Mechanism for the degradation of origin recognition complex containing Orc5p with a defective Walker A motif and its suppression by over-production of Orc4p in yeast cells

Masaki MAKISE*, Naoko TAKAHASHI†, Kazuya MATSUDA*, Fumiko YAMAIRI*, Keitarou SUZUKI*, Tomofusa TSUCHIYA† and Tohru MIZUSHIMA*¹

*Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan, and †Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700-8530, Japan

Orc5p is one of six subunits constituting the ORC (origin recognition complex), a possible initiator of chromosomal DNA replication in eukaryotes. Orc5p contains a Walker A motif. We recently reported that a strain of *Saccharomyces cerevisiae* having a mutation in Orc5p's Walker A motif (*orc5-A*), showed cell-cycle arrest at G₂/M and degradation of ORC at high temperatures (37°C). Over-production of Orc4p, another subunit of ORC, specifically suppressed these phenotypes [Takahashi, Yamaguchi, Yamairi, Makise, Takenaka, Tsuchiya and Mizushima (2004) J. Biol. Chem. 279, 8469–8477]. In the present study, we examined the mechanisms of ORC degradation and of its suppression by Orc4p over-production. In *orc5-A*, at high temperatures, ORC is degraded by proteasomes; either addition of a proteasome inhibitor, or introduction of a mutation of either *tan1-1* or *nobl-4* that inhibits proteasomes, prevented ORC degradation. Introduction

of the *tan1-1* mutation restored cell cycle progression, suggesting that the defect was due to ORC degradation by proteasomes. Yeast two-hybrid and co-immunoprecipitation analyses suggested that Orc5p interacts preferentially with Orc4p and that the *orc5-A* mutation diminishes this interaction. We suggest that this interaction is mediated by the C-terminal region of Orc4p, and the N-terminal region of Orc5p. Based on these observations, we consider that ATP binding to Orc5p is required for efficient interaction with Orc4p and that, in *orc5-A*, loss of this interaction at higher temperatures allows proteasomes to degrade ORC, causing growth defects. This model could also explain why over-production of Orc4p suppresses the *orc5-A* strain's phenotype.

Key words: DNA replication, origin recognition complex (ORC), Orc4p, Orc5p, Walker A motif, yeast.

INTRODUCTION

The initiation of chromosomal DNA replication must be tightly regulated, and co-ordinated with cell division, to replicate the genome just once per cell cycle. In *Escherichia coli*, adenine nucleotides bound to the DnaA protein, the initiator of chromosomal DNA replication, play a major role in this regulation. DnaA has a high affinity for both ATP and ADP. The ATP–DnaA complex is active for DNA replication both *in vivo* and *in vitro*, but the ADP–DnaA complex is inactive [1–3]. DnaA has intrinsic ATPase activity and thus inactivates itself, suppressing over-initiation [4,5]. Acidic phospholipids interact with DnaA via conserved basic amino acid residues, and this re-activates the ADP–DnaA complex, and suppresses over-initiation of DNA replication [6–10].

In eukaryotes, ORC (origin recognition complex), a possible initiator of chromosomal DNA replication, binds to ATP and ADP [11–14]. ORC was originally identified as a six-protein complex that specifically binds to *Saccharomyces cerevisiae* origins of chromosomal DNA replication [11]. Homologues have been found in various eukaryotes, including humans [15]. In *S. cerevisiae*, ORC's ATP-binding activity has been well characterized *in vitro*. ORC has two subunits (Orc1p and Orc5p), which bind ATP. Orc1p, but not Orc5p, has ATPase activity [12]. Orc5p, but not Orc1p, can bind to ADP [13]. ATP binding to Orc1p, but not to Orc5p, is essential for specific binding of ORC to origin DNA [12,14]. ATP binding to Orc5p increases the affinity of Orc1p for ATP [14]. In contrast with these biochemical studies,

ATP binding to ORC has not been examined extensively by genetic techniques, and therefore its role *in vivo* remains unknown.

We previously reported that a strain of *S. cerevisiae* having a mutation in the Walker A motif (consensus sequences for ATP-binding protein) of Orc5p (*orc5-A*) showed, at high temperatures, decreased levels of ORC, cell cycle arrest at G₂/M, and a growth defect [16]. In the present study, experiments using a proteasome inhibitor, and a mutation (*tan1-1* or *nobl-4*) that inhibits proteasomes, suggested that the decreased ORC level is mediated by degradation by proteasomes. Since the *tan1-1* mutation also suppressed the defects in cell cycle progression and cell growth in *orc5-A*, we presume these are also due to increased ORC degradation.

