Origin Single-stranded DNA Releases Sld3 Protein from the Mcm2–7 Complex, Allowing the GINS Tetramer to Bind the Mcm2–7 Complex

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The initiation of DNA replication involves the complex orchestration of proteins that begin replication fork unwinding (1–4). Replication fork unwinding is required for the generation of single-stranded DNA templates that are used by the DNA polymerases for copying the genomic information (5). The Mcm2–7 complex is a heterohexameric assembly that binds and hydrolyzes ATP and functions as the motor of the replication fork helicase (6–8). Sister Mcm2–7 rings load at an origin of replication in late M phase and G1 (9, 10). Recent evidence suggests that the sister Mcm2–7 rings separate upon recruitment of these factors to origins of replication (23). It has recently been shown that CDK-phosphorylated Sld2 binds tightly to origin single-stranded DNA (ssDNA) in vitro (26).

In this study, we find that Saccharomyces cerevisiae Sld3 binds tightly to single-stranded DNA found at an origin of replication. CDK-phosphorylated Sld3 also binds to origin ssDNA with similar high affinity. Origin ssDNA does not disrupt the interaction between Sld3 and Dpb11, and origin ssDNA does not disrupt the interaction between Sld3 and Cdc45. However, origin ssDNA disrupts the interaction between Sld3 and Mcm2–7, whereas origin ssDNA promotes the association between GINS and Mcm2–7. We also show that origin single-stranded DNA promotes the formation of the CMG complex. We conclude that origin single-stranded DNA releases Sld3 from Mcm2–7, allowing GINS to bind Mcm2–7.

EXPERIMENTAL PROCEDURES

Plasmids—Full-length CDC45 or SLD3 PCR product was cloned into the BamHI/Xhol site of either pET 41a or pET 33b.

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2The abbreviations used are: CMG, Cdc45-Mcm2–7-GINS; ssDNA, single-stranded DNA; CDK, cyclin-dependent kinase; DDK, Dbf4-dependent kinase; PKA, protein kinase A; dsDNA, double-stranded DNA.
to contain a GST tag or PKA recognition sequence at the N terminus of the protein, respectively. The stop codon was removed at the C terminus to allow for a read-through into the His6 tag.

Plasmids for the expression of GINS were a generous gift from Mike O’Donnell. These GINS plasmids (pET-Psf3 and Psf5, pLANT-Psf2, pCDF-His-Psf1) were used to simultaneously co-express all four subunits of GINS (30). PKA-GINS has a PKA tag at the N terminus of Psf1. CDK protein expression plasmids and purification methods were a generous gift of Dr. H. Araki (31).

**Protein Expression and Purification**—Dpb11 was purified as described (26). CDK was purified as described (31). The Mcm2–7 complex and PKA-Mcm2–7 complex (PKA-Mcm3) were purified as described (8, 32). PKA was a generous gift of Dr. Susan Taylor. Cdc45 (GST, PKA, and native), Sld3 (GST and PKA native), and GINS (PKA and native) were expressed and purified as follows.

**Expression**—The plasmids were transformed into Escherichia coli Rosetta 2 (BL21) (EMD Biosciences) and a colony from transformation was used to inoculate 6 liters of ZYM-5052 auto-inducing media (33) containing 100 μg/ml kanamycin and 34 μg/ml chloramphenicol. For GINS, the media also contained 20 μg/ml streptomycin sulfate and 100 μg/ml ampicillin. The cells were grown at 37 °C until an A600 of 1.0 was reached, then the temperature was then lowered to 14 °C, and the cells were allowed to express protein for 16 h.

**Purification of GST Proteins**—Harvested cells were lysed with a French press in 200 ml of 50 mm Tris-HCl, pH 8.0, 500 mm NaCl, and 50 mm imidazole. The lysate was then applied to a 20-ml NiSO4-charged chelating Sepharose fast flow resin (GE Healthcare). The column was washed with the same buffer. The protein was then eluted in 50 mm Tris-HCl, pH 8.0, 100 mm NaCl, 10% glycerol, and 250 mm imidazole. Peak fractions were pooled, and 4 ml of glutathione-Sepharose 4B (GE Healthcare) resin were added. The mixture was rotated gently on ice for 1 h and then poured into a column, washed with 50 mm Tris-HCl, pH 8.0, 100 mm NaCl, and 10% glycerol, and eluted with the same buffer containing 100 mm glutathione. Peak fractions were then pooled, dialyzed against 50 mm Tris-HCl, pH 8.0, 50 mm NaCl, and 10% glycerol, and applied to an 8-ml Source Q column (GE Healthcare). The protein was then eluted with a 20-ml linear gradient from 50 to 500 mm NaCl. Peak fractions were pooled and frozen.

