The Replication Initiation Protein Sld3/Treslin Orchestrates the Assembly of the Replication Fork Helicase during S Phase

Background: Sld3/Treslin are required for the initiation of DNA replication.
Results: Sld3 interaction with ssDNA is required for GINS attachment to Mcm2–7 in yeast cells. The biochemical functions identified for Sld3 are conserved in human Treslin.
Conclusion: Sld3/Treslin orchestrate the assembly of the replication fork helicase during S phase.
Significance: A conserved mechanism for eukaryotic replication initiation is proposed.

The initiation of DNA replication is a highly regulated process in eukaryotic cells, and central to the process of initiation is the assembly and activation of the replication fork helicase. The replication fork helicase is comprised of CMG (Cdc45, Mcm2–7, and GINS) in eukaryotic cells, and the mechanism underlying assembly of the CMG is studied in this article. Work from our laboratory has shown that Sld2, Sld3, and Dpb11, along with Sld5, Psf2, Psf3 (14–17). During G1, Sld2 (synthetic lethal with dpb11–1) and Sld3, and these phosphorylation events trigger the attachment of Sld3 and Sld2 to Dpb11 (DNA polymerase B-associated protein) to form a complex (5–8). DDK phosphorylates the Mcm2 (minichromosome maintenance protein 2), Mcm4, and Mcm6 subunits of the Mcm2–7 complex, and these phosphorylation events are critical for the assembly of Cdc45 with Mcm2–7 and activation of the replication fork helicase (9–13). The replication fork helicase unwinds parental duplex DNA at a replication fork, providing single-stranded DNA templates for the replication polymerases (1). The Mcm2–7 complex forms the motor of the replication fork helicase, which is called CMG and is composed of GINS, Cdc45, Mcm2, Mcm4, Mcm5, Mcm6, and Mcm7. CMG in human cells.

DNA replication is restricted to the S (synthesis) phase of the cell cycle and consists of three phases: initiation, elongation, and termination (1, 2). The initiation of DNA replication in S phase is managed by two S-phase specific kinases, S-CDK (S-phase cyclin-dependent kinase) and DDK (Dbf4-dependent kinase) (3, 4). S-CDK phosphorylates two proteins essential for replication initiation, Sld2 (synthetic lethal with dpb11–1) and Sld3, and these phosphorylation events trigger the attachment of Sld3 and Sld2 to Dpb11 (DNA polymerase B-associated protein) to form a complex (5–8). DDK phosphorylates the Mcm2 (minichromosome maintenance protein 2), Mcm4, and Mcm6 subunits of the Mcm2–7 complex, and these phosphorylation events are critical for the assembly of Cdc45 with Mcm2–7 and activation of the replication fork helicase (9–13). The replication fork helicase unwinds parental duplex DNA at a replication fork, providing single-stranded DNA templates for the replication polymerases (1). The Mcm2–7 complex forms the motor of the replication fork helicase, which is called CMG and is composed of GINS, Cdc45, Mcm2, Mcm4, Mcm5, Mcm6, and Mcm7. CMG in human cells.

This article has been withdrawn by the authors. The Dpb11 immunoblot from whole cell extracts in Fig. 4A and the Arp3 immunoblot from whole cell extracts in Fig. 4B were duplicated. Upon further investigation by the Journal, the Journal determined that the co-immunoprecipitation experiments shown in Fig. 4, A and B, were not performed appropriately.
**Experimental Procedures**

Antibodies—Antibodies directed against RPA were purchased from Pierce. Antibodies directed against Mcm2-1-160 and Mcm2-161-173-phosphoserine 164-phosphoserine 170 were validated as described (51). Antibodies directed against the FLAG, HA, or His epitopes were commercially purchased. Antibodies directed against Sld3, Dpb11, and Arp3 were validated as described (51). Antibodies directed against RPA were purchased from Zymo Research. Antibodies directed against Sld3, Dpb11, and Arp3 were validated as described (51). Antibodies directed against RPA were from Zymo Research. Antibodies directed against RPA were purchased from Zymo Research. Antibodies directed against RPA were purchased from Zymo Research. Antibodies directed against RPA were purchased from Zymo Research. Antibodies directed against RPA were purchased from Zymo Research.