In *orc5-A*, over-production of Orc4p, another subunit of ORC, can also suppress the defects seen at high temperature [16]. This suppression was specific to *orc5-A*: Orc4p did not restore function in *orc5-1*, another temperature-sensitive strain that has a mutation outside the ATP-binding domain [16]. Furthermore, the suppression was specific to Orc4p; over-production of each of the other subunits (Orc1p, Orc2p, Orc3p and Orc6p) did not restore function [16]. These results revealed that Orc4p and ATP binding to Orc5p are closely linked. One possibility is that Orc5p can preferentially interact with Orc4p via its Walker A motif domain. In the present study, we used yeast two-hybrid analysis to suggest that Orc5p's interaction with Orc4p depends on the Walker A motif of Orc5p. We therefore consider that ATP binding to Orc5p is important for Orc5p to interact with Orc4p, which in turn is important for the stability of ORC in cells, and hence DNA

Abbreviations used: AD, activation domain; BD, binding domain; HA, haemagglutinin; MG-132, carbobenzoxy-L-leucyl-L-leucyl-leucinal; ONPG, o-nitrophenyl β-D-galactopyranoside; ORC, origin recognition complex; ORF, open reading frame; SC, synthetic complete.

¹ To whom correspondence should be addressed (email mizu@gpo.kumamoto-u.ac.jp).

Table 1 Yeast strains

Strains	Genotypes	Reference
EGY48	<i>MATα trp1 ura3 his3 6lexAop-LEU2</i>	[18]
FY24	<i>MATα trp1Δ63 ura3-52 leu2Δ1</i>	[17]
<i>tan1-1</i>	<i>MATα ura3-52 leu2Δ1 tan1-1</i>	[17]
W303-1A	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	[19]
YY411	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 orc5-A</i>	[16]
YY412	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 orc5-A orc2-3HA::HIS3 orc6-3HA::TRP1</i>	The present study
Y138	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 nob1-4::LEU2</i>	[20]
NT411	<i>MATα trp1Δ63 ura3-52 leu2Δ1 orc5-A</i>	The present study
NT412	<i>MATα trp1Δ63 ura3-52 leu2Δ1 orc5-A tan1-1</i>	The present study
NT422	<i>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 orc5-A nob1-4::LEU2</i>	The present study

replication and cell growth. We also suggested that the Orc4p–Orc5p interaction is mediated by the C-terminal region of Orc4p and the N-terminal region of Orc5p that contains the Walker A motif.

EXPERIMENTAL

Strains, plasmids and medium

S. cerevisiae strains are listed in Table 1 [16–20]. Cells were cultured in SC (synthetic complete) medium.

The *orc5-A* gene (*orc5K43E*) was introduced into pRS406 to create pRS406-*orc5-A* [16]. This plasmid was transformed into FY24 and *tan1-1* strains and transformed cells were selected on SC agar plates lacking uracil. The resultant strains were transferred to plates containing 5-fluoro-orotic acid (the two-step gene replacement method) [21]. After checking by PCR, the resultant strains were named NT411 (FY24, *orc5-A*) and NT412 (FY24, *tan1-1*, *orc5-A*) respectively. We introduced the *nob1-4* mutation into the *orc5-A* mutant to construct NT422 strain (W303-1A, *orc5-A*, *nob1-4*).

Modifications of *ORC2* and *ORC6* genes with 3 $\times$ HA (haemagglutinin) were performed as described previously [22]. PCR was performed using pFA6a-3HA-TRP1 or pFA6a-3HA-His3MX6 plasmid as a template and primers containing DNA sequences for the C-terminal region of *ORC6* or *ORC2* gene respectively. The amplified DNA was transformed into YY411. The construct of the resultant strain (YY412) was confirmed by the colony PCR method.

FACS analysis of cellular DNA content

Samples were prepared as previously described [23,24], with the following modifications. Cells were pelleted by centrifugation, washed with sterilized water, and fixed in 70 % (v/v) ethanol for 12 h. Cells were again pelleted, resuspended in 50 mM sodium citrate, sonicated for 1 min, treated with 0.25 mg/ml RNaseA for 1 h at 50 °C, and then with 1 mg/ml proteinase K for 1 h at 50 °C. DNA was stained with 50 μ g/ml of propidium iodide, and 20000 cells from each sample were scanned with a FACSCalibur instrument (Becton Dickinson).

Assessment of ORC subunits in yeast chromatin

Yeast spheroplasts were lysed with Triton X-100, and samples were processed into soluble (supernatant) and chromatin (insoluble precipitate) fractions by centrifugation, as previously described [24]. Equivalent amounts (total protein) of chromatin fractions were subjected to electrophoresis on 7.5 or 10 % polyacrylamide gels containing SDS, transferred to a PVDF mem-

brane, and probed with monoclonal antibodies against Orc3p (SB3), Orc5p (SB5) and HA (12CA5) [24,25].