Histidine-tagged proteins were purified using the same procedure as GST proteins, except that the glutathione-Sepharose step was replaced by a size-exclusion chromatography purification step (Superose 6). Coomassie-stained polyacrylamide gels of the final purified products of Sld3, GST-Sld3, Cdc45, GST-Cdc45, and GINS are shown in supplemental Fig. 1, and we estimated the purity of each sample to be ~99%.

**Kinase Labeling**—CDK kinase labeling was performed in a 50-μl reaction containing 20 nm Sld3, 20 ng of CDK, 5 mm cold ATP, 5 μCi of [γ-32P]ATP (PerkinElmer Life Sciences, 6000 Ci/nmol) in 5 mm Tris-HCl, pH 8.5, 10 mm MgCl2, and 1 mm DTT. Reactions were incubated at 30 °C for 1 h. PKA kinase labeling was performed as described (32).

**Analytical Size Exclusion Chromatography**—Radiolabeled protein was mixed with unlabeled proteins as described in the figure legends in a final volume of 200 μl and incubated for 1 h at 30 °C in column buffer (50 mm Tris, pH 7.5, 100 mm NaCl, 1 mm EDTA, and 5% glycerol). The protein mixture was then subjected to 24 ml of Superose 6 (GE Healthcare) size exclusion chromatography, in the same column buffer. Each 250-μl fraction was then subjected to SDS-PAGE analysis and quantified using PhosphorImager software.

**GST Pulldown Assays**—The 100-μl GST pulldown reaction 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 μg/ml pepstatin, 0.1 mm PMSF, and 0.1 mg/ml BSA), and varying amounts of radiolabeled protein as described in each figure. Reactions were incubated at room temperature for 1 h. Following incubation, reactions were added to 40 μl of prepared glutathione-Sepharose and mixed gently. Binding of GST-tagged protein to the beads was performed for 20 min, with gentle mixing every few minutes. Once the binding was complete, the reaction mixture was aspirated, and the beads were washed twice with 0.5 ml GST-binding buffer. After the last wash, 30 μl of 5X SDS sample buffer were added to each reaction, and the samples were boiled for 10 min. Samples (20 μl) were then analyzed by SDS-PAGE.

**Biotin Pulldown Assay**—Biotinylated DNA conjugated to streptavidin-agarose magnetic beads (Dynal) were incubated 5 min at 30 °C with increasing concentrations of radiolabeled protein in a solution containing 0.1 mm EDTA, 0.2 mm DTT, 10 mm magnesium acetate, 10% glycerol, 40 μg/ml BSA, 100 mm NaCl, and 20 mm Tris-HCl, pH 7.5, in a final volume of 25 μl. Following the 5-min incubation, the beads were collected at room temperature using a magnet (Dynal). The supernatant was removed, and the beads were washed twice with a solution containing 0.1 mm EDTA, 0.2 mm DTT, 10 mm magnesium acetate, 10% glycerol, 40 μg/ml BSA, 100 mm NaCl, and 20 mm Tris-HCl, pH 7.5. The beads were collected with a magnet, the supernatant was removed, and the beads were boiled at 95 °C for 10 min in a solution containing 2% SDS, 2 mm DTT, 4% glycerol, 4 mm Tris-HCl, and 0.01% bromphenol blue. The reactions were analyzed by SDS-PAGE. The gel was dried for 1 h at 80 °C and exposed to a phosphorimaging screen for 1 h.

**Fluorescence Anisotropy**—Sld3 binding to DNA was measured by the increase in anisotropy as varying concentrations of Sld3 were incubated with 10 mm fluorescent DNA (5-FAM) conjugated to streptavidin-agarose magnetic beads (Dynal) were incubated 5 min at 30 °C with increasing concentrations of radiolabeled DNA in a solution containing 50 mm Tris-HCl, pH 7.5, 100 mm NaCl, 1 mm EDTA, and 5% glycerol. The protein mixture was then incubated at room temperature for 1 h. Following incubation, reactions were added to 40 μl of prepared glutathione-Sepharose and mixed gently. Binding of GST-tagged protein to the beads was performed for 20 min, with gentle mixing every few minutes. Once the binding was complete, the reaction mixture was aspirated, and the beads were washed twice with 0.5 ml GST-binding buffer. After the last wash, 30 μl of 5X SDS sample buffer were added to each reaction, and the samples were boiled for 10 min. Samples (20 μl) were then analyzed by SDS-PAGE.

