Assembly of the Cdc45-Mcm2–7-GINS complex in human cells requires the Ctf4/And-1, RecQL4, and Mcm10 proteins

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Contributed by Jerard Hurwitz, July 17, 2009 (sent for review July 1, 2009)

In eukaryotes, the activation of the prereplicative complex and assembly of an active DNA unwinding complex are critical but poorly understood steps required for the initiation of DNA replication. In this report, we have used bimolecular fluorescence complementation assays in HeLa cells to examine the interactions between Cdc45, Mcm2–7, and the GINS complex (collectively called the CMG complex), which seem to play a key role in the formation and progression of replication forks. Interactions between the CMG components were observed only after the G_1/S transition of the cell cycle and were abolished by treatment of cells with either a CDK inhibitor or siRNA against the Cdc7 kinase. Stable association of CMG required all three components of the CMG complex as well as RecQL4, Ctf4/And-1, and Mcm10. Surprisingly, depletion of TopBP1, a homologue of Dpb11 that plays an essential role in the chromatin loading of Cdc45 and GINS in yeast cells, did not significantly affect CMG complex formation. These results suggest that the proteins involved in the assembly of initiation complexes in human cells may differ somewhat from those in yeast systems.

bimolecular fluorescence complementation | Cdc45-Mcm-GINS complex | cyclin-dependent kinase | Cdc7-Dbf4 kinase

The initiation of eukaryotic DNA replication is a multistep process that requires the assembly of the prereplicative complex (pre-RC), activation of the pre-RC and formation of the replisome (1, 2). Pre-RC assembly occurs during the G_1 phase of the cell cycle by a stepwise recruitment of the origin recognition complex (ORC), Cdc6, Cdt1, and the Mcm2–7 complex onto DNA origins. During the G_1/S transition stage, the recruitment of other replication factors such as Cdc45 and GINS and the combined actions of two S phase promoting kinases, the cyclin-dependent (CDK) and Cdc7-Dbf4 (DDK) kinases, lead to the assembly of an active DNA helicase complex at replication origins. This activation results in the unwinding of replication origins, the recruitment of DNA polymerases and accessory factors and the assembly of the replisome for DNA synthesis.

In eukaryotic cells, the Mcm2–7 complex seems to be the catalytic core of the replicative helicase. It is essential for origin unwinding and replication fork progression (3–6). It has been shown that a variety of Mcm complexes, including the Mcm4/6/7 subcomplex (from many species), double hexameric complexes of Mcm homologues in Archaea and the budding yeast Mcm2–7 complex, exhibit DNA helicase activity in vitro (1, 7). In vivo, however, formation of an active helicase at replication origins requires the further recruitment of several factors including Cdc45 and GINS, and this activation process is governed by CDK and DDK (1).

Both Cdc45 and GINS are required for the establishment and progression of the replication fork (8, 9). These proteins are loaded onto origins during S phase and form a stable complex with Mcm2–7 (10). The complex seems to be a DNA unwinding complex and moves along DNA as part of the replication fork complex (6, 11). Consistent with these observations, a complex of Mcm2–7, Cdc45, and GINS (the CMG complex), purified from *Drosophila* embryos, has DNA helicase activity in vitro (12).

In addition to the above components, the assembly of the DNA unwinding complex also depends on additional factors including Dpb11/Cut5, Sld2, Sld3, and Mcm10. Mcm10 is required for the chromatin binding of Cdc45 and DNA polymerase α in yeast and *Xenopus* (13–16). Dpb11/Cut5 is essential for origin binding of GINS in *Saccharomyces cerevisiae* (17), and reported to be required for chromatin binding of Cdc45 and DNA polymerase α in *Xenopus* (18). In yeasts, Sld2, Sld3 and Dbp11/Cut5 play essential roles for the origin binding of Cdc45. Recent studies revealed that Sld2 and Sld3 are the only replicative proteins that must be phosphorylated by CDKs for the initiation of replication (19, 20). Thus, the combined action of these proteins is critical for the loading of Cdc45 and GINS onto replication origins and the assembly of the DNA unwinding complex but how these proteins act remains unclear.