Yeast two-hybrid analysis

Plasmids pSH18-34 (a reporter plasmid in which the *lacZ* gene is located downstream of a 6-fold repeated operator of the *lexA* gene), pEG202 [a plasmid to express BD fusions (binding domain fusions) under the control of the *ADH* promoter] and pJG4-5 [a plasmid to express AD fusions (activation domain fusions) under the control of a *GALI* promoter] were purchased from OriGene Technologies. In pJG4-5, the ampicillin resistance gene was replaced by a chloramphenicol resistance gene, to construct pJG4-5Cm^R. DNA fragments containing intact or partial ORF (open reading frame) for each ORC subunit were prepared by PCR and inserted into pEG202 or pJG4-5Cm^R.

EGY48 cells were transformed with pSH18-34, a pEG202 derivative and a pJG4-5Cm^R derivative.

β -Galactosidase assay: cells were collected by centrifugation, suspended with 1 ml of Z buffer (100 mM phosphate buffer, pH 7.0, 10 mM KCl, 1 mM MgSO₄ and 50 mM 2-mercaptoethanol) to which were added three drops of CHCl₃ and one drop of 0.1 % SDS. After mixing and incubation for 5 min at 37 °C, the β -galactosidase reaction was started by addition of 0.2 ml of ONPG (*o*-nitrophenyl β -D-galactopyranoside) and stopped by addition of 0.5 ml of 1 M Na₂CO₃. After centrifugation, the absorbance at 420 nm of supernatant was determined. One unit of β -galactosidase hydrolyses 1 μ mol of ONPG per minute.

Co-immunoprecipitation assay

Yeast whole cell extract was prepared by the standard glass beads method in the presence of protease inhibitors. Whole cell extract was incubated with Dynabeads Protein G (Dynal Biotech) conjugated with an antibody against HA (12CA5) for 1 h at 4 °C with rotation. After centrifugation, precipitates were washed and finally suspended with SDS sample buffer (150 mM Tris/HCl, pH 6.8, 50 % glycerol, 1.4 M 2-mercaptoethanol, 5 % SDS and 0.0025 % bromophenol blue). Samples were analysed by immunoblotting with an antibody against HA or LexA.

RESULTS

In *orc5-A*, ORC degradation depends on the proteasome system

Most intracellular proteins are degraded, in a controlled way, by the ubiquitin–proteasome system. Polyubiquitinated proteins are degraded by 26 S proteasomes, which are composed of a 20 S proteasome core and a 19 S regulatory subunit [26–29]. We here examined whether the proteasome system degrades ORC in *orc5-A*

Figure 1 Effect of a proteasome inhibitor on ORC degradation in *orc5-A* cells

YY411 and YY412 (in which Orc2p and Orc6p are tagged with HA) cells were cultured in SC medium to exponential phase at 24°C and further incubated in the presence or absence of MG-132 (10 µg/ml) for 1 h. Then the incubation temperature was changed to 37°C, and the culture was sampled as shown. Chromatin fractions were prepared and analysed by immunoblotting using monoclonal antibodies specific for Orc3p, Orc5p or HA. As loading controls, samples were stained with silver (A, B). Control samples (shown as 'C') were taken before the MG-132 treatment (A).

Figure 2 Effect of the *tan1-1* or *nob1-4* mutation on ORC degradation caused by the *orc5-A* mutation

FY24 (wild-type), NT411 (*orc5-A*) and NT412 (*orc5-A, tan1-1*) (A) or W303-1A (wild-type), YY411 (*orc5-A*) and NT422 (*orc5-A, nob1-4*) (B) cells were cultured in SC medium and sampled, and chromatin fractions were prepared and analysed by immunoblotting, as described for Figure 1.

(YY411) at high temperatures. After temperature shift-up (from 24 to 37°C), both Orc3p and Orc5p disappeared from *orc5-A* cells (Figure 1A), as described previously [16]. Pre-incubating cells with a proteasome inhibitor [MG-132 (the proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-leucinal)] [30,31] completely suppressed this disappearance (Figure 1A). We also examined the state of ORC subunits other than Orc3p and Orc5p under these conditions. As shown in Figure 1(B), Orc2p and Orc6p were decreased in *orc5-A* cells at 37°C and this phenotype was suppressed by pre-incubation of cells with MG-132. Although we could not detect Orc1p and Orc4p by immunoblotting even in the wild-type strain, we presume that all ORC subunits disappear from *orc5-A* cells at 37°C.

The intensity of the Orc5p band increased after addition of MG-132 (Figure 1A). This increase was diminished by pre-incubation of cells with cycloheximide (an inhibitor of protein synthesis; results not shown), suggesting that this increase is due to the stimulation by MG-132 of the expression of Orc5p.