**RESULTS**

**GST-Sld3 Binds to ARS1 Origin ssDNA**—Sld3 binds to early origins in G1 phase in budding yeast (18, 23). The proximity of Sld3 to origin DNA prompted us to investigate whether Sld3

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binds directly to origin DNA. A schematic of the ARS1 origin is shown in Fig. 1A. The ARS1 origin contains several elements that are important for the initiation of DNA replication: the A, B1, B2, and B3 elements (34, 35). The A element contains the ARS consensus sequence and is a binding site for the origin recognition complex. The B1, B2, and B3 elements encompass a readily unwound region positioned adjacent to the origin recognition complex binding site where the Mcm proteins bind (36–38). We divided the ARS1 origin into three segments: one encompassing the A and B1 sites, one encompassing the B2 and B3 sites, and one positioned adjacent to the B2 and B3 sites (Fig. 1A).

We purified Sld3 bearing a GST tag to homogeneity and studied its interaction with radiolabeled ssDNA and dsDNA from ARS1 using a GST pulldown assay. We analyzed any bound, radiolabeled DNA by denaturing gel (Fig. 1B). We then quantified the bound DNA with phosphorimaging and plotted the resulting average of similar experiments (Fig. 1C). GST-Sld3 pulls down the majority of ssARS1-2, the sequence of ARS1 encompasses the A and B1 regions. However, GST-Sld3 does not pull down the majority of ssARS1-1, the complementary strand of ssARS1-2, suggesting that GST-Sld3 binding to DNA is strand-specific. Furthermore, GST-Sld3 binds weakly to double-stranded DNA encompassing this same region (dsARS1-12), suggesting that Sld3 binding to DNA is specific for the single-stranded structure. GST-Sld3 also does not pull down the majority of ssARS1-3, ssARS1-4, or dsARS1-34, the single and double DNA strands encompassing the B2 and B3 regions, suggesting that GST-Sld3 binding to ARS1 is region-specific. GST does not pull down any of the input radiolabeled sequences, suggesting that interaction between GST-Sld3 and origin ssDNA depends upon Sld3.

We also studied the interaction of GST-Sld3 with the adjacent complementary single-stranded DNA sequences of ARS1 (ssARS1-5 and ssARS1-6) and found that Sld3 pulls down more than half of the input for one of these sequences (ssARS1-5),...
whereas it very weakly interacts with the complementary sequence (ssARS1-6). These data suggest that Sld3 binds to two distinct regions of ARS1 (ssARS1-2 and ssARS1-5). GST-Sld3 binds very weakly to the double-stranded DNA encompassing this region (dsARS1-56), consistent with the preference of Sld3 for single-stranded DNA.

CDK-phosphorylated Sld3 with No GST Tag Binds to ARS1 Origin ssDNA—To confirm that Sld3 binds to origin ssDNA, we next utilized a different experimental technique (Fig. 2). We purified Sld3 with no GST tag to examine whether Sld3 with no GST tag can bind to origin ssDNA. We then radiolabeled Sld3 with either PKA or CDK and pulled down the radiolabeled Sld3 with biotinylated DNA. The bound protein was then analyzed by SDS-PAGE and quantified with phosphorimaging. 8, results from experiments shown in A were quantified, averaged, and plotted. The data are mean ± S.E.

![FIGURE 2. CDK-phosphorylated Sld3 binds to origin ssDNA. A, Sld3 containing an N-terminal PKA tag was radiolabeled with either PKA or CDK. PKA-Sld3 and CDK-Sld3 were then matched for radioactive counts and total Sld3 concentration. Varying amounts of the proteins (0.1, 0.3, 1.0, or 3.0 pmol) were then incubated with 5 pmol of biotinylated ssARS1-2, ssARS1-4, dsARS1-12, or biotin. The bound radioactive Sld3 was analyzed by SDS-PAGE followed by phosphorimaging. B, results from experiments shown in A were quantified, averaged, and plotted. The data are mean ± S.E.](image)

![FIGURE 3. Native Sld3 binds to origin ssDNA with an apparent Kd of 53 nM. 10 nM fluorescently labeled (5-FAM) ssARS1-2, ssARS1-4, or dsARS1-12 was mixed with varying concentrations of native Sld3 and incubated for 20 min at room temperature. Polarized fluorescence intensities were measured at excitation and emission wavelengths of 496 and 538 nm, respectively, at room temperature. The data were plotted as the change in anisotropy versus protein concentration in nM, and the curve was fit with the equation, y = m1/(m0 + m1). The table underneath the graph shows the apparent Kd for Sld3 interaction with ssARS1-2, ssARS1-4, or dsARS1-12. The data are mean ± S.E.](image)
wild-type affinity. We therefore tested replacement of thymines with adenines (T to A), guanines with cytosines (G to C), or cytosines with adenines (C to A). Whereas the G-to-C or C-to-A substitutions resulted in no change in affinity of GST-Sld3, the T-to-A substitution completely disrupted binding to GST-Sld3 (Fig. 4A). These data suggest that Sld3 binds specifically to the thymines of ssARS1–2.

