The initiation of chromosomal DNA replication is tightly regulated to achieve genome replication just once per cell cycle and cyclin-dependent kinase (CDK) plays an important role in this process. Adenine nucleotides that bind to the origin recognition complex (ORC) are also suggested to be involved in this process. Of the six subunits of the *Saccharomyces cerevisiae* ORC (Orc1-6p), both Orc1p and Orc5p have ATP binding activity, and both Orc2p and Orc6p are phosphorylated by CDK in cells. In this study we constructed a series of yeast strains expressing phospho-mimetic mutants of Orc2p or Orc6p and found that expression of a Ser-188 mutant of Orc2p (Orc2-5Dp) delays G1-S transition and S phase progression and causes the accumulation of cells with 2C DNA content. Using antibody that specifically recognizes Ser-188-phosphorylated Orc2p, we showed that Ser-188 is phosphorylated by CDK in a cell cycle-regulated manner. Expression of Orc2-5Dp caused phosphorylation of Rad53p and inefficient loading of the six minichromosome maintenance proteins. These results suggest that the accumulation of cells with 2C DNA content is due to inefficient origin firing and induction of the cell cycle checkpoint response and that dephosphorylation of Ser-188 of Orc2p in late M or G1 phase may be involved in pre-RC formation. *In vitro*, a purified mutant ORC containing Orc2-5Dp lost Orc5p ATP binding activity. This is the first demonstration of a link between phosphorylation of the ORC and its ability to bind ATP, which may be important for the cell cycle-regulated initiation of DNA replication.

The initiation of chromosomal DNA replication is tightly regulated to replicate the genome just once per cell cycle. To achieve this, both induction of initiation at the G1-S boundary and inhibition of initiation in other phases of the cell cycle are required. The mechanisms governing this regulation in eukaryotes have been studied the most extensively in budding yeast (*Saccharomyces cerevisiae*), and we describe mostly events in budding yeast in this paper otherwise noticed. Cyclin-dependent protein kinases (CDKs)2 play essential roles in both the induction and inhibition of initiation; low CDK activity in late M and G1 phases is required to prepare for initiation of DNA replication, and high CDK activity in S, G2, and early M phases is required for suppression of re-initiation of DNA replication before cell division. This high CDK activity is also involved in initiation of DNA replication at the G1-S boundary (1–4).

Cell cycle-regulated formation of protein complexes on origins of chromosomal DNA replication is a key step in regulation of the initiation of DNA replication. In G1 phase (under low CDK activity), a protein complex called the “pre-replication complex (pre-RC)” is formed on each origin. The pre-RC contains several proteins including the origin recognition complex (ORC), Cdc6p, Cdt1p, and the six minichromosome maintenance proteins (MCM), Mcm2–7p. The ORC was originally identified as a six-protein complex that specifically bound to *S. cerevisiae* origins of DNA replication (5), and its homologues have been found in various eukaryotic species, including human (3). In this manuscript, “ORC” refers to *S. cerevisiae* ORC. The ORC is bound to chromatin at the origins of chromosomal DNA replication throughout the cell cycle and is thought to function as a “landing pad” for the assembly of pre-RC. At the G1-S boundary, CDK and another kinase (Cdc7p-Dbf4p) activate the pre-RC to initiate chromosomal DNA replication. After initiation, re-formation of the pre-RC is strictly prohibited to suppress re-initiation of DNA replication, and high CDK activity is essential for this process; artificial inhibition of CDK activity in G2 phase resulted in re-formation of pre-RC and re-initiation of DNA replication (6–8). The B type cyclin-CDK complex affects initiation of DNA replication through two distinct mechanisms, phosphorylation of, or direct binding to replication-related proteins (9, 10). Therefore, identification of the components of the protein complex present on origin DNA that are phosphorylated by CDK and an understanding of the role of this phosphorylation are important for understanding the mechanisms which ensure that replication occurs just once per cell cycle.

It has been suggested that Orc2p, Orc6p, Cdc6p, and MCM are phosphorylated by CDK in a cell cycle-regulated manner.

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2 The abbreviations used are: CDK, cyclin-dependent protein kinase; ORC, origin recognition complex; pre-RC, pre-replicative complex; MCM, minichromosome maintenance proteins; 5-FOA, 5-fluoroorotic acid; HA, hemagglutinin; α-factor, mating factor; ChIP, chromatin immunoprecipitation; ARS1, autonomously replicating sequence 1; GST, glutathione S-transferase; FACS, fluorescence-activated cell sorter.

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Masaki Makise, Masaya Takehara, Akihiko Kuniyasu, Nanako Matsui, Hitoshi Nakayama, and Tohru Mizushima
From the Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan

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Phosphorylation of Cdc6p or MCM seems to cause its degradation or nuclear exclusion, respectively (11–17). Furthermore, the expression of degradation-resistant Cdc6p and Mcm7p with an exogenous nuclear localization signal with the expression of mutant forms of Orc2p and Orc6p in which possible CDK-phosphorylated sites are mutated to be inert, induced reformation of the pre-RC and re-initiation of DNA replication without inhibition of CDK (18, 19). These results suggest that CDK-dependent phosphorylation of ORC, Cdc6p, and MCM play an important role in suppression of re-initiation of pre-RC and re-initiation of DNA replication. Furthermore, the role of dephosphorylation of ORC suppresses the re-initiation of DNA replication. Unclear which subunit (Orc2p or Orc6p) or which possible CDK-phosphorylated site is responsible for this regulation and how the phosphorylation of ORC suppresses the re-initiation of DNA replication. Furthermore, the role of dephosphorylation of ORC in late M or G1 phase cannot be ruled out by use of mutants that are inert for phosphorylation. To address these issues, characterization of phospho-mimetic mutants (in which particular CDK target amino acid residues are substituted with Asp or Glu) is useful. For example, analysis of a phospho-mimetic mutant of Sld2p suggested that phosphorylation of Sld2p is responsible for CDK-dependent initiation of DNA replication (20–22).

As is the case for the bacterial initiators of chromosomal DNA replication, DnaA (23–27), adenine nucleotides bound to the ORC seem to regulate the initiation of DNA replication. The ORC has two subunits (Orc1p and Orc5p), which bind ATP (28, 29). Orc1p, but not Orc5p, has ATPase activity, which stimulates the loading of MCM onto origins (30, 31). Orc5p, but not Orc1p, can bind ADP (32). The binding of ATP to Orc1p, but not to Orc5p, is essential for the specific binding of ORC to origin DNA (28, 29). ATP binding to Orc5p increases the affinity of Orc1p for ATP in vitro (29) and is important for maintaining the stability of ORC in vivo (33, 34). However, a link between phosphorylation of ORC and its ATP binding activity has not been previously established.