**Protein Purification—** Yeast Mcm2, Mcm3, and Mcm5 subunits, DDK, Sld2, Sld3 (wild-type and mutants), Cdc45, Dpb11, and CDK were purified as described (40, 42, 43, 51). Human PKA-Treslin, human Sld3, Treslin, GINS-Mcm3, and GINS-Mcm5 were subjected to additional purification (see below) chromatography. Human GINS-Mcm3, GINS-Mcm5, Treslin, Sld3, and Sld5 were subjected to additional purification (see below) chromatography. Human GINS-Mcm3, GINS-Mcm5, Treslin, Sld3, and Sld5 were subjected to additional purification (see below) chromatography. Human GINS-Mcm3, GINS-Mcm5, Treslin, Sld3, and Sld5 were subjected to additional purification (see below) chromatography. Human GINS-Mcm3, GINS-Mcm5, Treslin, Sld3, and Sld5 were subjected to additional purification (see below) chromatography. Human GINS-Mcm3, GINS-Mcm5, Treslin, Sld3, and Sld5 were subjected to additional purification (see below) chromatography.

**Plasmids—** cDNA for human Mcm3, Mcm5, and GINS were a very generous gift from Jerard Hurwitz (Sloan Kettering, New York, NY) (52). The Mcm3 and Mcm5 genes were recloned from baculovirus vector to pET41 (GST-Mcm3, GST-Mcm5). The GINS genes were recloned from baculovirus vector to pRSF (His-Psf2 and Sld5) and pDUET (His-Psf1 and Psf3). Human Treslin cDNA was a very generous gift from Dr. William Dunphy (Cal Tech, Pasadena, CA) (48, 49). Full-length human Treslin was recloned into pET33b (PKA-Treslin) or pET41a (GST-Treslin). The following plasmids were used for experiments: pIB401 (pSR415 CEN6/ARSH4 GALS::SLD3-6His LEU2), pIB403 (pSR415 CEN6/ARSH4 GALS::sld3-Q532A, F533A, S534A-6His LEU2), and pIB404 (pSR415 CEN6/ARSH4 GALS::sld3-V535A, S536A, D537A-6His LEU2).

**Yeast Cell Growth (Figs. 3, 4, and 5)—** Cells were grown overnight in -Leu media supplemented with raffinose (2%). When the cell density reached 6 × 10⁶ cells, the cells were spun down and resuspended in YPGal (0.15% galactose when galactose was indicated) and α-factor was added. The cells are grown for 3 h at 37 °C (when indicated), spun down, washed two times with buffer, and resuspended in fresh, pre-warmed (37 °C) YPGal media containing 50 μg/ml of Pronase at time 0. Time points are then taken for extract analyses.

**Fluorescence-activated Cell Sorting (FACS Analysis)—** FACS analysis was performed as described (43). 6 × 10⁶ cells/ml were treated with α-factor (Zymo Research) for 3 h. After extensive washes and the addition of 50 μg/ml of Pronase, the cells were incubated for the indicated time. Cell cycle progression was then analyzed by flow cytometry (FACS) stained with propidium iodide with FACS aria.


Chromatin Immunoprecipitation (ChIP)—Chromatin immunoprecipitation was performed as described (51). 6 x 10⁶ cells/ml were treated with α-factor (Zymo Research) for 3 h. Following extensive washes and the addition of 50 μg/ml of Pronase, cells were further incubated for 0 or 20 min at the indicated temperature of the experiment. We performed PCR with [α-³²P]dCTP as a component of the PCR to quantify the amplified DNA product. Formaldehyde cross-linked cells were lysed with glass beads in a Bead Beater. DNA was fragmented by sonication (Branson 450, 6 cycles of 15 s each). Antibody and magnetic protein A beads were added to the cleared lysate to immunoprecipitate the DNA. Immunoprecipitates were then washed extensively to remove nonspecific DNA. Eluted DNA was subjected to PCR analysis using primers directed against ARS305, ARS306, or a region midway between ARS305 and ARS306 as described (51). The radioactive band in the agarose gel, representing specific PCR amplified DNA product, was quantified by phosphorimaging and normalized by a reference standard run in the same gel. The reference standard was a PCR accomplished with known quantity of template DNA replacing immunoprecipitate.

Co-immunoprecipitation—Co-immunoprecipitation (Co-IP)² was performed as described (51). 6 x 10⁶ cells were treated with α-factor (Zymo Research) for 3 h. Cells were then subjected to extensive washes, followed by the addition of 50 μg/ml of Pronase. Cells (4 x 10⁹) were collected and lysed at 4 °C with 0.5%Churchill 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 0.7 mg/ml of pepstatin, 0.1 mg/ml of BSA. After the last wash, 30 μl of 5X SDS sample buffer was added to each reaction, and the samples were heated at 95 °C for 10 min.