Whereas the requirements for these proteins have been partially defined in yeasts, the factors involved in the chromatin loading of Cdc45 and GINS in mammalian cells are less clear. Homologues of Sld3 have not been identified as yet in mammalian systems. Although neutralizing antibodies against TopBP1, the human homologue of Dpb11/Cut5, were reported to efficiently inhibit replicative DNA synthesis in HeLa cell nuclei in vitro (21), the major role of human TopBP1 seems to be in the S phase checkpoint control (22). *RECQL4*, which is mutated in the Rothmund–Thomson Syndrome, and Mcm10 were shown to be required for the initiation of DNA replication in human cells, but their roles in the initiation process are still unknown (23–26). Ctf4/And-1, which is required for sister chromatid cohesion in yeasts (27), was also shown to be essential for the chromatin binding of DNA polymerase α and for the initiation of DNA replication in mammalian cells (25).

To study the assembly of the preinitiation complex in human cells, we have used a bimolecular fluorescence complementation (BiFC) assay (28). We found that the interactions between the Cdc45, Mcm2–7 and GINS proteins occurred only after the G_1/S transition of the cell cycle and were dependent upon the CDK and Cdc7 kinase, similar to those reported in other systems (1). In addition, the stable interactions between CMG proteins required the simultaneous presence of all three components of the CMG complex as well as Mcm10, RecQL4 and Ctf4, but not TopBP1. These findings, made using BiFC assays, were also substantiated by chromatin loading experiments. They suggest

Author contributions: J.H. and J.-K.L. designed research; J.-S.I., S.-H.K., A.F., and D.-S.J. performed research; J.H. and J.-K.L. analyzed data; and J.H. and J.-K.L. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0908039106/DCSupplemental.

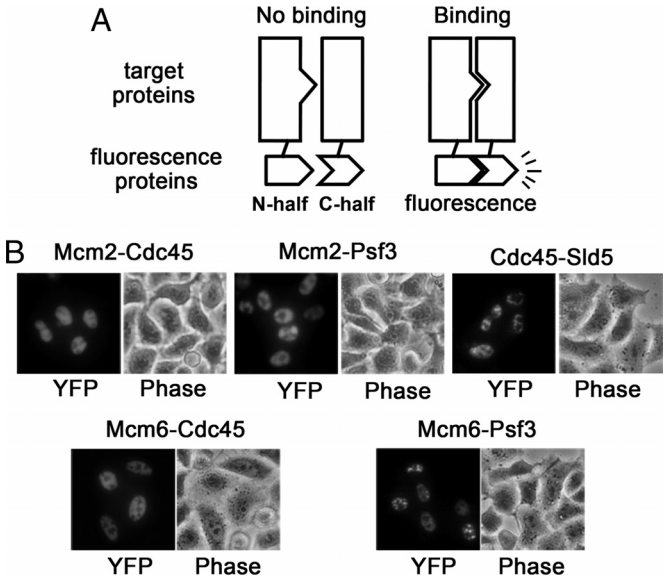

Fig. 1. Interactions between CMG proteins were examined by BiFC analysis in HeLa cells. (*A*) Principle of BiFC analysis. (*B*) HeLa cells were transfected with BiFC expression vectors fused to CMG component proteins as indicated; cells were then analyzed for fluorescence after incubation at 37 °C for 24 h.