There are 21 thymines in the 80-base sequence ssARS1–2. To determine whether Sld3 binds to a specific region of ssARS1–2, we replaced regions of ssARS1–2 with T-to-A substitutions. Replacement of seven or 14 thymines at the 3′ end of ssARS1–2 had little effect on GST-Sld3 binding (Fig. 4B). However, replacement of seven thymines at the 5′ end of ssARS1–2 reduced binding of GST-Sld3 to GST background levels (Fig. 4C). These data suggest that Sld3 binds to the seven 5′-thymines of ssARS1–2. These seven thymines are positioned in the region between the B1 and B2 elements of ARS1.

GST-Sld3 Binds to ARS305 Origin ssDNA—We next determined whether Sld3 can bind to a second origin of replication, ARS305 (Fig. 5, A and B). Complementary single-stranded DNA and double-stranded DNA were analyzed for binding to GST-Sld3. GST-Sld3 pulled down more than half of input radiolabeled ssARS305–1; however, GST-Sld3 does not pull down a large fraction of either ssARS305–2 or dsARS305–12. These data suggest that Sld3 binds to the single strands of two different origins of replication tested (ARS1 and ARS305). Further

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)
thermore, the data suggest that Sld3 binding to origin DNA is specific for single-stranded DNA.

We next addressed whether Sld3 contains two different binding sites for ssDNA: one site for ss\textit{ARS1-2} and a second site for ss\textit{ARS305–1}, or whether ss\textit{ARS1-2} and ss\textit{ARS305–1} compete with one another for the same Sld3-binding site. GST-Sld3 was used to pull down radiolabeled ss\textit{ARS1-2} in the presence of increasing amounts of unlabeled ss\textit{ARS305–1} (Fig. 5, C and D). As the amount of ss\textit{ARS305–1} was increased, there was a progressive decrease in the amount of ss\textit{ARS1-2} bound to GST-Sld3. These data suggest that ss\textit{ARS1-2} and ss\textit{ARS305–1} compete for the same Sld3-binding site. Sld3 binds to the seven \textit{5}’-thymines of ss\textit{ARS1-2}. ss\textit{ARS305–1} is thymine-rich (64% thymine, see Table 1), and the thymines present in the ss\textit{ARS305–1} sequence may provide some of the binding specificity for Sld3.

\textit{Origin ssDNA (ss\textit{ARS1-2}) Specifically Disrupts Interaction between Sld3 and Mcm2–7}—Sld3 binds to Dpb11 \textit{in vivo} when it is phosphorylated by CDK (24, 25). We phosphorylated Sld3 with CDK and tested its interaction with radiolabeled Dpb11 using a GST pulldown assay. We found that approximately one-third of Dpb11 bound to CDK-treated GST-Sld3 (Fig. 6, A and B). In contrast, very little Dpb11 bound to mock-treated GST-Sld3 (Fig. 6, A and B). We next studied whether ss\textit{ARS1-2} affects the interaction between Sld3 and Dpb11 (Fig. 6, C and D). We incubated CDK-treated-GST-Sld3 with radiolabeled Dpb11 and varying concentrations of ss\textit{ARS1-2}. When the ratio of ss\textit{ARS1-2} to Dpb11 was 3 or 10, there was no effect on radiolabeled Dpb11 interaction with CDK-treated GST-Sld3. These data suggest that origin ssDNA does not influence the interaction between CDK-treated Sld3 and Dpb11.

Sld3 binds to Cdc45 \textit{in vivo} during different phases of the cell cycle (18). Sld3 also binds to Cdc45 \textit{in vitro} (Fig. 6, E and F) (39). We next investigated whether ss\textit{ARS1-2} affects the interaction between Sld3 and Cdc45. GST-Cdc45 was used to pull-down radiolabeled PKA-Sld3 or CDK-Sld3. At molar ratios of DNA to Sld3 of 1, 3, or 10, there was little to no effect on Cdc45 interaction with PKA-Sld3 or CDK-Sld3 (Fig. 6, E and F). These data suggest that ss\textit{ARS1-2} does not influence the interaction between Sld3 and Cdc45.

Sld3 also binds directly to Mcm2–7 (Fig. 6 G) (39). We next investigated whether ss\textit{ARS1-2} affects the interaction between Sld3 and Mcm2–7. GST-Sld3 was used to pull down radiolabeled Mcm2–7 in the absence or presence of 5 mM ATP or the nonhydrolyzable analog ATP-\textit{γS}, and ss\textit{ARS1-2} was added in varying amounts to the pulldown reaction. ss\textit{ARS1-2} inhibited the interaction between GST-Sld3 and Mcm2–7 in a concentration-dependent manner (Fig. 6, G and H). The inhibition was similar in the absence or presence of 5 mM ATP or 5 mM ATP-\textit{γS}, suggesting that ss\textit{ARS1-2} disruption of Sld3-Mcm2–7 binding is independent of the nucleotide binding state of Mcm2–7.