In this study we constructed a series of yeast strains expressing phospho-mimetic mutant Orc2p or Orc6p for each possible CDK-phosphorylated site. We found that expression of a Ser-188 mutant of Orc2p, but not of other CDK-phosphorylated site mutants, delays cell growth, G1-S transition, S phase progression, and pre-RC formation. This suggests that Ser-188 is the key amino acid residue in CDK-dependent regulation of ORC. Thus, dephosphorylation of Ser-188 of Orc2p in late M or G1 phase may be involved in the formation of pre-RC. In vitro, the purified phospho-mimetic mutant ORC containing the Ser-188 Orc2p mutant retained the ATP binding activity of Orc1p and origin DNA binding activity but lost the ATP binding activity of Orc5p. We consider that phosphorylation of Ser-188 of Orc2p affects pre-RC formation by inhibiting the binding of ATP to Orc5p.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Plasmids, and Strains**—[α-32P]ATP (3000 Ci/mmol), [γ-32P]ATP (6000 Ci/mmol), and poly(dI-dC) were purchased from GE Healthcare, and 8-N3-[γ-32P]ATP was from ALT Bioscience. DNA fragments (290 bp) containing wild-type ARS1 and mutant ars1/A “B1” were prepared by PCR as described previously (35) and purified by polyacrylamide gel electrophoresis. DNA fragments were radiolabeled by T4 polynucleotide kinase and [γ-32P]ATP as described previously (29). The specific activity of each probe was ~4000 cpm/μmol DNA. Mouse monoclonal antibodies against Orc3p, Mcm2p, and hemagglutinin (HA) were gifts from Dr. Stillman (Cold Spring Harbor Laboratory). Mouse monoclonal antibody against FLAG was purchased from Sigma. Plasmids, pRS403, 405, 413, 416, and 425 were purchased from Invitrogen.

Plasmids pRS413-ORC2 and pRS416-ORC2 contain ORC2 (from −805 to +1909) ligated into the SacI-SalI site. To express HA-tagged Orc2p in cells, pRS413–3HA and pRS413-GAL1-3HA were first constructed. Plasmid pRS413–3HA has a triple HA epitope gene (3HA) in the Xhol-Apal site, and pRS413-GAL1-3HA has the GAL1 promoter (from −457 to −1) in the BamHI-EcoRV site. An expression plasmid, pRS413-ORC2-3HA (or pRS413-GAL1-ORC2-3HA), contains ORC2 ligated in-frame with 3HA. A plasmid pRS425-GAL1-ORC2-3HA was constructed by insertion of the BssHII-BssHII DNA fragment of pRS413-GAL1-ORC2-3HA into the same restriction enzyme site on pRS425. Plasmids pRS413-ORC6 and pRS416-ORC6 contain ORC6 (from −600 to +1808) cloned into the XbaI-Xhol site. Site-directed mutagenesis of the CDK consensus sites was done as described previously (36). DNA fragments containing orc2- (or orc6)-All-A were PCR-amplified using chromosomal DNA prepared from plL1095 and plL1096 cells (gifts from Dr. Li (University of California, San Francisco)) (18) as templates. A series of expression plasmids for mutant forms of orc were constructed by replacement of the wild-type ORC gene with each mutant orc gene.

* S. cerevisiae strains are listed in Table 1 (37, 38). These strains were cultured in YP medium (2% bactopeptone, 1% yeast extract, 2 mg/ml adenine hemisulfate) or synthetic complete medium containing glucose or galactose.

To disrupt the chromosomal ORC2 or ORC6, TRP1 was inserted between flanking sequences (40 bp) of the ORC2 or ORC6, and this DNA fragment was introduced into the DK186 diploid (resultant strains were YMM10 or YMM18, respectively). We confirmed that all tetrad showed only two viable spores. For plasmid shuffling, plasmid pRS413 (a low copy number plasmid containing HIS) (39) with each mutant orc2 or orc6 gene, was transformed into YMM10 or YMM18 cells by the lithium acetate method. The transformants were cultured on synthetic complete agar plates containing 2% glucose and 0.1% 5-fluoorotic acid (5-FOA) at 30 °C for 3 days.

YMM77 is a derivative of YMM10 in which orc2-1–3FLAG was integrated into the leu2 locus. We confirmed that all tetrad showed only two viable spores. For plasmid shuffling, plasmid pRS413 (a low copy number plasmid containing HIS) (39) with each mutant orc2 or orc6 gene, was transformed into YMM10 or YMM18 cells by the lithium acetate method. The transformants were cultured on synthetic complete agar plates containing 2% glucose and 0.1% 5-fluoorotic acid (5-FOA) at 30 °C for 3 days.

YMM77 is a derivative of YMM10 in which orc2-1–3FLAG was integrated into the leu2 locus. YMM84 and YMM76 are derivatives of YMM77 in which orc2-3HA and orc2-5d-3HA, respectively, were integrated into the his3 locus. We confirmed these integrations by PCR and Southern blot analysis.

**Preparation of Antibodies and ORC**—Rabbit polyclonal antibody against Ser-188-phosphorylated Orc2p (α-Ser(P)-188) was generated against synthetic peptide with the following sequence: NHDFTS[PO3]PPLKQII (40). The antibody was purified on a protein A-Sepharose column followed by Sulfolink Coupling Gel (Pierce) coupled with the synthetic peptide. The specificity of the antibody was tested by enzyme-linked
immunosorbent assay using the peptide and the peptide lacking the phosphorus at the serine residue (data not shown).

ORCs were co-expressed in S. cerevisiae cells infected with recombinant baculoviruses and purified as described (29). Baculovirus containing the phospho-mimetic orc2 gene was constructed using a BD BaculoGold™ transfection kit (BD Biosciences) according to the manufacturer's instructions.

Phosphorylation of ORC—Purification of yeast recombinant CDK (rGST-Cdc28-Clb5) was performed as described (21). The plasmid, pGEX6P-1/CDC28-CAK1-CKS1-CLB5, was gift from Dr. Araki (National Institute of Genetics). To obtain an active recombinant GST-Cdc28-Clb5 complex (rGST-Cdc28-Clb5), the cell extracts prepared from Escherichia coli Rosetta 2(DE) pLysS cells (Novagen) harboring the plasmid was applied to the glutathione-Sepharose column, and rGST-Cdc28-Clb5 was eluted with the buffer containing reduced glutathione.

For phosphorylation of ORC, ORC (30 pmol) and recombinant CDK (13.5 pmol) were incubated at 25 °C for 24 h in the buffer (600 μl) containing 50 mm Hepes-KOH, pH 7.5, 10 mm MgCl₂, 1 mm ATP. To remove ATP from ORC samples, ORC was precipitated with SP-Sepharose (GE Healthcare) and eluted with the buffer containing 50 mm Hepes-KOH, pH 7.5, 500 mm KCl, 5 mm Mg(OAc)₂, 1 mm EDTA, 1 mm EGTA, 0.02% v/v Nonidet P-40, and 10% v/v glycerol.