Samples (20 μl) were then analyzed by SDS-PAGE followed by phosphorimaging and quantitation. Experiments were performed in triplicate from a single pool of purified protein, and the mean ± S.E. is shown.

Biotin Pulldown Assays—The biotin pulldown assays were performed as described (40). Biotinylated DNA (4 pmol) conjugated to streptavidin-agarose magnetic beads was incubated with different concentrations of radiolabeled protein in a solution containing 20 mM Tris-HCl, pH 7.5, 100 μM EDTA, 10% glycerol, 40 μg/ml of BSA, 10 mM magnesium acetate, and 200 μM DTT in a final reaction volume of 25 μl. The reactions were incubated at 30 °C for 10 min. Following the incubation, the magnetic beads were collected at room temperature using a magnet (Dynal). The supernatant was removed and the beads were washed twice with a solution containing 2% SDS, 4% glycerol, 4 mM Tris-HCl, 2 mM DTT, and 0.1% β-mercaptoethanol. The reactions were analyzed by SDS-PAGE. The gel was dried at 80 °C for 1 h and then scanned with a phosphorimaging screen for 30 min.

Results

Steady-state DNA replication was performed as described (52). The GST pulldown assays were performed as described (51). GST pulldown reactions were in a volume of 100 μl and contained GST-tagged protein in GST-binding buffer (40 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 0.7 mg/ml of pepstatin, 0.1 mM PMSE, and 0.1 mg/ml of BSA) and varying amounts of radiolabeled protein as described in each figure legend. Reactions were incubated at 25 °C for 1 h. Following incubation, reactions were added to 40 μl of glutathione-Sepharose and gently mixed. Binding of GST-tagged protein to the protein was performed for 20 min with gentle mixing every 2 min. When the binding was complete, the beads were allowed to settle, the supernatant was removed, and the glutathione beads were washed twice with 0.5 ml of GST-binding buffer. After the last wash, 30 μl of 5X SDS sample buffer was added to each reaction, and the samples were heated to 95 °C for 10 min.

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Mcm3, Mcm5, and/or ssDNA. We found that one triple mutation, Sld3-Q532A, F533A, S534A (Sld3-m9), binds to both Mcm3 and Mcm5 at background levels (i.e. similar to GST). However, Sld3-m10 binds to Cdc45 (Fig. 2A), Mcm3, Mcm5, Sld2 (Fig. 2D), and ssDNA (Fig. 2C). Moreover, Sld3-m10 stimulates DDK phosphorylation of Mcm2 like wild-type Sld3 (Fig. 2F). These data suggest that Sld3-m9 is specifically defective for its interaction with Mcm3 and Mcm5, the double-mutant Mcm3-binding motif on Mcm3 and Mcm5, given the high degree of sequence similarity between Mcm3 and Mcm5. This makes sense.

We also found that one triple mutation, Sld3-Q532A, F533A, S534A (Sld3-m9), binds to ssDNA at background levels (Fig. 2E). However, Sld3-m9 binds to Mcm3 (Fig. 2A), Mcm5 (Fig. 2A), Cdc45 (Fig. 2B), Dpb11 (Fig. 2C), and Sld2 (Fig. 2D) like wild-type Sld3, and Sld3-m9 stimulates DDK phosphorylation of Mcm2 like wild-type Sld3 (Fig. 2F). These data suggest that Sld3-m9 is specifically defective in ssDNA binding, and Sld3-m9 is not defective in any other known biochemical function of Sld3.