Sld3 binding to ss\textit{ARS1-2} is disrupted by replacing the seven \textit{5}’-thymines with adenines (Fig. 4). These seven \textit{5}’-thymines are contained within the 20 \textit{5}’-nucleotides of the ss\textit{ARS1-2} sequence (supplemental Fig. 2). We next investigated whether a shorter region of ss\textit{ARS1-2} containing the seven \textit{5}’-thymines could dislodge Sld3 from Mcm2–7 (supplemental Fig. 2). GST-Sld3 was used to pull down radiolabeled Mcm2–7 in the pres-
Origin ssDNA Releases Sld3 from Mcm2–7

TABLE 1

| Name          | Sequence |
|---------------|----------|
| ARS1-1        | 5'ss/H11032-TTA CAT CTT GTT ATT TTA CAG ATT TTA TGT TTA GAT CTT TTA TGC TTG CTT TTC AAA AGG CCT GCA GGC AAG TGC ACA AA-3 |
| ARS1-12       | 5'ss/H11032-SS/ARS1-1 annealed to 5'ss/ARS1-2 |
| ARS1-3        | 5'ss/H11032-SS/ARS1-1 annealed to 5'ss/ARS1-2 |
| ARS1-4        | 5'ss/H11032-SS/ARS1-3 annealed to 5'ss/ARS1-4 |
| ARS1-56       | 5'ss/H11032-SS/ARS1-5 annealed to 5'ss/ARS1-6 |
| ARS305-1      | 5'ss/H11032-SS/ARS305-2 |
| ARS305-12     | 5'ss/H11032-SS/ARS305-1 annealed to 5'ss/ARS305-2 |

### Origin ssDNA Releases Sld3 from Mcm2–7

First, we conducted a number of control experiments. We studied the interaction between Sld3 and Mcm2–7 in the presence of increasing amounts of three different DNA species: full-length 80-mer ssARS1-2, 40-mer ssARS1-2, or 20-mer ssARS1-2. The 40-mer ssARS1-2 and the 20-mer ssARS1-2 were truncated from the 3' end, leaving the seven 5’-thymines intact in each of the sequences (supplemental Fig. 2A). The 40-mer ssARS1-2 disrupted the interaction between Sld3 and Mcm2–7 with similar efficiency as the 80-mer ssARS1-2 (supplemental Fig. 2, B and C). However, the 20-mer ssARS1-2 was less efficient than the 40-mer ssARS1-2 in dislodging Sld3 from Mcm2–7. These data suggest that 21–40 nucleotides of ssARS1-2 are required for efficient displacement of Sld3 from Mcm2–7.

Origin Single-stranded DNA (ssARS1-2) Releases Sld3 from Mcm2–7, Allowing GINS to Bind Mcm2–7—We next examined whether ssARS1-2 affects the interaction between GST-Mcm2–7 and GINS (Fig. 7, A and B). GST-Mcm2–7 pulls down the majority of input radiolabeled GINS in the absence or presence of 5 mM ATP. As ssARS1-2 is added to the GST pulldown at ssARS1-2 to Mcm2–7 ratios of 1, 3, 10, or 30, there is little effect on the interaction between GST-Mcm2–7 and radiolabeled GINS. These data suggest that ssARS1-2 does not disrupt the interaction between GINS and Mcm2–7 in the absence or presence of ATP.

We next examined whether ssARS1-2 affects the interaction between GST-Mcm2–7 and radiolabeled GINS in the presence of Sld3. When Sld3 is added to a GST-Mcm2–7 pulldown of GINS, Sld3 inhibits the interaction between GINS and Mcm2–7 (Fig. 7, C and D) (39). When ssARS1-2 is added to the GST-Mcm2–7 pulldown of radiolabeled GINS in the presence of competing Sld3, ssARS1-2 promotes the interaction between GINS and Mcm2–7 in a concentration-dependent manner (Fig. 7, C and D). This effect is similar in the presence or absence of 5 mM ATP. As a control, Sld3 does not bind directly to GINS (Fig. 8, A and B) (39). Cdc45 and Sld3 bind together in vitro (39) and in vivo (18), and thus, we also performed these experiments in the presence of Cdc45. When ssARS1-2 is added to the GST-Mcm2–7 pulldown of radiolabeled GINS in the presence of Cdc45 and Sld3, ssARS1-2 stimulates the interaction between GINS and Mcm2–7 in a concentration-dependent manner (Fig. 7, E and F). These data suggest that Cdc45 does not affect the ability of ssARS1-2 to stimulate GINS binding to Mcm2–7 in the presence of competing Sld3. All of the data discussed thus far indicate that ssARS1-2 releases Sld3 from Mcm2–7, allowing GINS to bind Mcm2–7.