UV-cross-linking Assay—UV-cross-linking experiments were done as described previously (28, 29). ORC (3 pmol) was incubated with 4 μM 8-Ne[γ-32P]ATP in the presence or absence of ARS1 DNA fragments at 4 °C for 5 min. Samples were placed on Parafilm and subjected to UV irradiation at 4 °C for 2 min. The photolabeling reaction was terminated by the addition of stop solution (70 μl) containing 0.1 mm dithiothreitol and 20 mm EDTA. Samples were precipitated by 20% trichloroacetic acid, washed with acetone, and separated by electrophoresis on a polyacrylamide gel (10%) containing SDS. Gels were stained with silver to identify each ORC subunit, and radiolabeled subunits were detected by autoradiography.

Filter Binding Assay—ORC was incubated with [α-32P]ATP or radiolabeled DNA fragments (200 fmol) at 30 °C for 5 min in 40 μl of buffer T (25 mm Tris-HCl, pH 7.6, 5 mm MgCl₂, 70 mm KCl, 5 mm dithiothreitol, and 5% (v/v) glycerol). In some of the ATP binding experiments, ORC was further incubated with DNA fragments at 30 °C for 5 min in the same buffer. Samples were passed through nitrocellulose membranes (Millipore HA, 0.45 μm) and washed with 5 ml of ice-cold buffer T twice. The radioactivity remaining on the filter was monitored with a liquid scintillation counter.

ATPase Assay—The ATPase activity of ORC was measured as described previously (28) with some modifications. ORC (0.3 pmol) was incubated with DNA fragments (6 pmol) in 10 μl of ATPase buffer (50 mm Hepes-KOH pH7.6, 150 mm KCl, 5 mm Mg(OAc)₂, 1 mm EDTA, 1 mm EGTA, 0.02% v/v Nonidet P-40, and 10 μM radiolabeled ATP) for 60 min at room temperature. The reaction was stopped by the addition of 2% w/v SDS (5 μl), and adenosine nucleotides were separated on polyethyleneimine cellulose F TLC plates (Merck).

Chromatin Binding Assay—Yeast spheroplasts were lysed with Triton X-100, and the samples were processed into soluble (supernatant) and chromatin (insoluble precipitate) fractions by centrifugation as previously described (41, 42). Equivalent amounts (total protein) of chromatin fractions were subjected to electrophoresis on a polyacrylamide gel (10%) containing SDS, transferred to polyvinylidene difluoride membranes, and probed with antibodies.

FACS Analysis—The samples were prepared as previously described (33) with some modifications. The cells were pelleted by centrifugation and fixed in 70% ethanol for 1 h. The cells

| Yeast strains used in this study |
|----------------------------------|
| **Table 1**                      |
| **Strains** | **Genotype** | **References** |
| W303-1A | MATa leu2-3,112 ura3-52 can1-100 ade2-1 his3-11 trpl-1 | Ref. 37 |
| DK186 |                       | Ref. 37 |
| YMM10 | DK186 orc2Δ::TRP1[pRS416-ORC2] | This study |
| YMM10-2 | YMM10[prS413] | This study |
| YMM10-3 | YMM10[prS413-ORC2] | This study |
| YMM10-4 | YMM10[prS413-orc2-All-D] | This study |
| YMM10-5 | YMM10[prS413-orc2-All-A] | This study |
| YMM10-6 | YMM10[prS413-orc2-2d] | This study |
| YMM10-7 | YMM10[prS413-orc2-2d] | This study |
| YMM10-8 | YMM10[prS413-orc2-12d] | This study |
| YMM10-9 | YMM10[prS413-orc2-4d] | This study |
| YMM10-10 | YMM10[prS413-orc2-5d] | This study |
| YMM10-11 | YMM10[prS413-orc2-6d] | This study |
| YMM10-12 | YMM10[prS413-orc2-6d] | This study |
| YMM10-13 | YMM10[prS413-orc2-456d] | This study |
| YMM10-14 | YMM10[prS413-orc2-12346d] | This study |
| YMM10-15 | YMM10[prS413-orc2-5a] | This study |
| YMM18 | DK186 orc2Δ::TRP1[pRS416-ORC6] | This study |
| YMM18-2 | YMM18[prS413] | This study |
| YMM18-3 | YMM18[prS413-ORC6] | This study |
| YMM18-4 | YMM18[prS413-orc2-All-D] | This study |
| YMM18-5 | YMM18[prS413-orc2-All-A] | This study |
| YMM69 | YMM69[prS413-GAL1-ORC2-3HA] | This study |
| YMM71-1 | YMM10[prS413-GAL1-ORC2-5d-3HA] | This study |
| YMM71-2 | YMM10[prS413-GAL1-ORC2-5a-3HA] | This study |
| YMM76 | YMM76[his3::pRS403-ORC2-3d-3HA] | This study |
| YMM77 | YMM77[his3::pRS403-ORC2-1-3FLAG] | This study |
| YMM84 | YMM77[his3::pRS403-ORC2-3HA] | This study |
| YMM87 | W303-1A[pRS425-GAL1-ORC2-3HA] | This study |
| YMM88 | W303-1A[cdc28-4[pRS425-GAL1-ORC2-3HA]] | This study |
were pelleted by centrifugation, washed with 50 mM sodium citrate once, pelleted again by centrifugation, incubated with 50 mM sodium citrate containing 0.25 mg/ml RNase A for 1 h at 50 °C, and then treated with 1 mg/ml proteinase K for 1 h at 50 °C. DNA was stained with 50 μg/ml propidium iodide at 4 °C for 1 h, and 20,000 cells from each sample were scanned with a FACS Calibur (BD Biosciences).

Gel Electrophoretic Mobility Shift Assay—A gel electrophoretic mobility shift assay was performed as described (32, 43) with some modifications. ORCs were incubated with adenine nucleotides for 5 min at 30 °C and with radiolabeled wild-type ARS1 or mutant ars1/A/B1 DNA fragments (100 fmol) for 5 min at 30 °C in 10 μl of buffer T containing 2 mg/ml of bovine serum albumin and 10 μg/ml poly(dI/dC) (nonspecific competitors). The reaction sample was loaded onto a 3.5% polyacrylamide gel containing 0.5 M Tris borate, pH 8.3, and 1 mM EDTA. The gel was electrophoresed for 1.5 h at a constant 200 V, dried, and autoradiographed.