To test further whether the proteasome system was responsible for the disappearance of ORC, we examined the effect of a mutation which disrupts the proteasome system. The *tan1-1* mutation is located in the *PRE8* gene, which encodes one of the subunits of

the 20 S proteasome core [17]. We introduced the *orc5-A* mutation into the *tan1-1* mutant, and into its corresponding wild-type strain (FY24), to construct strains NT412 and NT411 respectively (Table 1). Similar to experiments using the W303-1A genetic background, in NT411, the *orc5-A* mutation caused ORC disappearance at high temperatures (Figure 2A). However, ORC did not disappear from NT412 (FY24, *orc5-A, tan1-1*) cells, indicating that *tan1-1* stabilized ORC5-A (ORC containing the protein product of the *orc5-A* gene). Results in Figures 1(A) and 2(A) strongly suggest that in the *orc5-A* strain, ORC disappearance at high temperatures is mediated by the proteasome system. We confirmed that 37°C is a semi-permissive temperature for the *tan1-1* mutant; the mutant could grow at 37°C (Figure 4) but not at 38.5°C (results not shown) and results in Figure 2(A) suggested that the proteasome system is partially inactivated at 37°C in the mutant, this being consistent with results in a previous paper [17].

The *nob1-4* is a mutation in the gene encoding a protein that binds to the 19 S regulatory subunit and is essential for the ubiquitin-proteasome system. Furthermore, this mutation causes a temperature-sensitive growth phenotype [20]. We introduced the *orc5-A* mutation into the *nob1-4* mutant to construct strain NT422. As shown in Figure 2(B), both Orc3p and Orc5p were decreased

Figure 3 FACS analysis, showing the effect of the *tan1-1* mutation on cell cycle arrest caused by the *orc5-A* mutation

FY24 (wild-type), NT411 (*orc5-A*), the *tan1-1* strain and NT412 (*orc5-A*, *tan1-1*) cells were cultured in SC medium to exponential phase at 24°C, and then the temperature was increased to 37°C. The culture was sampled as indicated, and cellular DNA content was analysed by FACS.

Figure 4 The effect of the *tan1-1* mutation on temperature-sensitive growth caused by the *orc5-A* mutation

NT412 (*orc5-A*, *tan1-1*), NT411 (*orc5-A*), FY24 (wild-type) and the *tan1-1* strain were incubated on SC agar plates at 24°C or 37°C for 2 days.

in YY411 (W303-1A, *orc5-A*) but not in NT422 (W303-1A, *orc5-A*, *nobl-4*), confirming that the ORC disappearance at high temperatures in the *orc5-A* strain is mediated by the ubiquitin–proteasome system.

We next examined the effect of *tan1-1* on other characteristics of *orc5-A*. In a W303-1A genetic background, *orc5-A* caused cell cycle arrest at G₂/M phase at high temperatures [16]. FACS analysis of NT411 showed that cells with 2C DNA content (G₂/M phase) accumulated after temperature shift-up (Figure 3), which is consistent with results from YY411 (W303-1A, *orc5-A*) cells [16]. On the other hand, in NT412 (FY24, *orc5-A*, *tan1-1*) cells, populations of both G₁ and G₂/M cells (1C and 2C respectively) were observed even 10 h after temperature shift-up (Figure 3). In the *tan1-1* single mutant, cell cycle progression was not so affected, when compared with the wild-type strain, FY24 (Figure 3). Thus, in NT412, the *tan1-1* mutation suppressed cell cycle arrest at G₂/M phase. We therefore conclude that in *orc5-A*, cell cycle arrest is probably due to the proteasome-dependent degradation of ORC.

We also examined the effect of *tan1-1* on temperature-sensitive growth in *orc5-A* cells. As shown in Figure 4, cells containing the *orc5-A* mutation (NT411) could not grow at 37°C, as described

previously [16]. Interestingly, the *tan1-1* mutation completely restored ability to form colonies at 37°C (Figure 4), and NT412 cells showed a growth curve similar to that of the wild-type strain (results not shown). Cell growth of the *tan1-1* single mutant was similar to that of the wild-type strain (results not shown). All these results strongly suggest that in the *orc5-A* strain, the growth defect is due to proteasome-dependent degradation of ORC.

Yeast two-hybrid analysis of the interaction between Orc5p and Orc4p *in vivo*

Our previous studies showed that Orc5p interacts with Orc4p via the Walker A motif region [16]. We have used a yeast two-hybrid system to test this idea. The ORF encoding wild-type Orc5p, or Orc5-Ap, was fused to a *lexA* DNA-binding domain to construct BD fusions. The ORF encoding each of the ORC subunits (Orc1p–Orc6p) was fused to a B42-HA transcriptional activation domain, to construct AD fusions. Plasmids containing the AD fusion genes and the BD fusion genes are introduced into cells; if the fusion proteins interact, they activate expression of reporter genes, β -galactosidase and the *LEU2* gene product, which allows growth on SC agar plates without leucine [32].