We next used a solution-based assay, size exclusion chromatography, to determine whether ssARS1-2 promotes the interaction between GINS and Mcm2–7 in the presence of Sld3. First, we conducted a number of control experiments. We studied the interaction between Sld3 and GINS and found no interaction in the absence or presence of ssARS1-2 (Fig. 8, A and B). We also found that ssARS1-2 does not affect the migration of Mcm2–7, as expected for a low molecular weight DNA species (Fig. 8C). We next found that Sld3 can form an interaction with Mcm2–7 with 1:1 stoichiometry in the absence of DNA and that GINS can form an interaction with Mcm2–7 with 1:1 stoichiometry in the absence of DNA (Fig. 8, D–F) (39). However, in the presence of ssARS1-2, the interaction between Sld3 and Mcm2–7 is disrupted (Fig. 8, D and F), consistent with the GST.
pulldown results (Fig. 6, G and H). In contrast, ssARS1-2 does not directly affect the interaction between Sld3 and Dpb11 or between Sld3 and Cdc45. A, 2 pmol of CDK-treated or mock-treated (i.e. no kinase, buffer added only) GST-Sld3 or GST was incubated with varying amounts of PKA-radiolabeled Dpb11 (0.06, 0.2, 0.6, or 2 pmol). Bound radioactive Dpb11 was analyzed by SDS-PAGE. B, results from experiments shown in A were quantified, averaged, and plotted. C, 2 pmol of CDK-treated GST-Sld3 was incubated with 2 pmol of radiolabeled Dpb11 and varying amounts of ssARS1-2 (0, 6, or 20 pmol). Bound radioactive Dpb11 was analyzed by SDS-PAGE. D, results from experiments shown in C were quantified, averaged, and plotted. E, Sld3 containing an N-terminal PKA tag was radiolabeled with either PKA or CDK. PKA-Sld3 and CDK-Sld3 were then matched for radioactive counts and total Sld3 concentration. 2 pmol of PKA-Sld3 or CDK-Sld3 were then incubated with 2 pmol of GST-Cdc45 and varying concentrations of ssARS1-2 (0, 2, 6, or 20 pmol), and the bound radioactive Sld3 was analyzed by SDS-PAGE. F, results from experiments shown in E were quantified, averaged, and plotted. G, 2 pmol of radiolabeled Mcm2–7 in the presence or absence of 5 mM ATP or ATP–γS was incubated with 2 pmol of GST-Sld3 and varying concentrations of ssARS1-2 (0, 1, 2, 4, or 8 pmol). H, results from experiments shown in G were quantified, averaged, and plotted. The data are mean ± S.E.

We next studied a mixture of Sld3, GINS, and Mcm2–7 (Fig. 8, G–I). First, Sld3 was radiolabeled and incubated with unlabeled GINS and Mcm2–7 in the absence of ssARS1-2 (Fig. 8G). In the absence of ssARS1-2, two peaks of roughly equal size were observed, with one peak at an elution of ~720 kDa and a second peak at an elution of ~68 kDa. Because Sld3 does not bind directly to GINS, the data indicate that roughly half of the Sld3 is forming a complex with Mcm2–7 (high molecular mass peak), whereas the other half of Sld3 is free in solution (low molecular mass peak). We next incubated labeled GINS and labeled Mcm2–7 with Sld3 under identical conditions (Fig. 8H). Once again there are two peaks of roughly equal size in the absence of DNA, one of high molecular mass and one of low molecular mass. The high molecular mass peak is consistent
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FIGURE 7. Origin single-stranded DNA (ssARS1–2) promotes the association between GINS and Mcm2–7 in the presence of Sld3. A, ssARS1–2 does not disrupt the interaction between GINS and Mcm2–7. 2 pmol of GST-Mcm2–7 was incubated with 2 pmol of radiolabeled PKA-GINS and varying amounts of ssARS1–2 (0, 2, 6, 20, or 60 pmol) in the absence or presence of 5 mM ATP. Bound radioactive GINS was analyzed by SDS-PAGE. B, results from experiments shown in A were quantified, averaged, and plotted. C, 2 pmol of GST-Mcm2–7 was incubated with 2 pmol of radiolabeled GINS, the presence or absence of 10 pmol of Sld3, and varying amounts of ssARS1–2 (0, 5, 20, or 50 pmol) in the absence or presence of 5 mM ATP. Bound radioactive GINS was analyzed by SDS-PAGE. D, results from experiments shown in C were quantified, averaged, and plotted. E, 2 pmol of GST-Mcm2–7 was incubated with 2 pmol of radiolabeled PKA-GINS, 10 pmol of Cdc45, 10 pmol of Sld3, and varying amounts of ssARS1–2 (0, 5, 20, or 50 pmol) in the absence or presence of 5 mM ATP. Bound radioactive GINS was analyzed by SDS-PAGE. F, results from experiments shown in E were quantified, averaged, and plotted. The data are mean ± S.E.