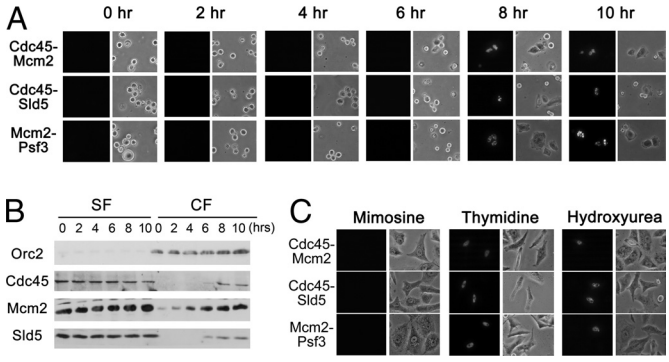

Fig. 2. Formation of the CMG complex occurs after the onset of the S phase during the cell cycle. (*A*) HeLa cells transfected with indicated expression vector pairs were treated with 100 ng/mL nocodazole for 24 h, and then released into the cell cycle in fresh medium without nocodazole. Cell fluorescence was examined at 2 h intervals, as indicated. (*B*) Chromatin fractions were prepared from HeLa cells synchronized as described in *A*, and the level of endogenous Orc2, Cdc45, Mcm2, and Sld5 proteins present in the supernatant function (SF) and chromatin fraction (CF) were analyzed by Western blotting. (*C*) HeLa cells were treated with 300 μM mimosine (for G_1 arrest) or 5 mM hydroxyurea (for early S arrest) for 12 h, and then transfected with the indicated BiFC expression vector pairs and incubated further for 24 h in the presence of mimosine or hydroxyurea. Cells subjected to thymidine double block (for G_1/S transition) were treated with 2.5 mM thymidine for 12 h and then released into the cell cycle in fresh medium for 14 h. These cells were transfected with the indicated BiFC expression vectors and incubated further for 24 h in a medium containing 2.5 mM thymidine.

that the proteins involved in the assembly of initiation complexes in human cells may differ somewhat from those in yeast systems.

Results

Detection of Stable Interactions Between CMG Proteins Using the BiFC Assay. To examine the assembly of critical protein complexes required for the initiation of DNA replication in human cells, we used BiFC assays to visualize these interactions. The basic principle of BiFC requires that two nonfluorescent fragments of a fluorescent protein associate to form a fluorescent complex (28). These interactions can be facilitated by fusing the nonfluorescent fragments to two different proteins that interact (Fig. 1*A*). Because a fluorescence signal does not occur in the absence of a stable interaction between the fused proteins, the emission of fluorescence reflects formation of a stable bimolecular complex between the two fused proteins.

We initiated the BiFC studies by examining the interactions between proteins constituting the CMG complex. For this purpose N-terminal (VN173: amino acids 1–173) or C-terminal (VN155: amino acids 155–238) fragments of the highly fluorescent mutated YFP (called Venus) were fused to the C terminus of indicated proteins. Vectors were constructed to express the fusion proteins, Mcm2-VN173, Mcm6-VN173, Sld5-VC155, Psf3-VC155, Cdc45-VN173 or Cdc45-VC155. When various combinations of these vectors were transfected into HeLa cells, strong fluorescent signals were observed in cells expressing Mcm2 and Cdc45, Mcm2 and Psf3, Cdc45 and Sld5, Mcm6 and Cdc45 or Mcm6 and Psf3 (Fig. 1*B*). Coexpression of Mcm2 and Sld5, Mcm6 and Sld5 or Cdc45 and Psf3, however, did not show the clear fluorescence signals. These results suggest direct interactions between Mcm2-Cdc45 or Psf3, Mcm6-Cdc45 or Psf3, and Cdc45-Sld5.

Interactions Between CMG Proteins Depend on the Cell Cycle, CDK, and Cdc7 Kinase. Previous studies in yeast and *Xenopus* systems showed that the loading of Cdc45 and GINS onto the pre-RC occurs only after the G_1/S transition, and requires both CDK and DDK activities. We tested whether the interaction between the CMG proteins showed identical requirements using the BiFC assay. For this purpose, cells transfected with BiFC expression vectors were arrested at G_2/M with nocodazole, and then analyzed for interactions every 2 h after being released into the cell cycle. As shown in Fig. 2*A*, fluorescence was detected in cells 8 h after their release into the cell cycle. The chromatin loading of Mcm2, Cdc45 and Sld5 was also monitored simultaneously to verify cell cycle progression. As shown in Fig. 2*B*, the loading of Mcm2 (reflecting Mcm2–7) revealed that a substantial number of cells were in G_1 phase 4 h after cells were released into the cell cycle, whereas the chromatin binding of Cdc45 and Sld5 showed that a significant percentage of cells were in S phase after 8 h (Fig. 2*B*); cells in G_2/M or G_1 (0, 2, and 4 h after release into the cell cycle) showed no fluorescence and thus no interactions between CMG proteins. Based on these results, interactions between CMG proteins seemed to occur only in S phase. To further verify these results, interactions between the CMG proteins were examined in cells treated with mimosine, thymidine or hydroxyurea. Cells arrested at G_1 with mimosine did not exhibit any fluorescent signal whereas cells fixed either in the G_1/S transition state (thymidine) or early S phase (hydroxyurea) did (Fig. 2*C*). Taken together, these results indicate that the interactions between CMG components occur after the onset of S phase.