Expression of sld3-m9 and sld3-m10 at Wild-type Levels Results in a Severe Growth Defect, and Markedly Slowed Progression through S Phase—To determine the role of the Sld3-Mcm3/Mcm5 and Sld3-ssDNA interaction on yeast cell function and DNA replication, we transformed plasmids harboring sld3-m9 or sld3-m10 under regulation by the GAL-S low-copy inducible promoter system (pRS415 vector) into cells harboring a temperature-sensitive degron for SLD3 (sld3-7 td, obtained from Karim Labib, University of Dundee, Dundee UK (35)). At the permissive temperature (25 °C) and in the absence of galactose, only the genomic copy of SLD3 is expressed. Under these conditions, only the plasmid copy of sld3 is expressed, and it is expressed at wild-type levels. Under these conditions, cells expressing sld3-m9 or sld3-m10 exhibit a slow growth defect compared with cells expressing SLD3-WT, suggesting that the Sld3-Mcm3/Mcm5 and Sld3-ssDNA interactions are required for yeast cell growth on agar plates (Fig. 3A, middle panel). We then varied the concentration of galactose to achieve wild-type levels of Sld3 protein as revealed by Western blot of whole cell extracts (Fig. 3B). Thus, under these conditions, only the plasmid copy of sld3 is expressed, and it is expressed at wild-type levels. Under these conditions, cells expressing sld3-m9 or sld3-m10 exhibited a severe growth defect compared with cells expressing SLD3-WT, suggesting that the Sld3-Mcm3/Mcm5 and Sld3-ssDNA interactions are required for yeast cell growth on agar plates (Fig. 3A, middle panel).

We next performed a similar experiment at the permissive temperature (25 °C) in the presence of galactose (Fig. 3A, right panel). Under these conditions, we are overexpressing mutant sld3 (Fig. 3B). Overexpression of sld3-m9 or sld3-m10 results in a severe growth defect, as detected by 10-fold serial dilutions on agar plates (Fig. 3A, right panel). These data suggest that sld3-m9 and sld3-m10 exhibit a dominant-negative severe growth defect.

To characterize the effect of expressing sld3-m9 or sld3-m10 at wild-type levels on DNA replication, we used similar conditions as those described for Fig. 3A, middle panel, in subsequent experiments (Figs. 3, C and D, 4, and 5). We next examined expression of phospho-Mcm2 levels with an antibody directed against Mcm2-phosphoserines 164 and 170, the DDK sites on Mcm2 (Fig. 3C) (51, 53). We found similar levels of Mcm2 phosphorylation for cells expressing wild-type levels of SLD3, sld3-m9, and sld3-m10.
m9, or sld3-m10, suggesting that the mutations do not affect DDK phosphorylation of Mcm2 in vivo.

Next, we performed FACS analysis to assess the rate of cell progression through S phase (Fig. 3D). We found that cells expressing sld3-m9 or sld3-m10 at wild-type levels exhibited markedly slowed progression through S phase, suggesting a defect in these cells for DNA replication. These data suggest that the Sld3-Mcm3/Mcm5 and Sld3-mDNA interactions are required for DNA replication in budding yeast cells.

Expression of Wild-type Levels of sld3-m9 Results in Substantially Diminished GINS-Mcm2–7 Interaction during S Phase, and Expression of Wild-type Levels of sld3-m10 Results in Substantially Diminished Cdc45-Mcm2–7 Interaction during S Phase—It has previously been shown that Sld3 interacts with Cdc45 in G1 and S phase when the cross-linking agent is added to budding yeast cells (22). Furthermore, Sld3 interacts with Dpb11 in S phase when the cross-linking agent is added to yeast cells (7, 8). To determine whether these known interactions were occurring in our mutant cells, we performed co-IP analysis of budding yeast cells expressing wild-type levels of SLD3-WT, sld3-m9, or sld3-m10. Cells were arrested in G1 with α-factor, and then released into medium lacking α-factor for 15, 30, or 45 min to assess complex formation during S phase. Cross-linking agent was added to these cells, but no hydroxyurea was added to avoid triggering the DNA damage response.

We found that the interaction between Sld3 and Cdc45, as assessed by co-IP analysis, was similar for cells expressing SLD3-WT, sld3-m9, and sld3-m10 (Fig. 4A). Furthermore, the interaction between Sld3 and Dpb11 was similar for cells expressing SLD3-WT, sld3-m9, and sld3-m10 (Fig. 4A). These data suggest that expression of sld3-m9 or sld3-m10 does not disrupt the interactions between Sld3 and Cdc45 or Sld3 and Dpb11 in the cell, consistent with the in vitro results (Fig. 2).

We next assessed the interaction between Sld3 and Mcm2 by co-immunoprecipitation analysis (Fig. 4B). Because Mcm2 exists in the Mcm2–7 complex in vivo, this experiment is probing the Sld3-Mcm2–7 interaction. For these experiments, no cross-linking agent was added, because the addition of the cross-linking agent may reveal indirect interactions among many different replication proteins. For cells expressing SLD3-WT and sld3-m9, the interaction between Sld3 and Mcm2 is clearly visible during 15-, 30-, and 45-min time points. In contrast, for cells expressing sld3-m10, there is a substantially diminished signal for Sld3-Mcm2 interaction. These data are consistent with the in vitro data, showing a decreased interaction between Sld3-m10 and Mcm3 or Mcm5.