Sld3 is required for the initiation of DNA replication, but Sld3 does not travel with the replication fork (18, 21). We found that Sld3 binds tightly to the single-stranded region of the ARS1 origin encompassing the A and B1 elements (ssARS1–2, K = 53 nM). Sld3 also binds to ssDNA positioned adjacent to the B2 and B3 elements (ssARS1–5), but Sld3 binds weakly to double-stranded ARS1 DNA. CDK-phosphorylated Sld3 binds to ssARS1–2 with similar high affinity as non-CDK-phosphorylated Sld3, suggesting that Sld3 is active in binding origin ssDNA during S phase. Sld3 also binds tightly to the single-stranded DNA of the ARS305 replication origin, but Sld3 does bind weakly to double-stranded ARS305. Replacement of the seven 5′-thymines in ssARS1–2 with adenines reduces Sld3 binding to background levels, suggesting that Sld3 binds to the seven 5′-thymines of ssARS1–2. These thymines are positioned between the B1 and B2 elements of ARS1. ssARS1–2 specifically inhibits the interaction between Sld3 and Mcm2–7, but ssARS1–2 does not inhibit the interaction between Sld3 and Dpb11 or between Sld3 and Cdc45. In a mixture of Sld3, GINS, and Mcm2–7, ssARS1–2 inhibits the interaction between Sld3 and Mcm2–7, whereas ssARS1–2 promotes the association between GINS and Mcm2–7. We also found that ssARS1–2 promotes the formation of the CMG complex. We conclude that ssARS1–2 releases Sld3 from Mcm2–7, allowing GINS to bind Mcm2–7.

CDK-phosphorylated Sld2 and Sld3 Bind to Single-stranded Origin DNA with High Affinity—In a previous study, we found that CDK-phosphorylated Sld2 binds to origin single-stranded DNA with high affinity (26). CDK-phosphorylated Sld2 binds to ssARS1–1, the complementary strand to ssARS1–2 (26). These data suggest that Sld2 and Sld3 may bind to complementary single-stranded DNA strands at an origin of replication.
Fig. 10). The region of DNA adjacent to the origin recognition complex binding site is AT-rich and prone to spontaneous melting (37, 38). Furthermore, the Mcm proteins initiate unwinding in the neighboring region of DNA (36, 40). Moreover, the CMG complex binds to ssDNA, not dsDNA, and the CMG complex unwinds DNA by a steric exclusion mechanism (13).

It is interesting that mutation of seven 5’-thymines of ssARS1-2 to adenines abolishes binding of Sld3 to DNA. Similarly, mutation of the thymines of ssARS1-1 to adenines abolishes binding of Sld2 to origin ssDNA (26). Origin sequences are AT-rich (34, 36, 37), and the preference of binding of Sld2 and Sld3 to thymines may reflect a mechanism to ensure that Sld2 and Sld3 will bind to the AT-rich regions of origin DNA. Previously, we found that Sld2 and Dpb11 stimulate the annealing of ssARS1-1 to ssARS1-2 (26). However, if Sld3 is bound to ssARS1-2, then this annealing activity may be inhibited in vivo.

Role of Sld3 in Initiation of DNA Replication—Sld3 and GINS compete with one another to bind Mcm2–7, and ssARS1-2 influences this competition. When Sld3 and GINS compete for Mcm2–7 binding in the presence of ssARS1-2, ssARS1-2 binds to Sld3. The interaction between Sld3 and ssARS1-2 releases Sld3 from Mcm2–7, allowing GINS to bind Mcm2–7. The mixture was then subjected to size-exclusion chromatography as described under “Experimental Procedures.” The radioactive counts in each fraction were used to calculate the pmol of Sld3 in each fraction. Sld3 (pmol) was then plotted versus the elution of molecular weight standards. B, E, and H, 100 pmol of 32P-labeled GINS was incubated in the absence or presence of 100 pmol of Sld3, 100 pmol of Mcm2–7, or 200 pmol of ssARS1-2. The mixture was then subjected to size-exclusion chromatography as described under “Experimental Procedures.” C, F, and I, 100 pmol of 32P-labeled Mcm2–7 was incubated in the absence or presence of 100 pmol of Sld3, 100 pmol of GINS, or 200 pmol ssARS1-2. The mixture was then subjected to size-exclusion chromatography as described under “Experimental Procedures.” The data are mean ± S.E.
Sld3 is to block the interaction between GINS and Mcm2–7. However, when single-stranded DNA is exposed during S phase, Sld3 releases its grip on Mcm2–7, allowing GINS to bind to Mcm2–7. Treslin and Ticrr have recently been identified as possible Sld3 homologs in higher eukaryotes (41, 42). Future work may reveal whether Treslin or Ticrr bind directly to origin ssDNA or whether some other protein fulfills this function in humans.

