line was synchronized by hydroxyurea arrest, and GST-ASK1 was pulled down from the Triton-soluble extracts at 0 h (early S) and 9 h (G2/M) after release. Western blotting confirmed the presence of GST-ASK1 protein. More GST-ASK1 was pulled down from the G2/M extracts (Fig. 1A, lanes 3 and 4). In the same fraction, huCdc7 protein was detected and its amount was roughly proportional to that of GST-ASK1. In control, the pull down from HeLa cells expressing GST alone indicated the presence of an equal amount of GST protein in early S and G2/M extracts but no huCdc7 was detected (Fig. 1A, lanes 1 and 2). Endogenous ASKL1 protein was immunoprecipitated next with affinity-purified anti-ASKL1 antibody from Triton-soluble extracts of asynchronous culture (Fig. 1B, left panel, lane 1), and Cdc7 was detected in the immunoprecipitates (Fig. 1B, right panel, lane 4). The interaction was detected at any stages of cell cycles, but ASKL1-bound fractions of huCdc7 appeared to increase during S phase (Fig. 1C, lanes 2 and 3). These results indicate that endogenous huCdc7 forms a complex with ASKL1 throughout the cell cycle.

Expression of Drf1/ASK1 during Cell Cycle—Transcription of human ASK is known to fluctuate during the cell cycle, increasing at late G1 and staying at a high level during the S phase (16). HeLa cells were synchronized, and the expression of Drf1/ASK1 was examined by Northern analyses during cell cycle progression. After release from hydroxyurea arrest, the Drf1/ASK1 transcript increased at 8–10 h after release when the cells were in late S to G2 and started to increase again at the next late S phase (24 h after release) (Supplemental Fig. S3A). In the release from arrest at mitosis by TN-16 treatment, transcription of Drf1/ASK1 was high at the time of release and decreased as the cell cycle progressed into G2/S. It increased again in the next late S phase (Supplemental Fig. S3B). This result indicates that transcription of Drf1/ASK1 is regulated during cell cycle and increases at the late stage of DNA synthesis through G2 and mitosis. Transcription of Drf1/ASK1 was detected in various other cell lines including human kidney-derived 293 cells and human amnion-derived FL cells, and its level in these cell lines also increased after arrest with TN-16 (data not shown).

Affinity-purified anti-ASKL1 antibody was used to identify the endogenous Drf1/ASK1 protein. Triton-soluble and -insoluble fractions were prepared from asynchronously growing HeLa cells and were examined by Western blotting. The bands migrating slightly faster than FLAG-tagged ASKL1 expressed in COS7 cells were identified in the Triton-soluble fraction. To confirm that this band indeed represents the endogenous Drf1/ASK1 protein, we conducted siRNA experiments. We have designed four different siRNA derived from different portions of Drf1/ASK1 as well as control siRNA derived from the luciferase gene. The intensities of the putative Drf1/ASK1 protein bands decreased with ASKL1-1 and ASKL1-2 siRNA but not with other siRNAs (Fig. 2A). This result strongly indicates that they represent the endogenous ASKL1 protein. We were not able to determine the cause for the presence of two bands.

Drf1/ASK1 protein level was examined in cells in which cell cycle was synchronized by release from double thymidine block-induced G1/S arrest. The Drf1/ASK1 protein was detected almost exclusively in the nuclear-soluble fraction (Fig. 2B). The protein level increased as the cells entered late S through G2/M (9–12 h), slightly decreased during G2/S (18 h), and increased again at next late S to G2/M (24 h) (Supplemental Fig. S3C). Drf1/ASK1 protein was detected in the cytoplasmic fraction at a low level but was not detected in the Triton-soluble chromatin-enriched fractions throughout the cell cycle (data not shown). These results indicate that the Drf1/ASK1 protein level oscillates during cell cycle and increases at late S to G2/M and that the protein is almost exclusively present in the nuclear-soluble fraction.