Chromatin immunoprecipitation (ChIP) Assay—A ChIP assay was done as described previously (44) with some modifications. Cells were cross-linked with 1% formaldehyde for 15 min at 25 °C. After the addition of 125 mM (final concentration) of buffer T containing 0.5 M Tris borate, pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate (w/v), 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 1 μg/ml leupeptin, and 2 μg/ml pepstatin A. Samples were sonicated 30 times for 10 s (to achieve an average fragment size of 0.5–1 kilobases). Immunoprecipitation was performed with magnetic beads which were coated with protein G (Dynal) and antibody against HA or Mcm2p. Precipitates were washed, processed for DNA purification, and subjected to PCR. The PCR cycles included an initial denaturation step of 0.5 min at 95 °C, which was followed by 35 cycles of a denaturation step for 0.5 min at 95 °C, an annealing step for 0.5 min at 50 °C, a polymerization step for 1 min at 72 °C, and a final extension for 4 min at 72 °C. The PCR products were separated on a 3% agarose gel and visualized under UV after ethidium bromide staining.

RESULTS

Site-directed Mutational Analysis of Orc2p and Orc6p Phosphorylation—It was reported that Orc2p and Orc6p have six (Ser-16, Ser-24, Thr-70, Thr-174, Ser-188, and Ser-206) and four (Ser-106, Ser-116, Ser-123, and Thr-146) consensus CDK phosphorylation sites ((S/T)P, respectively (Fig. 1A). Expression of mutant forms of Orc2p and Orc6p in which these amino acid residues were substituted with Ala caused re-initiation of DNA replication in G2 phase when a degradation-resistant mutant of Cdc6p and a Mcm7p with an exogenous nuclear localization signal were expressed simultaneously (18, 19). We constructed a phospho-mimetic mutant of Orc2p, Orc2-All-Dp, in which all of the consensus CDK phosphorylation sites were mutated to Asp. Orc6-All-Dp was constructed in a similar way. We also constructed Orc2-All-Ab and Orc6-All-Ab in which the consensus CDK phosphorylation sites were mutated to Ala. Each mutant gene was inserted into a plasmid, and the plasmid was transformed into strain YMM10 (or YMM18) which contains a chromosomal ORC2 or ORC6 deletion and an alternative wild-type gene on a plasmid with the URA3 selectable maker. When the transformants were grown on agar plates containing 5-FOA, the URA3 plasmid was selected against and lost, causing cells to rely solely on the mutant orc2 or orc6 gene (plasmid-shuffling analysis). YMM10 (or YMM18) cells containing plasmid carrying the wild-type genes could grow, and those containing vector only could not grow on the 5-FOA-containing plates (Fig. 1B). The mutations in the CDK sites of the various Orc2p mutants are indicated, and the results of a plasmid-shuffling assay for each Orc2p mutant are shown in (C).

FIGURE 1. Plasmid shuffling assay for phospho-mimetic orc2 and orc6 mutants. A, the positions of consensus CDK phosphorylation sites ((S/T)P) in Orc2p and Orc6p are shown. B, strains were spread onto synthetic complete agar plates in the arrangement depicted in the right panel with or without 5-FOA and incubated at 30 °C for 3 days. C, the mutations in the CDK sites of the various Orc2p mutants are indicated, and the results of a plasmid-shuffling assay for each Orc2p mutant are shown (C).
other hand, YMM18 expressing Orc6-All-Dp could grow even on agar plates containing 5-FOA (Fig. 1B). Plasmid shuffling analysis also showed that both of the Ala substitution mutants can support cell growth (Fig. 1B), as described previously (18). Based on results in Fig. 1, we consider that dephosphorylation of Orc2p but not of Orc6p is required for cell growth and cell cycle progression.

To identify the amino acid residue responsible for the phenotype exhibited by cells expressing Orc2-All-Dp, we constructed a series of mutant Orc2p, as shown in Fig. 1C, and analyzed the function of each mutant protein by plasmid shuffling analysis. Results show that the S188D Orc2p mutant (Orc2-5Dp) but not the variants of Orc2p with mutations in the other consensus CDK phosphorylation sites is unable to support cell growth (Fig. 1C). Furthermore, a mutant Orc2p in which all of the consensus CDK phosphorylation sites except the Ser-188 are replaced with Asp (Orc2-12346Dp) could support cell growth (Fig. 1C). Thus, we concluded that Ser-188 is an important target of CDK for controlling cell cycle progression, and phosphorylation of this amino acid residue may block cell cycle progression.

To detect phosphorylation of Ser-188 of Orc2p in vivo, we prepared polyclonal antibody that recognizes Ser-188-phosphorylated Orc2p (α-Ser(P)-188). As shown in Fig. 2A, α-Ser(P)-188 detected a band with the same migration as that detected by antibody against HA in cells expressing wild-type HA-tagged Orc2p but not in cells expressing HA-tagged Ser-188 Orc2p mutant (Orc2-5Dp or Orc2-5Ap). The band was not detected in the wild-type Orc2p-containing sample that had been treated with λ-protein phosphatase (Fig. 2B). Based on these results, we conclude that α-Ser(P)-188 can specifically recognize Ser-188-phosphorylated Orc2p. As shown in Fig. 2C, Ser-188-phosphorylated Orc2p was detected in asynchronously growing cells, hydroxyurea-blocked cells and nocodazole-blocked cells but not in α mating factor (α-factor)-blocked cells, suggesting that Ser-188 of Orc2p is phosphorylated at the G₁-S boundary and dephosphorylated in late M or G₂ phase. This pattern correlates well with that of cell cycle-regulated phosphorylation of Orc2p, as judged by an upward band shift (18). We also found that Ser-188-phosphorylated Orc2p was not detected in a temperature-sensitivecdc28 mutant at a non-permissive temperature (Fig. 2D), suggesting that the phosphorylation of Ser-188 of Orc2p is catalyzed by CDK.

**Function of Orc2-5Dp in Cells—**
To understand the role of phosphorylation of Orc2p at Ser-188 and the function of Orc2-5Dp in cells, we constructed a YMM76 strain in which the wild-type ORC2 is deleted, FLAG-tagged Orc2-1p is expressed under control of the GAL1 promoter and Orc2-5Dp is constitutively expressed. Because Orc2-1p is rapidly degraded at high temperatures (45), YMM76 cells cultured in galactose-free medium at 37 °C rely on Orc2-5Dp. YMM84 cells express wild-type Orc2p instead of Orc2-5Dp, and YMM77 strain was used as the control.