We also tested whether interactions between CMG proteins depended on CDK and/or Cdc7 kinase (Fig. 3 *A–D*). When cells were treated with the CDK inhibitor, roscovitine (Fig. 3*A*), or transfected with siRNA against the Cdc7 kinase (Fig. 3*C*), which effectively reduced the level of Cdc7 (Fig. 3*D*), interactions between those proteins were not detected. As shown, treatment with roscovitine or Cdc7 siRNA did not alter the expression of Cdc45, Mcm2, or GINS components (Fig. 3 *B* and *D*).

Taken together, BiFC analyses revealed cell cycle- and CDK/Cdc7-dependent interactions between CMG proteins, confirming and extending observations made in both yeasts and *Xenopus* systems (1).

Stable Interactions Between CMG Proteins Require All Three Components of the CMG Complex. We next examined whether the depletion of Cdc45, Mcm2–7 or GINS affected the interaction between the

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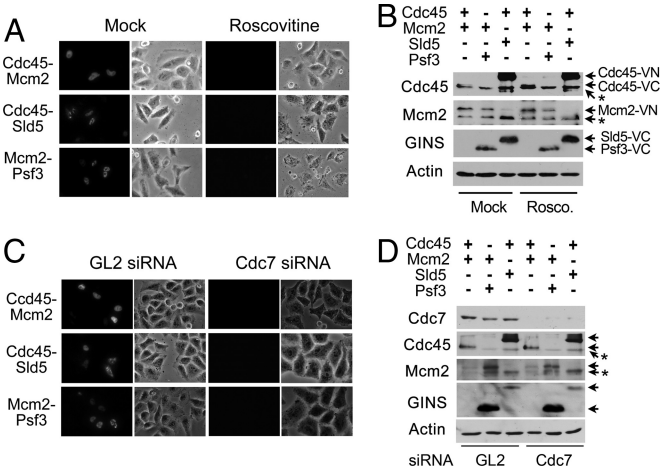

Fig. 3. Interactions between CMG proteins depends upon CDK and Cdc7 kinase. (*A* and *B*) HeLa cells were transfected with BiFC expression vectors as indicated, and incubated for 24 h in the presence of 10 μM roscovitine. Cell fluorescence was examined under the microscope (*A*) and the expression of transfected proteins was analyzed by Western blot analysis using whole cell lysates (*B*). (*C* and *D*) HeLa cells were cotransfected with GL2 siRNA (25) as control or Cdc7 siRNA (120 nM), and the BiFC expression vectors as indicated. After incubation at 37 °C for 24 h, cell fluorescence (*C*) and expression of transfected proteins (*D*) were analyzed. Actin was used as a loading control. Asterisks in *C* and *D* indicate the endogenous protein bands of Cdc45 or Mcm2.

remaining proteins. As shown in Fig. 4*A*, targeted siRNA depletion of Sld5 abolished the interaction between Mcm2 and Cdc45. Similarly, the interaction between Cdc45-Sld5 or Mcm2-Psf3 was not detected after depletion of Mcm2 or Cdc45, respectively (Fig. 4*A*). We also observed that the chromatin binding of Cdc45 was prevented by the depletion of Sld5, and Cdc45 depletion also prevented the chromatin binding of Sld5 (Fig. 4*B*). These observations were not due to alterations in the stability of proteins or activation of the ATR/ATM-dependent checkpoint pathways because the cellular levels of fluorescence fusion proteins were not altered by the siRNA treatments (Fig. 4*C*) and the addition of caffeine did not rescue the interactions between the CMG proteins (Fig. 4*A Right*). Possibly, the interactions between two CMG components may not form a stable complex in solution or on replication origins. These findings indicate that the assembly of a stable complex requires the simultaneous presence of the three CMG components.