To examine whether the protein level oscillates in a manner independent from transcriptional regulation, stably expressed GST-ASK1[minimum] protein under the control of a constitutive promoter was examined. The cells were released from hydroxyurea arrest and were fractionated into cytoplasm and nuclear-soluble and nuclear pellet fractions. The levels of the stably expressed proteins were detected by anti-GST antibody (Supplemental Fig. S3D). The majority of GST protein was detected in cytoplasmic fractions, and a small amount was
detected in nuclear-soluble fractions at a constant level throughout the cell cycle. In contrast, GST-ASKL1 protein was detected in both cytoplasmic and nuclear-soluble fractions but not in nuclear pellet fractions. The total amount of GST-ASKL1 protein peaked at late S to G2/M phase. The protein level in nuclear-soluble fractions reached maximum at 6–8 h after release (late S to G2), decreased at 10–12 h (M to G1), and increased again at 24 h (late S), whereas the protein level in cytoplasm reached maximum at 10 h after release. In controls, RPA p34 subunits were detected in nuclear pellet (enriched in chromatin-bound proteins) only during S phase and I/H9260/H9252 protein was recovered only in cytoplasm fractions, verifying the procedure of cell fractionation. Because the transcription of the exogenous GST-ASKL1 is under a constitutive promoter (30), the result indicates that the level of Drf1/ASKL1 protein is regulated at post-transcriptional levels as well.

Potential Roles of Drf1/ASKL1 during Cell Cycle Progression—To obtain insight into the roles of Drf1/ASKL1 in human cell cycle progression, we have examined the effect of reducing the level of Drf1/ASKL1, ASK, and Cdc7 proteins in HeLa cells. The knockdown of target proteins or transcript was confirmed by immunoblotting or RT-PCR in HeLa cells treated with siRNA specific to ASKL1, ASK, or Cdc7. Drf1/ASKL1 and Cdc7 protein levels were reduced in Drf1/ASKL1 and Cdc7 siRNA-treated cells, respectively (Fig. 2C, lanes 3 and 4, and 7 and 8). Drf1/ASKL1 protein level was reduced also in Cdc7 siRNA-treated cells (Fig. 2C, lane 7). Reduction of ASK expression was confirmed by RT-PCR with ASK siRNA-treated cells (Fig. 2D, lanes 3 and 4).
Depletion of Drf1/ASKL1 leads to retardation of S phase progression. A, cells treated with ASKL1 siRNA sense strand (control) or ASKL1 siRNA were synchronized by double thymidine block and released into S phase with the protocol shown. BrdUrd was added to the culture for 30 min before the harvest. DNA contents (B) and BrdUrd incorporation (C) of the siRNA-treated HeLa cells in A were analyzed by flow cytometry at 0, 3, 6, 9, 10.5, and 12 h after release. In C, DNA contents (x axis) and BrdUrd incorporation (y axis) were plotted. D, fractions of...
Depletion of ASKL1 Slows Down Mitotic Progression and Inhibits Release from Mitosis—We previously noted that mitotic cells seemed to be increased when siRNA for ASKL1 was transfected into HeLa cells. Quantification of each cell cycle phase of the FACS data revealed that G2/M phase cells in ASKL1 siRNA- and control siRNA-treated cells were 33.1 and 11.6%, whereas G1 cell populations were 29.0 and 41.7%, respectively (Fig. 2, F and G), suggesting the increase in mitotic cells after ASKL1 siRNA treatment in HeLa cells. To strictly distinguish the cells at interphase including G2 and those at mitosis, siRNA-treated cells were subjected to detailed microscopic observation. The precise stage of mitosis at which the mitotic cells were arrested was determined by visualizing chromatin structures with Hoechst 33342 staining. The results indicated that the increase in mitotic cells in ASKL1 or Cdc7 siRNA-treated cells was statistically valid (Fig. 4A).