First, we studied wild-type Orc5p. Co-expression of a BD fusion of *ORC5* (BD-*ORC5*) and an AD fusion of *ORC4* (AD-*ORC4*) caused a much higher level of the β -galactosidase activity than the vector control, suggesting that Orc5p and Orc4p interact with each other (Figure 5A). Co-expression of BD-*ORC5* and AD-*ORC2* also caused high level of β -galactosidase activity, showing that Orc5p and Orc2p interact with each other (Figure 5A). Co-expression of BD-*ORC5* and AD fused to *ORC6* or other *ORC* genes (*ORC1*, *ORC3*) caused little or negligible respectively, stimulation for β -galactosidase activity, suggesting that Orc5p does not interact strongly with these subunits (Figure 5A). We confirmed, by immunoblotting analysis, that all AD and BD fusion proteins were expressed approximately equally (results not shown). Therefore, in cells, Orc5p seems to have the high affinity for Orc4p. It also interacts with itself (Figure 5A).

We next studied the *orc5-A* mutant. Co-expression of BD-*orc5-A* and AD-*ORC4* caused much the same level of β -galactosidase activity as the vector control (Figure 5A), suggesting that the mutation destroys Orc5p's ability to interact with Orc4p. We confirmed that BD-*ORC5* and BD-*orc5-A* were expressed approximately equally (results not shown), and conclude that the interaction between Orc4p and Orc5p requires the ATP-binding domain of Orc5p. Results in Figure 5(A) also suggest that the interaction between Orc5p with itself, and with Orc2p, also requires the ATP-binding domain of Orc5p.

For biochemical confirmation of the interaction between Orc5p and Orc4p, we performed co-immunoprecipitation experiments by use of crude whole cell extracts. Whole cell extracts were prepared from yeast expressing both AD-*ORC4* (HA-*Orc4p*) and BD-*ORC5* (LexA-*Orc5p*) or BD-*orc5-A* (LexA-*Orc5-Ap*), immunoprecipitated with an antibody against HA and detected by immunoblotting with an antibody against HA or LexA. As shown in Figure 5(B), approximately equal amounts of HA-*Orc4p* and LexA-*Orc5p* or LexA-*Orc5-Ap* were expressed in each strain and approximately equal amounts of HA-*Orc4p* were precipitated using the antibody against HA, suggesting that co-immunoprecipitation experiments worked well under the conditions. A significant amount of LexA-*Orc5p* was co-precipitated with HA-*Orc4p*, showing that Orc5p physically interacts with Orc4p under the conditions. A smaller amount of LexA-*Orc5-Ap* than of LexA-*Orc5p* was co-precipitated with HA-*Orc4p*, showing that the interaction of Orc4p with Orc5-Ap is weaker than that with LexA-*Orc5p*. These results suggest that the physical

Figure 5 Interaction between Orc5p and Orc4p, and its dependency on the ATP-binding domain of Orc5p

(A) EGY48 cells were co-transfected with pSH18-34, a pEG202 derivative (pEG202 alone, or pEG202-*ORC5* or pEG202-*orc5-A*; BD fusions), and a pJG4-5Cm^R derivative (pJG4-5Cm^R alone, or pJG4-5Cm^R-*ORC1*, -*ORC2*, -*ORC3*, -*ORC4*, -*ORC5* or -*ORC6*; AD fusions). The activity of β -galactosidase in cells was determined and expressed as total units in 1 ml of yeast cell culture with D_{600} (attenuance) value as 1.0. (B) Whole cell extract was prepared from EGY48 cells having pSH18-34, pEG202 (vector) or its derivative (pEG202-*ORC5* or pEG202-*orc5-A*) and pJG4-5Cm^R-*ORC4* and precipitated with or without antibody against HA (Orc4p is tagged with HA in pJG4-5Cm^R-*ORC4*). Each sample [7.5% input of whole cell extract and precipitates with (+) or without (-) antibody against HA] was analysed on the same gel by immunoblotting with an antibody against HA or LexA.

interaction between Orc4p and Orc5p requires the ATP-binding domain of Orc5p.

For further confirmation that Orc5p's interaction with Orc4p depends on the ATP-binding domain of Orc5p, we examined colony formation on SC agar plates without leucine. Co-expression of BD-*ORC5* and AD-*ORC4* permitted colony formation, confirming that Orc4p and Orc5p interact (results not shown). Cells co-expressing BD-*orc5-A* and AD-*ORC4* fusions had only a weak ability to form colonies, especially at higher temperatures, suggesting that Orc5-Ap and Orc4p might interact weakly (results not shown). There was no clear difference between BD-*ORC5* and BD-*orc5-A* for colony formation on SC agar plates with leucine at any temperatures (results not shown). These results confirm that Orc4p interacts weakly with Orc5-Ap, compared with its interaction with Orc5p, especially at high temperatures. Since in *ORC5-A* (ORC containing the protein product of the *orc5-A* gene), Orc5p loses its ability to bind to ATP [12], ATP binding to Orc5p is presumably important for Orc5p and Orc4p to interact.