We characterized these strains by a chromatin binding assay. As shown in Fig. 3A, incubation of the strains (YMM84 (ORC2), YMM76 (orc2-5d), and YMM77 (orc2Δ)) at 37 °C in glucose-containing medium caused the rapid disappearance of FLAG-tagged Orc2-1p. Expression of HA-tagged wild-type Orc2p or Orc2-5Dp was induced under these conditions, suggesting that yeast cells have an as yet an unknown mechanism for keeping the amount of Orc2p at a constant level. A similar mechanism seems to exist in human cells based on a recent report (46). Results similar to those in Fig. 3A were observed when total cell lysates were used instead of chromatin fractions (data not shown). The results in Fig. 3A also show that Orc2-5Dp (maybe as a complex with other subunits of ORC) can bind to chromatin as efficiently as wild-type Orc2p. Along with the degradation of FLAG-tagged Orc2-1p, Orc3p was degraded in YMM77 but not as distinctly in YMM76 cells (Fig. 3A), supporting the idea that Orc2-5Dp can form a complex with other ORC subunits because the ORC becomes unstable when one of its subunits is missing (47).
these cells were blocked at G2/M phase. Similar accumulation of Orc2-1p was suppressed by incubating asynchronously cultured cells with 2C DNA content (Fig. 3, A). FACS analysis showed that incubation of YMM76 cells in glucose for the indicated periods. Whole cell extracts were prepared and analyzed by immunoblotting with antibody against Orc2p, suggesting that Orc2-5Dp is as stable as wild-type Orc2p. Thus, expression of Orc2-5Dp in late M or early G1 phase seems to suppress G1-S transition and S phase progression, maybe through inhibiting pre-RC formation.

It is well known that various checkpoint responses exist to achieve proper cell cycle progression. For example, DNA replication stress, DNA damage, or defects in spindle attachment to centromeres induce the checkpoint response to arrest cell cycle progression. Asynchronous cultured cells (YMM84, YMM76, and YMM69) were further incubated at 37 °C in YP medium containing galactose for the indicated periods. FLAG-tagged Orc2-1p, HA-tagged Orc2p (or Orc2-5Dp), and Orc3p in chromatin fractions were detected by immunoblotting with antibody against FLAG, HA, or Orc3p. A, for loading control, the gel was stained with silver. B, cell cycle progression was examined by FACS analysis. C, YMM69 (GAL1-ORC2-3HA) and YMM71-1 (GAL1-orc2-5d-3HA) cells grown in YP medium containing galactose were further incubated at 37 °C in YP medium containing glucose for the indicated periods. Whole cell extracts were prepared and analyzed by immunoblotting with antibody against HA and Orc3p.

FACS analysis showed that incubation of YMM76 cells in glucose-containing medium at 37 °C causes accumulation of cells with 2C DNA content (Fig. 3B), suggesting that most of these cells were blocked at G2/M phase. Similar accumulation was observed in YMM77 cells, as described previously (45). Furthermore, we examined the stability of Orc2-5Dp in cells. As shown in Fig. 3C, after the expression of Orc2-5Dp or wild-type Orc2p was shut off, the time-course profile of degradation of Orc2-5Dp was indistinguishable from that of wild-type Orc2p, suggesting that Orc2-5Dp is as stable as wild-type Orc2p.

We performed block and release experiments to examine the cell cycle progression in detail. Asynchronous cultured cells were synchronized at G1 or G2/M phase by incubation with α-factor (Fig. 4, D and E) or nocodazole (Fig. 4, B and C), respectively, expression of Orc2-1p was suppressed by incubating cells in galactose-free medium, and then the cells were released into fresh medium (Fig. 4A). As shown in Fig. 4B, G1/M-G1 transition was indistinguishable between the YMM76 and YMM84 strains. However, the following G1-S transition and S phase progression was a little slower in the YMM76 than in the YMM84 strain (see the FACS data for 50 or 60 min after the release; Fig. 4B). The delay was more apparent in the orc2Δ strain (Fig. 4B). A chromatin binding assay confirmed that Orc2-1p disappeared before the release, and approximately equal amounts of wild-type Orc2p and Orc2-5Dp were loaded on the chromatin (Fig. 4C). Results similar to those in Fig. 4C were observed when total cell lysates were used instead of chromatin fractions (data not shown). On the other hand, data from α-factor block experiments showed that the G1-S transition and S phase progression was not so different between these two strains (YMM76 and YMM84) (Fig. 4D), suggesting that Orc2-5Dp can support the initiation of DNA replication after the point of the cell cycle which is blocked by α-factor; thus, after pre-RC formation. Thus, expression of Orc2-5Dp in late M or early G1 phase seems to suppress G1-S transition and S phase progression, maybe through inhibiting pre-RC formation.

The delay was more apparent in the orc2Δ strain (Fig. 4B). A chromatin binding assay confirmed that Orc2-1p disappeared before the release, and approximately equal amounts of wild-type Orc2p and Orc2-5Dp were loaded on the chromatin (Fig. 4C). Results similar to those in Fig. 4C were observed when total cell lysates were used instead of chromatin fractions (data not shown). On the other hand, data from α-factor block experiments showed that the G1-S transition and S phase progression was not so different between these two strains (YMM76 and YMM84) (Fig. 4D), suggesting that Orc2-5Dp can support the initiation of DNA replication after the point of the cell cycle which is blocked by α-factor; thus, after pre-RC formation. Thus, expression of Orc2-5Dp in late M or early G1 phase seems to suppress G1-S transition and S phase progression, maybe through inhibiting pre-RC formation.

To examine the activity of Orc2-5Dp in origin binding and pre-RC formation, we performed the experiments shown in Fig. 4A; asynchronously cultured cells (YMM84, YMM76, and YMM77) were synchronized at G1/M phase, expression of Orc2-1p was suppressed, and cells were released into fresh medium with α-factor to re-synchronize at G1 phase. FACS analysis showed that these synchronizations were well controlled (Fig. 5B). Under the same conditions we performed a ChIP assay; HA-tagged Orc2p or Orc2-5Dp was precipitated, and co-precipitated DNA species were monitored by PCR. As shown in Fig. 5C, both ARS1 (origin) and LEU2 (control) DNA fragments were approximately equally amplified when total DNA prepared from these strains was used as a template, but relative higher amplification of ARS1 DNA fragments was observed when DNA immunoprecipitated with an antibody against HA was used as a template, showing that a ChIP assay system had been established. Not only LEU2 but also ARS1 DNA fragments were amplified equally between the YMM84 and YMM76 strains (Fig. 5C). On the other hand,
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less efficient amplification of ARS1 DNA fragments was observed in the YMM77 strain (Fig. 5C). These results suggest that ORC containing Orc2-5Dp can specifically bind to origin DNA in a similar way to wild-type ORC.