We then determined whether Cdc45 is recruited to Mcm2 in wild-type and mutant cells by co-immunoprecipitation analysis (Fig. 4B). Because Mcm2 exists in the Mcm2–7 complex in vivo, this experiment is probing the Sld3-Mcm2–7 interaction. For these experiments, no cross-linking agent was added, because the addition of the cross-linking agent may reveal indirect interactions among many different replication proteins. For cells expressing SLD3-WT and sld3-m9, the interaction between Sld3 and Mcm2 is clearly visible during 15-, 30-, and 45-min time points. In contrast, for cells expressing sld3-m10, there is a substantially diminished signal for Sld3-Mcm2 interaction. These data are consistent with the in vitro data, showing a decreased interaction between Sld3-m10 and Mcm3 or Mcm5.
sld3-m9 during the 15- and 30-min time points, and for sld3-m9 during the 45-min time point. In contrast, no signal is visible for cells expressing sld3-m10. These data are consistent with in vitro data and in vivo data showing that Sld3 is required for Cdc45 recruitment to Mcm2–7 (22, 54).

Next, we investigated whether the GINS-Mcm2–7 interaction is disrupted in mutant cells compared with wild-type cells (Fig. 4B). A co-IP signal is clearly visible for the interaction between Psf2 (a subunit of GINS) and Mcm2 during S phase in wild-type cells and cells expressing sld3-m10. In contrast, no GINS-Mcm2–7 co-IP signal is visible in cells expressing sld3-m9. These data suggest that the Sld3-ssDNA interaction is important for the S-phase interaction between GINS and Mcm2–7. These data are also consistent with the in vitro data, demonstrating that ssDNA releases Sld3 from Mcm2–7, allowing GINS to bind Mcm2–7 by a passive, sequestration mechanism (42). Moreover, a co-IP signal is also visible at the 0 time point (G1 phase) for cells expressing sld3-m10, but not wild-type cells. These data suggest that for cells expressing sld3-m10, GINS associates prematurely with Mcm2–7 in G1. Alternatively, we cannot rule out the possibility that GINS association with Mcm2–7 for sld3-m10 at time 0 is the result of lack of GINS-Mcm2–7 dissociation from the previous cell cycle. These data are also consistent with the in vitro data showing that GINS and Sld3 compete with one another for binding the Mcm3/Mcm5 subunits of Mcm2–7 (54).

FIGURE 3. Expression of sld3-m9 and sld3-m10 at wild-type levels in budding yeast results in a severe growth defect, and markedly slowed progression through S phase. A. 10-fold serial dilution analysis of budding yeast sld3–7 td (sld3-temperature-sensitive degron) cells expressing SLD3-WT, vector, sld3-m9, or sld3-m10 from the GAL-S plasmid inducible promoter system (pRS415). The growth conditions are described at the top, and the plasmid insert is described at the left. B, Western analysis of whole cell extracts from cells used in A, probing with antibody directed against Sld3 or Arp3 (loading control). C, similar to B, except probing with antibody directed against DDK-phosphorylated Mcm2 (top gel) or Mcm2 (bottom gel). D, FACS analysis of cells described in A (37 °C plus galactose), using propidium iodide as a stain for DNA content.
Expression of sld3-m9 and sld3-m10 at Wild-type Levels Results in a Slightly Reduced RPA-ChIP Signal at Early Origins of Replication—The binding of RPA to replication origins occurs as a result of origin melting and the initiation of bidirectional helicase movement. To assess whether origin melting was occurring in our mutant cells, we performed chromatin immunoprecipitation with antibodies directed against RPA (RPA-ChIP, Fig. 5). We then probed by quantitative PCR for the relative enrichment of early origin sequences ARS305 or ARS306, or a genomic region midway between these origins, as previously described (32). We arrested the cells in G1 with α-factor and released into medium lacking α-factor for the indicated times. Hydroxyurea was not used in these experiments. We found that the RPA-ChIP signal for cells expressing wild-type levels of sld3-m9 and sld3-m10 was about half that of cells expressing SLD3-WT at ARS305 and ARS306 (Fig. 5). The reduced signal may be caused by a decrease in origin melting, or a failure of bidirectional helicase movement. Given that the helicase does not properly assemble in cells expressing sld3-m9 or sld3-m10 (Fig. 4B), we speculate that the decrease in signal may be the result of no helicase movement. Thus, the data suggest that origin melting may be occurring in cells expressing sld3-m9 or sld3-m10. Thus, the Sld3-Mcm3/Mcm5 and Sld3-ssDNA interactions may be required for helicase assembly and helicase movement, but not origin melting.