We then tried to determine the domain of each subunit (Orc5p and Orc4p) involved in the interaction. As shown in Figure 6(A), we created three fragments (N-terminal, middle and C-terminal) of Orc5p and Orc4p, constructed fusions, and examined the interaction between these fragments and an intact subunit. Co-expression of BD-*ORC5* and AD-*ORC4* caused a high level of β -galactosidase activity (Figure 6B). Co-expression of BD-N-terminal *ORC5* (termed *ORC51*) and AD-*ORC4* gave an even higher level of β -galactosidase activity. The other fragments of Orc5p seemed not to interact with Orc4p (Figure 6B). Co-expression of BD-*ORC4* and AD-*ORC5* caused the high level of β -galactosidase activity (Figure 6C). Co-expression of BD-C-terminal *ORC4* (termed *ORC43*) and AD-*ORC5* gave the lower but significant level of β -galactosidase activity (Figure 6C). The other fragments of Orc4p seemed not to interact with Orc5p. We confirmed that all fusion proteins were expressed approximately equally (results not shown). These results suggest that the N-terminal region of Orc5p interacts with the C-terminal region of Orc4p.

To test this idea, we examined the interaction between Orc43p and Orc51p using a yeast two-hybrid system. As shown in Figure 6(D), we could not detect the significant interaction between BD-*ORC43* and AD-*ORC51* or BD-*ORC51* and AD-*ORC43*. We confirmed that all fusion proteins were expressed

approximately equally (results not shown). We consider a possibility that although the N-terminal region of Orc5p interacts with the C-terminal region of Orc4p, full-length of Orc5p and Orc4p are necessary to recognize the counterpart.

DISCUSSION

In the present study, we examined the mechanism of ORC disappearance from the *orc5-A* strain at high temperatures. Experiments with a chemical proteasome inhibitor, or with the *tan1-1* or *nobl-4* mutant, strongly suggested that ORC degradation is mediated by the proteasome system. At present, it is not clear whether Orc5p is the direct target of polyubiquitination, because we could not detect the polyubiquitination of Orc5-Ap in cells under our experimental conditions (results not shown). Observation that the introduction of the *tan1-1* mutation in the *orc5-A* mutant restored cell growth at high temperatures surprised us, because we considered that the growth defects were primarily due to a loss of function or structure of Orc5-A, and that elimination by the proteasome system was secondary to this. Precise identification of defects of the double mutant (*orc5-A*, *tan1-1*) is important in order to understand the role of ATP-binding to Orc5p in cells. Although we tried to find these, the mutant's colony formation ability at 37°C, and cell viability after 37°C incubation, were much the same as in wild-type cells.

FACS analysis revealed that in the *orc5-A* strain, at high temperatures, the cell cycle arrests at G₂/M. This arrest was also suppressed by *tan1-1*, suggesting that it is also due to ORC degradation by proteasomes. In human cells, one ORC subunit, Orc6p, plays an important role in chromosome segregation and cytokinesis. Orc6p localized to kinetochores during mitosis, and inhibition of Orc6p expression resulted in multi-polar spindles, aberrant mitosis and the formation of multinucleated cells [33]. Orc6p could also be required for chromosome segregation and cytokinesis in yeast, and this could explain why ORC degradation in the *orc5-A* mutant causes cell cycle arrest.

Analysis with a yeast two-hybrid system suggested that Orc5p preferentially interacts with Orc4p. These *in vivo* results are consistent with results from previous studies using other approaches *in vitro*. Lee and Bell reported, using protein-DNA cross-linking, that Orc4p and Orc5p are located very close to each other on

Figure 6 Mapping domains involved in the interaction between Orc5p and Orc4p, by using a yeast two-hybrid assay

Schematic diagram of Orc5p, Orc4p and their partial fragments (**A**). Amino acid residues of full-length Orc5p and Orc4p and their partial fragments were numbered (**A**). EGY48 cells co-transfected with pSH18-34, pJG4-5Cm^R derivatives and pEG202 derivatives. The activity of β -galactosidase in cells was expressed as described in the legend of Figure 5 (**B–D**).

origins of replication. They also showed that when ORC was purified from over-producing cells, lack of Orc5p led to a loss of Orc4p from purified ORC [34]. Therefore it seems that Orc4p and Orc5p interact both *in vitro* and *in vivo*. Kneissl et al. [35] studied the interaction between subunits of mouse ORC by use of a yeast two-hybrid system. The interaction between Orc4p and Orc5p was weak, compared with interactions between other subunits [35]. Thus the structure of ORC seems to be different in yeast and mouse. Analysis with a yeast two-hybrid system also suggested the Orc5p–Orc5p interaction and its dependence on ATP binding to Orc5p. We previously reported that ORC forms a multimer on origin DNA *in vitro* under the conditions of high concentrations of ORC [25]. It is an interesting idea that ORC forms a multimer *in vivo* under some conditions. Further *in vivo* analysis on the state of ORC at distinct phases of the cell cycle or under some environmental conditions is necessary to test this idea.