We performed a chromatin binding assay to monitor pre-RC formation; the amounts of ORC and MCM in the chromatin fractions were monitored by immunoblotting. As shown in Fig. 5D, the amount of Orc2-5Dp in chromatin fractions was much the same as that of wild-type Orc2p, confirming that ORC containing Orc2-5Dp can bind to origin DNA in an equivalent way to wild-type ORC. On the other hand, the amount of Mcm2p in the chromatin fractions of YMM76 was less than that in YMM84 at G1 phase, suggesting that MCM loading on chromatin and pre-RC formation is inhibited in YMM76. Unfortunately, because ChIP assay for any subunits of MCM did not work in our strains, we could not confirm the results of chromatin binding assay by ChIP assay. These results suggest that ORC containing Orc2-5Dp is inefficient for pre-RC formation,

FIGURE 4. Cell cycle progression in orc2-5d strain. A, experimental outline and timing of sampling. YMM84 (GAL1–2-1-FLAG, ORC2-3HA), YMM76 (GAL-orc2-1-FLAG, orc2-5d-3HA), and YMM77 (GAL-orc2-1-FLAG) cells were incubated in YP medium containing galactose (Gal) and nocodazole (Noc) (B and C) or α-factor (α) (D–F) at 30 °C for 30 min and then incubated in YP medium containing glucose (Glu) and the same concentration of each blocker at 37 °C for 120 min. Cells were released into YP medium containing glucose and incubated at 37 °C for the indicated periods. Samples were analyzed by FACS (B and D). FLAG-tagged Orc2-1p and HA-tagged Orc2p (or Orc2-5Dp) in the chromatin fractions were detected as described in the legend of Fig. 3 (C and E). Whole cell extracts were analyzed by immunoblotting with antibody against Rad53p. F, the arrow and vertical line indicate unphosphorylated and phosphorylated, respectively, Rad53p.
We also compared the DNA binding activities of ORC2-5D, wild-type ORC, ORC-1A (an ORC that contains the mutant Orc1p with a defective Walker A motif), and ORC-5A (an ORC that contains the mutant Orc5p with a defective Walker A motif) by a gel electrophoretic mobility shift assay. As shown in Fig. 6C, in the presence of ATP, wild-type ORC and ORC-5A but not ORC-1A bound to wild-type ARS1 but not to mutant ars1/A − B1 DNA fragments. All of these ORCs did not bind to any DNA fragments in the presence of ADP, as described previously (32). The DNA binding properties of ORC2-5D were indistinguishable from that of wild-type ORC even in the gel electrophoretic mobility shift assay (Fig. 6C), confirming that ORC2-5D can specifically bind to origin DNA.

The ATP binding properties of the wild-type ORC and ORC2-5D were also compared using a filter binding assay. Both types of ORC showed high affinity for ATP, but the number of ATP molecules bound to each ORC2-5D was less than to wild-type ORC in the presence of high concentrations of ATP (Fig. 7A). Scatchard plot analysis showed that the $K_d$ values of wild-type ORC and ORC2-5D for ATP were 17 and 16 nm, respectively, and that the ATP binding sites per wild-type ORC and ORC2-5D were 0.35 and 0.18, respectively. These results suggest that ATP binding to either Orc1p or Orc5p is inhibited in ORC2-5D, and we speculated that ATP binding to Orc5p is inhibited in ORC2-5D, because ORC2-5D can specifically bind to origin DNA (Fig. 6), which is independent of Orc5p ATP binding.

To test this idea, we performed UV-cross-linking analysis using radiolabeled 8-N$_2$-ATP. As shown in Fig. 7B, the results for the wild-type ORC were similar to those reported previously (28); Orc1p, Orc4p, and Orc5p were labeled in the presence of origin DNA fragments, and the labeling of Orc1p and Orc4p was less in the absence of origin DNA fragments. For ORC2-5D, Orc1p and Orc4p but not Orc5p were labeled in the presence of origin DNA fragments, supporting the idea that ATP binding to Orc5p is inhibited in ORC2-5D.

It has been reported that origin DNA fragments stimulate ATP binding to Orc1p (28, 29). We, therefore, examined the effect of ARS1 and mutant ars1/A − B1 DNA fragments on ATP binding to wild-type ORC and ORC2-5D. As shown in Fig. 7C, ARS1 DNA fragments increased the amount of ATP bound to wild-type ORC, and mutant ars1/A − B1 DNA fragments produced a smaller increase, as reported previ-
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We measured the ATPase activity. As shown in Fig. 7D, ORC2-5D showed a similar level of ATPase activity as wild-type ORC, and ORC-1A showed no ATPase activity, as reported previously (28). It has been reported that double-stranded ARS1 DNA fragments inhibit the ATPase activity of ORC (28). Here we examined the effect of double-stranded ARS1 DNA fragments on the ATPase activity of ORC2-5D. ARS1 DNA fragments inhibited the ATPase activity of wild-type ORC and ORC2-5D in a similar way (Fig. 7D). The results suggest that the orc2-5d mutation affects neither the ATPase activity of ORC nor origin DNA binding to ORC. All of the data from the in vitro experiments show that although ORC2-5D has normal origin DNA binding, ATP binding to Orc1p, and ATPase activities, it is inert for ATP binding to Orc5p.

We also examined the effect of Ser-188 phosphorylation of Orc2p on biochemical characters ORC. For phosphorylation of ORC, wild-type ORC was treated with yeast recombinant CDK (rGST-Cdc28-CIb5). This treatment increased the band intensity of Orc2p in immunoblotting analysis with α-Ser(P)-188 (Fig. 8B) without apparent degradation of each subunit of ORC (Fig. 8A). A filter binding assay revealed that phosphorylation of wild-type ORC decreased the amount of bound ATP (Fig. 8C). UV-crosslinking analysis revealed that phosphorylation of wild-type ORC inhibited the labeling of Orc5p in both the presence and absence of origin DNA fragments (Fig. 8D). On the other hand, phosphorylation of wild-type ORC did not affect its binding to wild-type ARS1 DNA fragments (Fig. 8E). Results in Fig. 8 suggest phosphorylated wild-type ORC is less active for ATP binding to Orc5p than unphosphorylated wild-type ORC. Combining data in Figs. 6–8, it is suggested that Ser-188 phosphorylation of Orc2p of wild-type ORC inhibits ATP binding to Orc5p.