Single-stranded DNA or GINS Disrupts the Interaction between Treslin and Mcm3 or Mcm5—We next wanted to determine whether the biochemical mechanism revealed for yeast Sld3 is conserved for the human homolog, Treslin. First, we determined whether Treslin binds to ssDNA, like yeast Sld3. We used biotinylated beads coupled to DNA of different sequences and structures, and pulled down radiolabeled Treslin (Fig. 6A). In a separate set of experiments, we used GST-
Chromatin immunoprecipitation with antibodies directed against RPA

FIGURE 5. Expression of sld3-m9 and sld3-m10 at wild-type levels in budding yeast results in a slightly reduced RPA-ChIP signal at early replication origins. Chromatin immunoprecipitation was performed using cells as described in the legend to Fig. 4A (middle panel) at the restrictive temperature, in the presence of galactose. Cells were arrested with hydroxyurea (H) and then released for 20 min (S phase cells); an immunoprecipitate was probed for DNA binding. The data suggest that Treslin has a preference for ssDNA at a region midway between two origins of replication. Results from repeated experiments were quantified and plotted.

Discussion

We identified point mutations of Sld3 that are specifically defective for interaction with either Mcm3/Mcm5 (sld3-m10) or ssDNA (sld3-m9). Wild-type expression of sld3-m9 or sld3-m10 in an sld3-temperature sensitive degron (sld3-7 td) at the restrictive temperature resulted in severe growth and DNA replication defects, suggesting that the interaction between Sld3 and Mcm3/Mcm5 or Sld3 and ssDNA are required for DNA synthesis. We investigated the mechanism for the replication defect in these cells, and found that cells expressing sld3-m10 are defective for Cdc45 recruitment to Mcm2–7 during S phase, whereas cells expressing sld3-m9 are defective for GINS recruitment during S phase. Mutant cells exhibited modest to severe growth defects at replication origins, suggesting that the S appear to be productive in these cells. DDK phosphorylation of the N terminus of Mcm2, accounting for the rescue of Cdc45 recruitment in the mutant of Sld3 defective for the interaction with Mcm2–7. Phosphorylation of Mcm2 has been characterized (50), and its phenotype is different from that reported in this article (sld3-m9 and sld3-m10).

We next determined whether Treslin binds to Mcm3 and/or Mcm5 in vitro, like Sld3. Indeed, we did find that purified human Treslin interacted with human Mcm3 or human Mcm5 using a GST pulldown assay (Fig. 6, C–H). We then wanted to determine whether human GINS competes with Treslin for binding to Mcm3 or Mcm5, because GINS binds to Mcm3 and Mcm5. Indeed, we found competition between GINS and Treslin for Mcm3 binding (Fig. 6C) and Mcm5 binding (Fig. 6F). These data suggest that the mechanism of Sld3 and GINS competing for Mcm3/Mcm5 binding is conserved from yeast to human, at least in vitro.

We examined whether ssDNA or dsDNA releases Treslin from Mcm3 or Mcm5, analogous to the observation for yeast Sld3 (42). We found that random 80-mer ssDNA dislodges Treslin from either Mcm3 (Fig. 6D) or Mcm5 (Fig. 6G), but random dsDNA does not dislodge Treslin from Mcm3 (Fig. 6E) or Mcm5 (Fig. 6H). These data suggest that the mechanism for ssDNA-mediated release of Sld3 from Mcm3/Mcm5 during S phase, elucidated in budding yeast, may be conserved for humans as well.

A Model for Replication Initiation in Budding Yeast—We propose the following model for replication initiation in budding yeast cells, based upon the data presented here and elsewhere (Fig. 7). During G1, the Mcm2–7 complex loads as a double hexamer to encircle double-stranded DNA (Fig. 7A). Sld3 is also bound to Sld7 (24), and recently the crystal structure of the Sld3–Sld7 interaction domains demonstrates a tetramer of two Sld3 subunits and two Sld7 subunits (55). Sld7, however, is not required for cell growth (24), whereas Sld3 is required for cell growth (22).