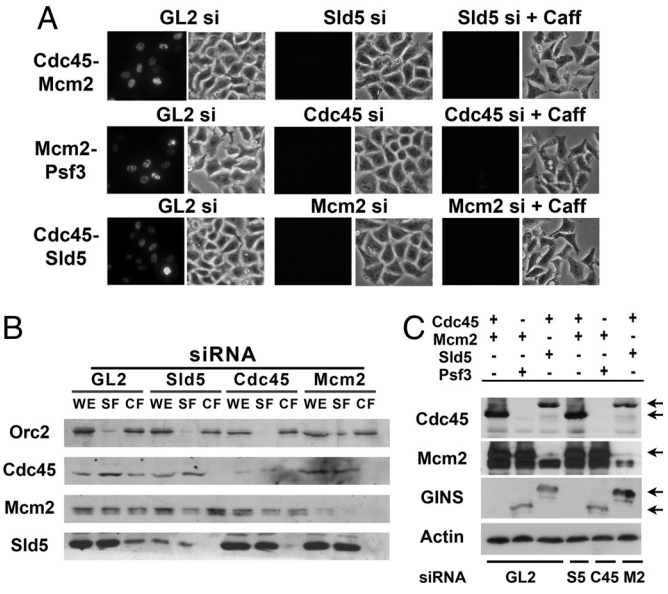

Fig. 4. Stable interaction between CMG proteins requires all three proteins of the CMG complex. HeLa cells were cotransfected with siRNA and BiFC expression vectors as indicated. After incubation at 37 °C for 24 h in the presence or absence of 5 mM caffeine, cell fluorescence (*A*) and expression of transfected proteins (*B*) were analyzed. Actin was used as a loading control. (*C*) HeLa cells were transfected with siRNAs as indicated. After incubation at 37 °C for 24 h, the levels of endogenous Orc2, Cdc45, Mcm2, and Sld5 proteins present in whole cell extract (WE), supernatant fraction (SF), and chromatin fraction (CF) were analyzed by Western blotting.

Mcm10, RecQL4, and Ctf4 but Not TopBP1 Are Required for the Assembly of CMG Complex. To identify other factors possibly required for the assembly of the CMG complex, we examined whether depletion of Mcm10, Ctf4, RecQL4, or TopBP1 affected formation of the complex in HeLa cells (Fig. 5). Western blot analyses indicated that the cellular levels of these proteins were markedly reduced after two consecutive siRNA treatments (Fig. 5 *B* and *E*). BiFC revealed that the interactions between CMG proteins were markedly reduced by depletion of Mcm10, Ctf4, or RecQL4 (Fig. 5*A Left*). The expression levels of transfected CMG proteins were not significantly altered by the siRNA treatments (Fig. 5*B*), and the addition of caffeine did not rescue the interactions between CMG proteins (Fig. 5*A Right*). These results indicate that the defects of CMG assembly shown in these cells are due to the siRNA targeted loss of the proteins, and not mediated by checkpoint activation or alteration in the protein levels of the CMG proteins. We noted that the loading of the CMG proteins onto chromatin was also prevented by depletion of Mcm10, Ctf4 or RecQL4 (Fig. 5*C*). Furthermore, the depletion of Mcm10 or Ctf4 reduced the chromatin binding of RecQL4, and the chromatin binding of Mcm10 was also decreased by depletion of Ctf4 or RecQL4. These results suggest that these proteins are all required for the chromatin recruitment of Cdc45 and GINS proteins and the assembly of the CMG complex. On the other hand, siRNA targeted depletion of TopBP1 did not affect formation of the CMG complex (Fig. 5*A*) as well as the chromatin loading of the CMG components (Fig. 5*D*). The depletion of TopBP1 protein seemed to be almost complete because checkpoint activation (measured by Chk1 phosphorylation) after UV irradiation was blocked (Fig. 5*E*). Taken together, these results suggest that Mcm10, RecQL4 and Ctf4 are all required for the assembly of CMG complex in human cells, whereas TopBP1, which is known to be essential for the formation of the initiation complex in yeast cells, is not required for the production of CMG complex in human cells.