The result in Fig. 2A suggests that ORC2-5D cannot be detected in immunoblotting analysis with α-Ser(P)-188. As shown in supplemental Fig. S1A, ORC2-5D (3 pmol) was not detected in immunoblotting analysis with α-Ser(P)-188 under the conditions in which wild-type ORC (0.3 pmol) was detected, supporting the idea mentioned above. On the other hand, treatment of wild-type ORC with λ-protein phosphatase decreased the band intensity in this analysis (data not shown), suggesting that wild-type ORC purified from S9 cells is partially phosphorylated at Ser-188 of Orc2p. Therefore, we compared wild-type ORC treated with λ-protein phosphatase to ORC2-5D in immunoblotting analysis with α-Ser(P)-188. As shown in supplemental Fig. S1B, wild-type ORC treated with λ-protein phosphatase is less reactive than ORC2-5D in this assay. These results suggest that ORC2-5D is more reactive to α-Ser(P)-188 than Ser-188-unphosphorylated wild-type ORC; however, it is less reactive than Ser-188-phosphorylated wild-type ORC, suggesting that α-Ser(P)-188 preferentially recognizes phosphorylated Ser-188, comparing to Asp-188 in Orc2p.
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FIGURE 7. ATP binding activity of ORC2-5D. A, wild-type ORC (ORC-W) and ORC2-5D were incubated with radiolabeled ATP as indicated, and the amount of ATP attached to the ORC was determined by the filter binding assay. Values are the mean ± S.D. (n = 3). B, wild-type ORC (W) and ORC2-5D (2–5D) (3 pmol) were incubated with 0.5 μM Orc5p-ORC (3 pmol) or Orc5p-ORC2-5D (3 pmol) were incubated with 4 μM 8-azido-ATP in the presence or absence of 0.5 pmol of ARS1 DNA fragments at 30 °C for 5 min, and the amount of ATP attached to the ORC was determined by the filter binding assay. Values are the mean ± S.D. (n = 3). C, wild-type ORC (W) and ORC2-5D (0.5 pmol) were incubated with 0.5 μM radiolabeled ATP in the presence or absence of 0.5 pmol of ARS1 DNA fragments at 30 °C for 5 min, and the amount of ATP attached to the ORC was determined by the filter binding assay. Values are the mean ± S.D. (n = 3). D, wild-type ORC (ORC-W), ORC2-5D, and ORC1A (0.5 pmol) were incubated with 10 μM radiolabeled ATP in the presence or absence of 6 pmol of ARS1 DNA fragments. ATPase activity is shown as ATP hydrolyzed/min/pmol of ORC. Values are the mean ± S.D. (n = 3).

DISCUSSION

Using mutant forms of Orc2p and Orc6p in which all the consensus CDK phosphorylated sites are substituted with Ala, which are unable to be phosphorylated, Nguyen et al. (18) suggested that phosphorylation of these subunits is important for suppression of re-initiation of chromosomal DNA replication at G2/M phase. Expression of these Orc2p and Orc6p mutants with the degradation-resistant mutant Cdc6p and the Mcm7p with exogenous nuclear localization signal induces re-initiation of chromosomal DNA replication (18). However, it is unclear which subunit and which amino acid residues are responsible for this regulation. Furthermore, the role of dephosphorylation of ORC in late M or G1 phase cannot be revealed with this type of mutant proteins. In this study, by using mutant forms of Orc2p and Orc6p in which the CDK phosphorylated sites are substituted with Asp, phospho-mimetic mutants of Orc2p and Orc6p, we have suggested that phosphorylation of Orc2p but not of Orc6p is important for the regulation of cell cycle progression. Furthermore, analysis of phospho-mimetic Orc2p mutants for each of these CDK phosphorylated sites suggested that of these sites, Ser-188 of Orc2p is important. Ser-188 is phosphorylated at the G1/S boundary and dephosphorylated at the late M or G1 phase, and expression of the phospho-mimetic Orc2p mutant for this amino acid residue (Orc2-5Dp) delayed cell cycle progression. Furthermore, we found that expression of Orc2-5Dp delayed the G1/S transition (and S phase progression), suggesting that dephosphorylation of Ser-188 of Orc2p is important for pre-RC formation. These are the first data suggesting that dephosphorylation of ORC is involved in regulation of initiation of DNA replication. It is reasonable to speculate that the phosphorylation of Ser-188 of Orc2p at the G1/S boundary is involved in suppression of re-initiation of DNA replication at the G1/M phase.

The YMM76 strain, which expresses Orc2-5Dp instead of wild-type Orc2p, showed a phenotype of accumulation of cells with 2C DNA content, suggesting that cell cycle progression is delayed at the G1/M phase. We also showed that phosphorylation of Rad53p is stimulated in the YMM76 strain, suggesting that some Rad53p-mediated cell cycle checkpoint responses are induced in this strain. It has been reported that ORC dysfunction causes less efficient formation of pre-RC, which in turn induces DNA damage, replication arrest, and spindle assembly checkpoint responses, resulting in cell cycle arrest at the G1/M phase (45, 50–52). Thus, it seems that checkpoint responses induced by inefficient formation of the pre-RC due to expression of Orc2-5Dp is responsible for the phenotype of accumulation of cells with 2C DNA content. It is also possible that the phenotype is due to a defect in sister-chromatid cohesion, because a recent report showed that ORC plays an important role in this process (45).

Biochemical analysis of ORC2-5D provided surprising results that ORC2-5D is defective in Orc5p ATP binding, suggesting that the phosphorylation of Ser-188 of Orc2p inhibits ATP binding to Orc5p. Data with Ser-188-phosphorylated wild-type ORC supported this idea. Both ATP binding and phosphorylation of ORC is important for regulating ORC function, and this is the first evidence for a link between them. ORC2-5D binds to origin DNA normally both in vitro and in vivo, and ORC2-5D retains the high affinity of Orc1p for ATP and ATPase activity.
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A–E, wild-type ORC was incubated with or without recombinant CDK (rCDK). One pmol of wild-type ORC was electrophoresed and analyzed with silver stain (A) or immunoblotting with α-Ser(P)-188 (α-pS188; B) as described in the legend of Figs. 6 or 2, respectively. C, ATP binding to ORC (1 pmol) and 0.5 μM ATP was examined as described in the legend of Fig. 7. Values are the mean ± S.D. (n = 3). D, UV-cross-linking analysis was done for ORC (2 pmol) as described in the legend of Fig. 7. E, gel electrophoretic mobility shift assay was done as described in the legend of Fig. 6.