What Releases Sld3 from Cdc45?—The Sld3-Cdc45 interaction may be disrupted by some mechanism in vivo as Sld3 does not travel with the replication fork, whereas Cdc45 does travel with the replication fork (21). Our data suggest that Cdc45 is more stable in a ternary complex with GINS and Mcm2–7 than in a binary complex with free Sld3 (Fig. 9). Thus, the formation of the CMG complex may itself promote the eventual release of Sld3 from Cdc45. Furthermore, excess GINS (6-fold excess relative to Sld3) will substantially disrupt (>80%) the interaction between Sld3 and Cdc45 in vitro (39). There are ~1430 molecules of Psf1 and ~2210 molecules of Psf3 in the cell, in comparison with ~125 molecules of Sld3 in the cell (43). The excess of GINS compared with Sld3 in the cell may be sufficient to disrupt the interaction between Sld3 and Cdc45 in vivo. Thus, the formation of the CMG complex may promote the eventual release of Sld3 from Cdc45, or excess cellular GINS may be sufficient to disrupt the interaction between Sld3 and Cdc45 in vivo. On the other hand, there are ~1730 molecules of Cdc45 in the cell, compared with ~125 molecules of Sld3 (43). Because Cdc45 is in excess compared with Sld3, the disruption of the Sld3-Cdc45 interaction may not be critical for origin firing.

Model for Initiation of DNA Replication (Fig. 10)—The origin recognition complex, Cdc6, and Cdt1 load sister Mcm2–7 hexameric rings around double-stranded DNA at an origin of DNA replication. This process requires the interaction of Cdc6 with Cdt1 and the Mcm2–7 complex. The Mcm2–7 complex then promotes the unwinding of DNA, allowing the replication fork to move forward. Cdc6 and Cdt1 are degraded after replication initiation, allowing the Mcm2–7 complex to enter the CMG complex. This complex then promotes the formation of the origin recognition complex, allowing the replication fork to continue moving forward.
GINS and Cdc45 bind tightly to Mcm2–7, the Cdc45-Mcm2–Mcm2–7, exposing a binding site on Mcm2–7 for GINS. Once single-stranded DNA exposed at an origin during S phase (26).

phosphorylated Sld2 and Sld3 bind to the complementary single-stranded DNA at an origin by a CDK-mediated event. The DNA melts in the region adjacent to the origin recognition complex, allowing Sld3 and Sld2 to bind to the single strands of the origin. Binding of Sld3 to single-stranded DNA at an origin releases Sld3 from Mcm2–7, allowing Mcm2–7 to bind to GINS. The Cdc45-Mcm2–7-GINS (CMG) complexes separate and move bidirectionally to initiate bidirectional DNA unwinding. Sld3 and Sld2 do not migrate with the replication fork.

replication during late M phase and G1 phase (Fig. 10) (9, 10). Cdc45 and Sld3 bind to early replication origins during G1, (23). Sld3 may bind directly to the Mcm2–7 complex in this recruitment event. During S phase, DDK and CDK are active. DDK phosphorylates Mcm4, and this phosphorylation event alleviates a critical inhibitory activity of the N-terminal region of Mcm4 (29). DDK phosphorylation of Mcm4 also promotes the interaction between Cdc45 and Mcm2–7 (28). CDK phosphorylates Sld2 and Sld3, and these phosphorylation events promote the interaction of Sld2 and Sld3 with Dpb11 (24, 25). CDK-phosphorylated Sld2 and Sld3 bind to the complementary single-stranded DNA exposed at an origin during S phase (26). Binding of Sld3 to single-stranded DNA releases Sld3 from Mcm2–7, exposing a binding site on Mcm2–7 for GINS. Once GINS and Cdc45 bind tightly to Mcm2–7, the Cdc45-Mcm2–7-GINS complex becomes active as a helicase, unwinding DNA bidirectionally to initiation DNA replication (11, 13).

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FIGURE 10. Model for the initiation of DNA replication. Two sister Mcm2–7 complexes are loaded onto an early replication origin in late M phase and G1. Mcm2–7-GINS (CMG) complexes separate and move bidirectionally to initiate bidirectional DNA unwinding. Sld3 and Sld2 do not migrate with the replication fork.