In the next set of experiments, siRNA treatment was combined with mitotic cell arrest. HeLa cells transfected with siRNA were blocked by nocodazole and then released into cell cycle for 4 h. The cells were observed by phase-contrast microscopy (Fig. 4C). In comparison with the control untransfected cells in which the majority of the population exited the M phase and entered G1 phase, ASKL1- and Cdc7-depleted cells at 4 h after release from double-thymidine block (Fig. 4B). The delay of M phase was observed also with Cdc7 depletion under the similar condition (data not shown).

In the next set of experiments, siRNA treatment was combined with mitotic cell arrest. HeLa cells transfected with siRNA were blocked by nocodazole and then released into cell cycle for 4 h. The cells were observed by phase-contrast microscopy (Fig. 4C). In comparison with the control untransfected cells in which the majority of the population exited the M phase and entered G1 phase, ASKL1- and Cdc7-depleted cells at 4 h after nocodazole release displayed increased fractions of mitotic population: 89.5, 26.2, and 13.8% in Cdc7 siRNA-treated, ASKL1 siRNA-treated, or untreated cells, respectively (Fig. 4D). These results indicate that mitotic progression is delayed or inhibited in Cdc7 or ASKL1 siRNA-treated HeLa cells. The magnified images of aberrant mitotic cells at 4 h after nocodazole release included those arrested at various stages of mitosis (data not shown), indicating that the arrest was not at a specific stage of mitosis. The population analyses of mitotic stages in ASKL1 or Cdc7 siRNA-treated asynchronous cells also supported the conclusion that mitotic cells at a particular stage were not accumulated (Supplemental Fig. S4A). In addition, the multinucleated population also increased in ASKL1 and Cdc7 siRNA-treated cells (Supplemental Fig. S4B), suggesting a defect in nuclear division or cytokinesis.

BrdUrd-positive cells in control or ASKL1 siRNA-treated cells of C are presented at each time point. The gate definition of BrdUrd-positive cells is shown by a polygon in C. Open column, control; filled column, ASKL1 siRNA. This experiment was conducted three times with similar results, and representative data are shown. E, cells treated with siRNA as indicated were synchronized by nocodazole block/release with the protocol shown. F, flow cytometry analyses of DNA contents of the siRNA-treated HeLa cells in E at 0, 3, 6, 12, 24, and 36 h after release. G, siRNA-treated HeLa cells at the times indicated after release from nocodazole block were stained with anti-BrdUrd antibody (upper of each pair of rows) or with 4′,6-diamidino-2-phenylindole (lower of each pair of rows). Upper pair, luciferase siRNA; middle pair, Cdc7 siRNA; lower pair, ASKL1 siRNA. H, the percent of BrdUrd-positive cells at each time point in the cell populations of G were counted and presented. The gray and black segments of the columns indicated weakly and strongly BrdUrd-positive cells. In A and E, the shaded zones indicate the times when cells are growing in the presence of 2.5 mM thymidine or 50 ng/ml nocodazole, respectively, whereas the dark gray zones indicate the times when transfection is conducted.
Double-stranded DNA Breaks Are Generated in ASKL1 siRNA-treated Cells—The above results indicate that S phase progression is retarded by depletion of ASKL1 protein in HeLa cells, suggesting a possibility that replication forks are stalled. Therefore, we have examined the induction of DNA damages by using γH2AX antibody, which detects double-stranded DNA breaks. γH2AX signal was induced by hydroxyurea or UV treatment of control cells (Fig. 5, lanes 4 and 6). In contrast, in ASKL1 siRNA-treated cells, γH2AX signals were detected without the damaging agents (Fig. 5, lane 8). Similarly, damage-independent γH2AX signal was detected in ASK siRNA-treated cells (Fig. 5, lane 14). We next examined whether Chk2 is activated in these cells. The antibody that recognizes the phosphorylated Thr68 residues detected Chk2 only after hydroxyurea or UV treatment in control cells (Fig. 5, lanes 3 and 5), whereas it reacted even without DNA damages in ASKL1- or ASK-treated cells (Fig. 5, lanes 7 and 13). On the other hand, Chk1 was not activated by ASKL1 siRNA treatment without
hydroxyurea or UV treatment (Fig. 5, lane 7). Cdc2 tyrosine 15 phosphorylation did not increase nor was the CyclinB-Cdc2 kinase activity inhibited in response to ASKL1 siRNA (data not shown), suggesting that G2/M checkpoint signal is not generated. These results indicate that down-regulation of ASKL1 in HeLa cells indeed arrests the replication fork progression, which leads to the generation of double-stranded DNA breaks or unusual DNA structures and activation of Chk2 kinase, although mitotic inhibition signals are not induced.