Discussion

In eukaryotes, the activation of the pre-RC at origins involves a complex set of reactions. A critical step in this activation process is the loading of Cdc45 and GINS onto the pre-RC leading to the formation of the CMG complex, which has been proposed to be the replicative fork helicase. In this study we examined the production of the CMG complex in human cells using BiFC. In this assay formation of a stable complex between two target proteins, each fused to a nonfluorescent fragment of a fluorescent protein, leads to a fluorescent signal when the two nonfluorescent fragments are closely juxtaposed. As shown here, the BiFC technique is highly sensitive and can be used effectively to monitor the formation of the CMG complex.

The experiments described here revealed that formation of the CMG complex is cell cycle-dependent and requires the action of two kinases, CDK and DDK, which is similar to findings reported for the activation of the pre-RC in both the yeast and *Xenopus* systems (1). These experiments were carried out with a number of different protein pairs such as Mcm2-Psf3 or Cdc45-Sld5 that yielded strong fluorescent signals when coexpressed in HeLa

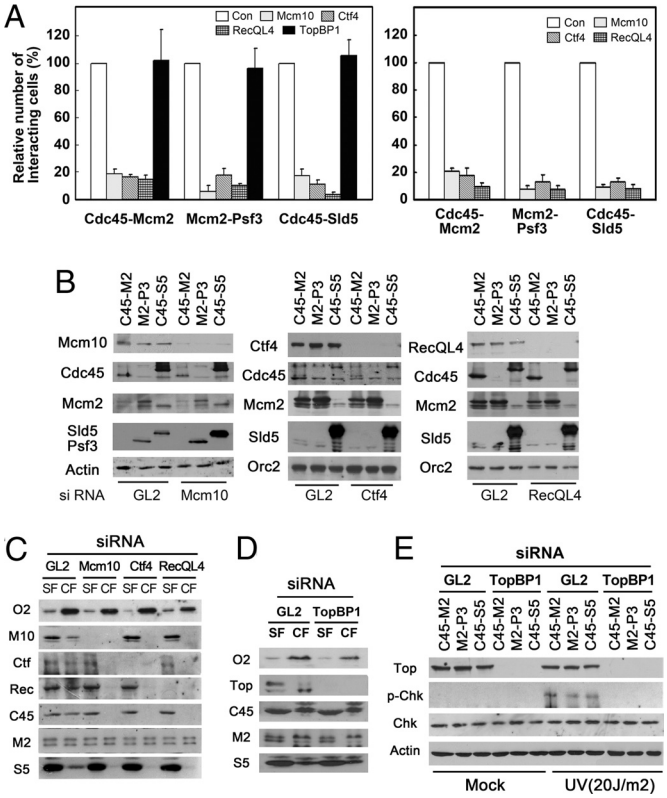

Fig. 5. RecQL4, Ctf4, and Mcm10 play essential roles in the assembly of the CMG complex. (*A*) HeLa cells were transfected with siRNAs as indicated, and incubated for 24 h. Cells were then cotransfected with the same siRNAs and BiFC expression vectors as indicated. After incubation of transfected cells in the absence (*Left*) or the presence (*Right*) of 5 mM caffeine for 24 h, the percentage of cells showing interactions between CMG proteins was counted. Each bar represents the mean value of three independent experiments, and the error bar indicates the standard error observed between experiments. (*B*) Whole cell extracts were prepared from cells cotransfected with siRNAs and BiFC expression vectors as in *A*, and the expression of CMG proteins was analyzed by Western blotting. (*C* and *D*) HeLa cells were transfected with siRNAs as indicated, and the loading of endogenous CMG proteins onto chromatin was examined by chromatin fractionation followed by Western blot analysis. SF and CF denote supernatant and chromatin fraction, respectively. (*E*) HeLa cells were transfected with TopBP1 siRNA and BiFC vectors as described in *A*. After incubation for 24 h, cells were irradiated with UV (20 J/m2), and incubated further for 4 h. Whole cell extracts were prepared from these UV-irradiated cells or mock irradiated cells, and the activation of the Chk1 pathway was analyzed by Western blotting using phosphor-specific antibody against the Chk1 protein. C45, Cdc45; M2, Mcm2; P3, Psf3; S5, Sld5; O2, Orc2; M10, Mcm10; Ctf, Ctf4; Rec, RecQL4.