Furthermore, we have previously suggested that Orc2p phosphorylation does not affect its binding to other ORC subunits (36). Thus, the mutation in ORC2–5D does not seem to drastically affect the higher order structure of ORC and non-specifically diminish ORC function. At present, it is not clear how Orc2p phosphorylation affects the binding of ATP to Orc5p. Because our previous yeast two-hybrid analysis showed that Orc2p has a strong interaction with Orc5p (36), it is possible that phosphorylation of Orc2p affects the structure of Orc5p resulting in its losing its affinity for ATP. We recently reported that ORC-5A (ORC containing a mutant Orc5p with a defective Walker A motif) is unstable in cells due to degradation by the ubiquitin-proteasome system (33, 34). However, we have shown here that ORC2–5D is stable in cells. Because Orc5p–5p showed decreased affinity for Orc4p by yeast two-hybrid analysis (36), one possibility is that the mutation in ORC-5A affects its interaction with Orc4p in a manner that is independent of ATP binding to Orc5p, which is responsible for its instability in cells. Supporting this notion, we have previously shown that overexpression of Orc4p suppresses the growth defect phenotype of the orc2-5d strain (33) but not that of the orc2-5d strain (data not shown in this paper). One remaining unsolved question is whether the defect in ATP binding to Orc5p is responsible for the phenotype exhibited by the orc2-5d strain, such as a slow G1-S transition, induction of phosphorylation of Rad33p, and inefficient loading of MCM onto chromatin. It was shown that ORC can directly interact with Cdc6p, Cdt1p, and Mcm2p and that these interactions are important for pre-RC formation (35, 55, 56). Analysis of a suppressor mutant for the orc2-5d strain and biochemical analysis of the effects of ATP binding to Orc5p on the interactions between ORC–Cdc6p, ORC–Cdt1p, and ORC–MCM will be important to address this issue. Cell cycle-regulated fluctuation in CDK activity is a key event for regulation of initiation of DNA replication. Activation of CDK at the G1-S boundary is important for initiation of DNA replication through phosphorylation of Sld2p and Sld3p (20–22). Maintenance of this high level of CDK activity is important for suppression of re-initiation at G1/M phase through phosphorylation of Orc2p, Cdc6p, and MCM. The results of this study also suggest that dephosphorylation of Orc2p at late M or G1 phase is important for the formation of pre-RC.

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REFERENCES

1. Kelly, T. J., and Brown, G. W. (2000) Annu. Rev. Biochem. 69, 829–880
2. Bell, S. P., and Dutta, A. (2002) Annu. Rev. Biochem. 71, 333–374
3. Dutta, A., and Bell, S. P. (1997) Annu. Rev. Cell Dev. Biol. 13, 293–332
4. Diffley, J. F. (2004) Curr. Biol. 14, 778–786
5. Bell, S. P., and Stillman, B. (1992) Nature 357, 128–134
6. Dahmann, C., Diffley, J. F., and Nasmyth, K. A. (1995) Curr. Biol. 5, 1257–1269
7. Noton, E., and Diffley, J. F. (2000) Mol. Cell 5, 85–95
8. Weinreich, M., Liang, C., Chen, H. H., and Stillman, B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11211–11217
9. Mimura, S., Seki, T., Tanaka, S., and Diffley, J. F. (2004) Nature 431, 1118–1123
10. Wilmes, G. M., Archambault, V., Austin, R. J., Jacobson, M. D., Bell, S. P., and Cross, F. R. (2004) Genes Dev. 18, 981–991
11. Elssasser, S., Chi, Y., Yang, P., and Campbell, J. L. (1999) Mol. Biol. Cell 10, 3263–3277
12. Sanchez, M., Calzada, A., and Bueno, A. (1999) J. Biol. Chem. 274, 9092–9097
13. Drury, L. S., Perkins, G., and Diffley, J. F. (1997) EMBO J. 16, 5966–5976
14. Drury, L. S., Perkins, G., and Diffley, J. F. (2000) Curr. Biol. 10, 231–240
15. Nguyen, V. Q., Co, C., Irie, K., and Li, J. J. (2000) Curr. Biol. 10, 195–205
16. Liku, M. E., Nguyen, V. Q., Rosales, A. W., Irie, K., and Li, J. J. (2005) Mol. Biol. Cell 16, 5026–5039
17. Labib, K., Diffley, J. F., and Kearsey, S. F. (1999) Nat. Cell Biol. 1, 415–422
18. Nguyen, V. Q., Co, C., and Li, J. J. (2001) Nature 411, 1068–1073
19. Archambault, V., Ikui, A. E., Drapkin, B. I., and Cross, F. R. (2005) Mol. Biol. Cell 16, 6707–6721
20. Tanaka, S., Umemori, T., Hirai, K., Muramatsu, S., Kamimura, Y., and Araki, H. (2007) Nature 445, 328–332
21. Tak, Y. S., Tanaka, Y., Endo, S., Kamimura, Y., and Araki, H. (2006) EMBO J. 25, 1087–1996
22. Masumoto, H., Muramatsu, S., Kamimura, Y., and Araki, H. (2002) Nature 415, 651–655
23. Sekimizu, K., Bramhill, D., and Kornberg, A. (1987) Cell 50, 259–265
24. Mizushima, T., Sasaki, S., Ohishi, H., Kobayashi, M., Katayama, T., Miki, T., Maeda, M., and Sekimizu, K. (1996) J. Biol. Chem. 271, 25178–25183
25. Mizushima, T., Takaki, T., Kubota, T., Tsuichia, T., Miki, T., Katayama, T., and Sekimizu, K. (1998) J. Biol. Chem. 273, 20847–20851
26. Katayama, T., Kubota, T., Kurokawa, K., Crooke, E., and Sekimizu, K.
Phosphorylation and ATP Binding of ORC

(1998) Cell 94, 61–71
27. Mizushima, T., Nishida, S., Kurokawa, K., Katayama, T., Miki, T., and Sekimizu, K. (1997) EMBO J. 16, 3724–3730
28. Klemm, R. D., Austin, R. J., and Bell, S. P. (1997) Cell 88, 493–502
29. Makise, M., Takenaka, H., Kuwae, W., Takahashi, N., Tsuchiya, T., and Mizushima, T. (2003) J. Biol. Chem. 278, 46440–46445
30. Bowers, J. L., Randell, J. C., Chen, S., and Bell, S. P. (2004) Mol. Cell 16, 967–978
31. Randell, J. C., Bowers, J. L., Rodriguez, H. K., and Bell, S. P. (2006) Mol. Cell 21, 29–39
32. Takenaka, H., Makise, M., Kuwae, W., Takahashi, N., Tsuchiya, T., and Mizushima, T. (2007) FEBS Lett. 552, 259–263
33. Liang, C., Weinreich, M., and Stillman, B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2224–2228
34. Rao, H., and Stillman, B. (1999) Science 285, 817–823
35. Semple, J. W., Da-Silva, L. F., Jervis, E. J., Ah-Kee, J., Al-Attar, H., Kummer, L., Heikkila, J. I., Pasero, P., and Duncker, B. P. (2006) EMBO J. 25, 5150–5158
36. Asano, T., Makise, M., Takehara, M., and Mizushima, T. (2007) FEBS Lett. 578, 2417–2423
37. Harvey, S. L., and Kellogg, D. R. (2003) Curr. Biol. 13, 264–275