Sld3 binds directly to Mcm2–7 during S phase, and this interaction is required for the recruitment of Cdc45 to Mcm2–7.
Sld3/Treslin Regulates Helicase Assembly

FIGURE 6. Human GINS or single-stranded DNA disrupts the interaction between Treslin and Mcm3 or Mcm5. A, similar to Fig. 1C. 10 pmol of biotin-DNA was used to pulldown various amounts of radiolabeled Treslin, as described under “Experimental Procedures.” Treslin had an N-terminal tag with a protein kinase A consensus sequence (LLRASV). The PKA tag was used to radiolabel the Treslin protein with $^{32}$P for subsequent quantification. The products of the pull down were analyzed by SDS-PAGE, followed by phosphorimaging, and identical experiments were averaged and plotted. B, similar to Fig. 1A. 10 pmol of GST-Treslin was used to pulldown various amounts of radiolabeled DNA. The sequences of the random DNA are described under “Experimental Procedures.” C, 3 pmol of GST-Mcm3 was used to pull down various amounts of radiolabeled Treslin in the presence of various amounts of unlabeled GINS protein. The products of the pull down were analyzed by SDS-PAGE, followed by phosphorimaging, and identical experiments were averaged and plotted. D, similar to C, except random 80-mer ssDNA (strand 1) was used instead of GINS. E, similar to C, except random 80-mer dsDNA was used instead of GINS. F, similar to C, except GST-Mcm5 was used instead of GST-Mcm3. G, similar to D, except random 80-mer dsDNA was used instead of GINS. H, similar to E, except GST-Mcm5 was used instead of GST-Mcm3.

A Model for Replication Initiation in Human Cells—Treslin, like Sld3, binds to Dpb11 in a CDK-dependent manner (46, 48, 49). This interaction may be important for the recruitment of GINS to replication origins, analogous to the situation in yeast. However, as in yeast, Treslin blocks the interaction between GINS and Mcm3 and Mcm5 (Fig. 6). Thus, it appears that GINS is not recruited directly to Mcm2–7 by Treslin in human cells, analogous to the situation for yeast. Treslin interacts with ssDNA, similar to Sld3, and the interaction between Treslin and ssDNA releases Treslin from Mcm3 and Mcm5 (Fig. 6). Thus, in both yeast and human, the interaction between GINS and Dpb11 blocks this interaction face (40, 43) (Fig. 4).

Next, DDK phosphorylation of Mcm2 stimulates the opening of the Mcm2-Mcm5 gate, thereby allowing the extrusion of T-rich ssDNA from the central channel of Mcm2–7 (51) (Fig. 7C). The extrusion of T-rich ssDNA from the central channel of Mcm2–7 generates an ssDNA-binding platform for Sld2, Sld3, and Dpb11 (40, 43) (Fig. 4). Binding of Sld3-Sld2-Dpb11 to ssDNA releases this complex from Mcm2–7, thereby exposing the binding site for GINS on Mcm2–7 (40, 43) (Fig. 4). Thus, by this passive sequestration mechanism, GINS now binds directly to Mcm2–7 to complete the formation of the CMG complex (Fig. 7D). The CMG complex is now fully assembled and closed, and it can now translocate along DNA and melt the parental duplex (45).

(7B). DDK is also required for the timely recruitment of Cdc45 to Mcm2–7 (11, 24, 26, 28–30, 56). Moreover, Sld3 stimulates the DDK phosphorylation of Mcm2 (50). Sld3 is in a complex with Sld2 and Dpb11, and Dpb11 is bound directly to GINS (7, 8, 57). Thus, GINS is positioned at a replication origin at this stage, as observed by cross-linking studies, but GINS does not bind directly with Mcm2–7 because Sld3, Sld2, and Dpb11 block this interaction face (40, 43) (Fig. 4).
and Mcm2–7 may depend upon the extrusion of ssDNA from the central channel of Mcm2–7. In a separate study, we found that Treslin, like Sld3, stimulates DDK phosphorylation of Mcm2–7, opens the Mcm2–Mcm5 gate, allowing for the extrusion of ssDNA from the central channel of Mcm2–7. Sld3/Treslin facilitates its interaction with Cdc45 on the chromatin.

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