cells. However, coexpression of Mcm2 and Sld5 or Cdc45 and Psf3 failed to yield fluorescent signals. These findings suggest that Mcm2 (as part of the Mcm2–7 complex) either interacts specifically with Psf3 (as part of the GINS complex) and not Sld5 or that its interaction with Sld5 fails to juxtapose the two truncated fluorescent fragments fused on the two proteins. Furthermore, formation of the CMG complex required Mcm10, RecQL4 and Ctf4. Surprisingly, TopBP1, the human homologue of Dpb11/Cut5, did not seem to be required for CMG complex formation in HeLa cells.

The assembly of the CMG complex has been studied in *Xenopus* and most extensively in yeasts. In the latter, Dpb11/Cut5, Sld2, Sld3 and Mcm10 are all required for the loading of Cdc45 and GINS onto pre-RCs (1). Extensive CDK-mediated phosphorylation of Sld2 and Sld3 was shown to be required for their interaction with Dpb11 (19, 20). Presumably this Sld2, Sld3 and Dpb11 complex in the presence of Mcm10 and other components, lead to CMG formation. In *Xenopus* and in yeasts, both Cdc45 and GINS seem to be stably loaded onto the pre-RC in a mutually dependent manner (10, 17). Using BiFC, we also found that Cdc45, GINS and Mcm2–7 were all required to form the CMG complex in human cells. However, the requirements for CMG complex formation in mammalian cells may differ from those described in yeasts. A homologue of the yeast *SLD3* has not as yet been identified in mammalian cells and TopBP1, the homologue of Dpb11/Cut5, does not seem to play a critical role in the initiation of DNA replication. Furthermore, Ctf4, which is not essential for the viability of budding yeast, is essential for the initiation of DNA replication in human cells.

RecQL4, which has been identified as a homologue of Sld2, seems to be required for the initiation of DNA replication in *Xenopus* and *Drosophia* (23, 24). The N-terminal fragment of RecQL4, which possesses limited homology to yeast Sld2, partially restored the replication activity of *Xenopus* egg extracts suggesting a conserved role for this protein as an essential factor in the assembly of the initiation complex. However, studies with *Xenopus* extracts showed that the loading of RecQL4 onto chromatin occurred after the chromatin loading of Cdc45 and GINS (24). In keeping with this finding, depletion of RecQL4 from *Xenopus* extracts did not affect the chromatin loading of Cdc45 and GINS. These results suggest that in *Xenopus* RecQL4 may play a role in DNA replication after the chromatin loading of Cdc45 and GINS. However, when we depleted the RecQL4 protein in HeLa cells, CMG complex formation was significantly inhibited, in contrast to observations made in *Xenopus* and more in accord with those made in yeasts and *Drosophila*. The reasons for these discrepancies are unclear.