**DISCUSSION**

Cdc7-Dbf4 kinase complex plays crucial roles in regulating initiation and progression of DNA replication, and their functions appear to be conserved throughout evolution (15, 19). Dbf4 resembles cyclin molecules in that it is an essential regulatory subunit for the Cdc7 catalytic subunit (11) and that its expression is cell cycle-regulated, although there is no homology on the level of their primary structures. It has not been known whether Dbf4 forms a protein family, each member of which plays distinct functions. Recently, a second set of the Cdc7-Dbf4-related kinase complex, Spo4-Spo6, was identified to bind to chromatin during DNA replication (31), human Drf1/ASKL1 was not detected in chromatin-enriched Triton-insoluble fractions, even after DNA replication block or DNA damages (data not shown).

**FIG. 5. Induction of double-stranded DNA breaks and Chk2 activation by ASKL1 siRNA in HeLa cells.** HeLa cells were transfected once with control (ASKL1 sense siRNA, lanes 1–6), ASKL1 siRNA (lanes 7–12), or ASKL siRNA (lanes 13–18). Lanes 1, 2, 7, 8, 13, and 14 (NT), untreated cells. Lanes 3, 4, 9, 10, 15, and 16 (hydroxyurea HU), cells incubated with hydroxyurea (5 mM) for last 6 h before harvest. Lanes 5, 6, 11, 12, 17, and 18 (UV), cells irradiated with UV (50 J/m²) at 2 h before harvest. Untreated or ASKL1 siRNA-treated cells were recovered at 48 h after transfection, whereas with ASKL siRNA transfection (lanes 13–18), cells were harvested at 24 h after transfection to avoid cell death caused by this siRNA. Triton-soluble (S) and -insoluble (P) extracts were prepared from each sample, and proteins were detected by immunoblotting with antibodies indicated. NT, transfection without siRNA.
tion fork block. However, this is not likely because we did not detect an increase of Cdc2 tyrosine 15 phosphorylation or Cdc2 kinase inhibition by ASKL1 siRNA (data not shown). Morphological analyses of mitotic cells in ASKL1 siRNA-treated populations indicated they are not arrested at a particular stage of mitosis, although cells at prometaphase are slightly more abundant (Supplemental Fig. S4A). We also detected an increase of multinucleated cells (Supplemental Fig. S4B), which may result from aberrant nuclear division or the failure of cytokinesis. Similar mitotic delay and increase of multinucleated cells were observed with Cdc7 siRNA.

ASKL1 forms a complex with Cdc7 in nuclei, and the amounts of this complex increase at late S through G2/M. Cdc7-ASKL1 may phosphorylate the mitotic apparatus to facilitate the progression of M phase. Alternatively, the loss of ASKL1 may affect the proper processing of stalled replication forks, which may lead to altered chromatin structures of replicated molecules, including impairment of sister chromatid cohesion or of chromatin condensation. These defects in chromatin structures may directly or indirectly affect the processes of mitosis. In fact, we have shown previously (21) that sister chromatid cohesion is partially impaired in a fission yeast hsk1ts mutant. Furthermore, we recently obtained evidence showing that Hsk1 is required also for mitosis of fission yeast. These results indicate that the roles of Cdc7 during M phase may be conserved.

In summary, our results show that huCdc7 is regulated by two distinct activation subunits, ASK and ASKL1. ASKL1 may not be essential for DNA replication but may play supplemental roles in S phase progression and/or emergency roles in restoration of stalled replication forks as well as roles in M phase progression in conjunction with Cdc7 kinase.

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