We suggest here, from use of the yeast two-hybrid system, that the interaction between Orc4p and Orc5p involves an ATP-binding site on Orc5p, and thus presumably depends on ATP bound to Orc5p; since purified Orc5p alone (without other subunits) does not bind ATP *in vitro*, we could not test the dependence of Orc4p–Orc5p interaction on ATP. In the *orc5-A* strain, both the defect in Orc4p–Orc5p interaction, and enhanced ORC degradation, were apparent at high temperatures. Loss of the Orc4p–Orc5p interaction has probably led to the degradation of ORC. This idea is interesting because it is connected to another novel idea that ORC degradation is regulated by ATP binding to Orc5p even in wild-type cells. However, up to now, there is no reported evidence suggesting that wild-type ORC becomes unstable

under some conditions. Furthermore, we previously reported that the ATP–Orc5p complex is relatively stable compared with the Orc1p–ATP complex [36]. Again, further analysis of the stability of wild-type ORC at distinct phases of the cell cycle or under some environmental conditions is necessary to test this idea.

We thank Dr Bruce Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, U.S.A.) for providing antibodies against ORC, and Dr Kenji Kohno (Laboratory of Molecular and Cell Genetics, Nara Institute of Science and Technology, Nara, Japan) and Dr Akio Toh-E (Department of Biological Sciences, University of Tokyo, Tokyo, Japan) for providing yeast strains. The present study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, by the Asahi Glass Foundation, by the Naito Foundation and by the Kato Memorial Foundation. M. M. and N. T. are Research Fellows of the Japan Society for the Promotion of Science.

REFERENCES

- 1 Sekimizu, K., Bramhill, D. and Kornberg, A. (1987) ATP activates DnaA protein in initiating replication of plasmids bearing the origin of the *E. coli* chromosome. *Cell* **50**, 259–265.
- 2 Mizushima, T., Sasaki, S., Ohishi, H., Kobayashi, M., Katayama, T., Miki, T., Maeda, M. and Sekimizu, K. (1996) Molecular design of inhibitors of *in vitro* oriC DNA replication based on the potential to block the ATP binding of DnaA protein. *J. Biol. Chem.* **271**, 25178–25183.
- 3 Mizushima, T., Takaki, T., Kubota, T., Tsuchiya, T., Miki, T., Katayama, T. and Sekimizu, K. (1998) Site-directed mutational analysis for the ATP binding of DnaA protein. Functions of two conserved amino acids (Lys-178 and Asp-235) located in the ATP-binding domain of DnaA protein *in vitro* and *in vivo*. *J. Biol. Chem.* **273**, 20847–20851.
- 4 Katayama, T., Kubota, T., Kurokawa, K., Crooke, E. and Sekimizu, K. (1998) The initiator function of DnaA protein is negatively regulated by the sliding clamp of the *E. coli* chromosomal replicase. *Cell* **94**, 61–71.