In this study, the depletion of TopBP1 in HeLa cells did not significantly affect the formation of the CMG complex or the loading of Cdc45 and GINS onto chromatin (Fig. 5). Because several studies in yeasts showed an important role for Dpb11 in the loading of Cdc45 and GINS, this result was surprising. Although we cannot rule out that low levels of TopBP1 present in depleted cells might be ample to support formation of the CMG complex, the level of TopBP1 present in siRNA treated cells detected by Western blotting was <4% of the level in control siRNA treated cells. Furthermore, the checkpoint activity of these depleted cells was significantly reduced, indicating that the depletion of TopBP1 prevented checkpoint activation (Fig. 5*E*). Although there are reports suggesting that TopBP1 plays an essential role in replication of human cells (21, 29), our findings that TopBP1 depletion did not affect CMG complex formation are more consistent with other observations showing that TopBP1-depleted cells supported significant levels of DNA synthesis (30, 31). These findings do not rule out the possibility that mammalian cells may use multiple pathways for the assembly of the CMG complex or that TopBP1 plays a role in DNA replication after the CMG complex is assembled.

Ctf4 was shown recently to be required for the initiation of DNA replication by recruiting DNA polymerase α to chromatin (28). Whereas Ctf4 is not essential for the viability of budding yeast, Mc11, a homologue in *Schizosaccharomyces pombe*, is essential and required for the maintenance of genome integrity (32). Genetic and physical interactions between Ctf4/Mcl1 and DNA replication proteins including DNA polymerase α, have also been reported (32, 33). As shown here, Ctf4 seemed to play a critical role in the assembly of CMG complex in human cells. Ctf4 was reported to interact with Mcm10 in human and *Xenopus* systems, and the loading of Ctf4 onto chromatin was found to require Mcm10 (25), which is also essential for CMG complex formation. In human cells, the depletion of Ctf4, as well as Mcm10, leads to the instability of DNA polymerase α (26). These findings suggest that these two proteins may act jointly in the assembly of the CMG complex.

Materials and Methods

Construction of Expression Vectors for BIFC. Various cDNA fragments containing the full ORF of *CDC45, MCM2, MCM6, SLD5*, and *PSF3* were amplified by PCR, and cloned into the EcoRI and KpnI sites of pBiFC-VN173 (for fusion with the N-terminal 1–173 of Venus) or pBiFC-VC155 (for fusion with the C-terminal 155–238 of Venus) plasmids. These vectors (kindly supplied by C.-D. Hu, Purdue University, West Lafayette, IN) were used after removal of a FLAG-tag at their N termini. The sequences of the primers used for PCR are in Table S1.

Cell Culture and Reagents. HeLa, human cervical adenocarcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics, and maintained at 37 °C in a humidified incubator containing 5% CO_2. All siRNAs were chemically synthesized by Samchully Pharm (Daejon, Korea). Transfections of siRNAs (120 nM) were performed using oligofectamine (Invitrogen) following the manufacturer's instruction. For cotransfection of siRNA and BiFC plasmid DNAs, lipofectamine-PLUS (Invitrogen) was used. The sense strand sequences of siRNAs used in this study are in Table S2.

Chromatin Fractionation and Immunoblotting. Cells were lysed in buffer containing 50 mM Hepes-NaOH, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM ATP, 50 mM NaF, 1 mM sodium vanadate and protease inhibitors. After incubation at 4 °C for 10 min, the chromatin fraction was separated from the soluble fraction by centrifugation at 15,000 $\times$ *g* for 10 min. To prepare whole cell extracts for immunoblotting, cells were resuspended in buffer containing 50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 1 mM sodium vanadate and protease inhibitors. Cells were disrupted by sonication and proteins were measured using the Bradford assay. $\approx$30 μg of protein was subjected to SDS/PAGE. Commercial antibodies and their sources are as follows: anti-Sld5 antibodies (Strategic Diagnostics); anti-Cdc7 and anti-Mcm10 antibodies (Abcam); anti-RecQL4 and anti-p-Chk1 (Cell Signaling); anti-Ctf4 and anti-TopBP1 (Biolegend and Bethyl-Lab, respectively); anti-Mcm2, anti-Cdc45, anti-Chk1, and anti-Actin antibodies (Santa Cruz Biotechnology); and anti-Orc2 (BD Biosciences).

ACKNOWLEDGMENTS. This work was supported by Korea Research Foundation Grant KRF-2006-311-C00112 and by National Institutes of Health Grant 5RO1-GM034559.

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