- 5 Mizushima, T., Nishida, S., Kurokawa, K., Katayama, T., Miki, T. and Sekimizu, K. (1997) Negative control of DNA replication by hydrolysis of ATP bound to DnaA protein, the initiator of chromosomal DNA replication in *Escherichia coli*. *EMBO J.* **16**, 3724–3730
- 6 Makise, M., Mima, S., Tsuchiya, T. and Mizushima, T. (2001) Molecular mechanism for functional interaction between DnaA protein and acidic phospholipids: identification of important amino acids. *J. Biol. Chem.* **276**, 7450–7456
- 7 Makise, M., Mima, S., Tsuchiya, T. and Mizushima, T. (2000) Identification of amino acids involved in the functional interaction between DnaA protein and acidic phospholipids. *J. Biol. Chem.* **275**, 4513–4518
- 8 Makise, M., Mima, S., Katsu, T., Tsuchiya, T. and Mizushima, T. (2002) Acidic phospholipids inhibit the DNA-binding activity of DnaA protein, the initiator of chromosomal DNA replication in *Escherichia coli*. *Mol. Microbiol.* **46**, 245–256
- 9 Sekimizu, K. and Kornberg, A. (1988) Cardiolipin activation of DnaA protein, the initiation protein of replication in *Escherichia coli*. *J. Biol. Chem.* **263**, 7131–7135
- 10 Xia, W. and Dowhan, W. (1995) *In vivo* evidence for the involvement of anionic phospholipids in initiation of DNA replication in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 783–787
- 11 Bell, S. P. and Stillman, B. (1992) ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature* **357**, 128–134
- 12 Klemm, R. D., Austin, R. J. and Bell, S. P. (1997) Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. *Cell* **88**, 493–502
- 13 Takenaka, H., Makise, M., Kuwae, W., Takahashi, N., Tsuchiya, T. and Mizushima, T. (2004) ADP-binding to origin recognition complex of *Saccharomyces cerevisiae*. *J. Mol. Biol.* **340**, 29–37
- 14 Makise, M., Takenaka, H., Kuwae, W., Takahashi, N., Tsuchiya, T. and Mizushima, T. (2003) Kinetics of ATP-binding to origin recognition complex of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **278**, 46440–46445
- 15 Dutta, A. and Bell, S. P. (1997) Initiation of DNA replication in eukaryotic cells. *Annu. Rev. Cell Dev. Biol.* **13**, 293–332
- 16 Takahashi, N., Yamaguchi, Y., Yamairi, F., Makise, M., Takenaka, H., Tsuchiya, T. and Mizushima, T. (2004) Analysis on origin recognition complex containing Orc5p with defective Walker A motif. *J. Biol. Chem.* **279**, 8469–8477
- 17 Kimata, Y., Higashio, H. and Kohno, K. (2000) Impaired proteasome function rescues thermosensitivity of yeast cells lacking the coatomer subunit epsilon-COP. *J. Biol. Chem.* **275**, 10655–10660
- 18 Estojak, J., Brent, R. and Golemis, E. A. (1995) Correlation of two-hybrid affinity data with *in vitro* measurements. *Mol. Cell. Biol.* **15**, 5820–5829
- 19 Thomas, B. J. and Rothstein, R. (1989) Elevated recombination rates in transcriptionally active DNA. *Cell* **56**, 619–630
- 20 Tone, Y. and Toh-E, A. (2002) Nob1p is required for biogenesis of the 26S proteasome and degraded upon its maturation in *Saccharomyces cerevisiae*. *Genes Dev.* **16**, 3142–3157
- 21 Scherer, S. and Davis, R. W. (1979) Replacement of chromosome segments with altered DNA sequences constructed *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4951–4955
- 22 Longtine, M. S., McKenzie, III, A., Demarini, D. J., Shah, N. G., Wach, A., Brachet, A., Philippsen, P. and Pringle, J. R. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* **14**, 953–961
- 23 Takahashi, N., Tsutsumi, S., Tsuchiya, T., Stillman, B. and Mizushima, T. (2002) Functions of sensor 1 and sensor 2 regions of *Saccharomyces cerevisiae* Cdc6p *in vivo* and *in vitro*. *J. Biol. Chem.* **277**, 16033–16040
- 24 Liang, C. and Stillman, B. (1997) Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in *cdc6* mutants. *Genes Dev.* **11**, 3375–3386
- 25 Mizushima, T., Takahashi, N. and Stillman, B. (2000) Cdc6p modulates the structure and DNA binding activity of the origin recognition complex *in vitro*. *Genes Dev.* **14**, 1631–1641
- 26 Pickart, C. M. (2001) Ubiquitin enters the new millennium. *Mol. Cell* **8**, 499–504
- 27 Coux, O., Tanaka, K. and Goldberg, A. L. (1996) Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.* **65**, 801–847
- 28 Hershko, A. and Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425–479
- 29 Wilkinson, K. D. (1995) Roles of ubiquitinylation in proteolysis and cellular regulation. *Annu. Rev. Nutr.* **15**, 161–189
- 30 Saito, Y., Tsubuki, S., Ito, H. and Kawashima, S. (1990) The structure–function relationship between peptide aldehyde derivatives on initiation of neurite outgrowth in PC12h cells. *Neurosci. Lett.* **120**, 1–4
- 31 Jensen, T. J., Loo, M. A., Pind, S., Williams, D. B., Goldberg, A. L. and Riordan, J. R. (1995) Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell* **83**, 129–135
- 32 Fields, S. and Song, O. (1989) A novel genetic system to detect protein–protein interactions. *Nature* **340**, 245–246
- 33 Prasanth, S. G., Prasanth, K. V. and Stillman, B. (2002) Orc6 involved in DNA replication, chromosome segregation, and cytokinesis. *Science* **297**, 1026–1031
- 34 Lee, D. G. and Bell, S. P. (1997) Architecture of the yeast origin recognition complex bound to origins of DNA replication. *Mol. Cell. Biol.* **17**, 7159–7168
- 35 Kneissl, M., Putter, V., Szalay, A. A. and Grummt, F. (2003) Interaction and assembly of murine pre-replicative complex proteins in yeast and mouse cells. *J. Mol. Biol.* **327**, 111–128
- 36 Makise, M., Takenaka, H., Kuwae, W., Takahashi, N., Tsuchiya, T. and Mizushima, T. (2003) Kinetics of ATP binding to the origin recognition complex of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **278**, 46440–46445

Received 6 June 2006/9 November 2006; accepted 15 November 2006

Published as BJ Immediate Publication 15 November 2004, doi:10.1042/BJ20060841