RESEARCH ARTICLE

Yeast two-hybrid analysis of the origin recognition complex of *Saccharomyces cerevisiae*: interaction between subunits and identification of binding proteins

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Abstract

Origin recognition complex (ORC), a six-protein complex (Orc1p–6p), is the most likely initiator of chromosomal DNA replication in eukaryotes. Although ORC of *Saccharomyces cerevisiae* has been studied extensively from biochemical and genetic perspectives, its quaternary structure remains unknown. Previous studies suggested that ORC has functions other than DNA replication, such as gene silencing, but the molecular mechanisms of these functions have not been determined. In this study, we used yeast two-hybrid analysis to examine the interaction between ORC subunits and to search for ORC-binding proteins. As well as the known Orc4p–Orc5p interaction, we revealed strong interactions between Orc2p and Ord3p (2p–3p), Orc2p and Ord5p (2p–5p), Orc2p and Ord6p (2p–6p) and Orc3p and Ord6p (3p–6p) and weaker interactions between Orc1p and Ord4p (1p–4p), Orc3p and Ord4p (3p–4p), Orc2p and Ord3p (3p–5p) and Orc5p and Ord3p (5p–6p). These results suggest that 2p–3p–6p may form a core complex. Orc2p and Orc6p are phosphorylated *in vivo*, regulating initiation of DNA replication. However, replacing the phosphorylated amino acid residues with others that cannot be phosphorylated, or that mimic phosphorylation, did not affect subunit interactions. We also identified several proteins that interact with ORC subunits; Sir4p and Mad1p interact with Orc2p; Cac1p and Ykr077wp with Orc3p; Rrm3p and Swi6p with Orc5p; and Mih1p with Orc6p. We discuss roles of these interactions in functions of ORC.

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. To understand this regulation, it is important to study the structure and function of the initiator of chromosomal DNA replication. In eukaryotes, Origin recognition complex (ORC) is the most likely initiator of chromosomal DNA replication. ORC was originally identified as a six-protein complex (Orc1p–6p) that specifically binds to *Saccharomyces cerevisiae* origins of chromosomal DNA replication (Bell & Stillman, 1992). ORC homologues have been found in various eukaryotes, including humans (Dutta & Bell, 1997); but that from *S. cerevisiae* has been studied the most extensively, both biochemically and genetically. In this manuscript 'ORC' refers to *S. cerevisiae* ORC, unless otherwise stated.

ORC binds to chromatin at origins of chromosomal DNA replication throughout the cell cycle. It recruits other DNA replication-related proteins (such as Cdc6p, MCM (minichromosome maintenance complex) and Cdt1p) to origin DNA to form a prereplicative complex (pre-RC) (Mendez & Stillman, 2003). Adenine nucleotides bound to ORC play an important role in the functions and structure of ORC. ORC has two subunits (Orc1p and Orc5p), which bind to ATP (Klemm *et al.*, 1997). Orc1p, but not Orc5p, has ATPase activity (Klemm *et al.*, 1997). ATP-binding to Orc1p is essential for the specific binding of ORC to origin DNA *in vitro* (Klemm *et al.*, 1997; Makise *et al.*, 2003), whereas ATP-binding to Orc5p is important for the stability of ORC *in vivo* (Makise *et al.*, 2003, 2007). The ATPase activity of ORC is involved in formation of the pre-RC (Klemm & Bell, 2001; Bowers *et al.*, 2004; Randell *et al.*, 2006). Phosphorylation of ORC is also important: cell cycle-regulated

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phosphorylation of Orc2p and Orc6p by the cyclin-dependent protein kinase (Cdc28p) was observed *in vivo*, and this blocks reinitiation of DNA replication in G2/M phase (Nguyen *et al.*, 2001; Weinreich *et al.*, 2001; Archambault *et al.*, 2005). In contrast to our understanding of the functions of ORC described above, our understanding of its structure is poor. For example, interactions between subunits of ORC are not well known. Understanding the structure of ORC is important to further elucidate its function.

Genetic analyses show that ORC has other functions besides DNA replication. For example, some temperature-sensitive *orc* mutants were defective in gene silencing or chromatin condensation at mitosis (Bell *et al.*, 1993; Foss *et al.*, 1993; Suter *et al.*, 2004; Shimada & Gasser, 2007). As ORC must interact with Cdc6p to initiate DNA replication, ORC-binding proteins may also be required for these other functions of ORC.

The yeast two-hybrid system is a powerful genetic technique to search for proteins that bind to distinct targets, and to reveal interaction between subunits of protein complexes. This type of analysis has been used to study mouse and maize ORC (Kneissl *et al.*, 2003; Witmer *et al.*, 2003), but not *S. cerevisiae* ORC. We recently used it to reveal a strong interaction between *S. cerevisiae* Orc4p and Orc5p (Makise *et al.*, 2007). This interaction depended on ATP-binding to Orc5p, and was important for ORC stability (Makise *et al.*, 2007). In this study, we have analyzed interactions between all subunits of ORC, and thus propose an interaction map for ORC. We also suggest that ORC phosphorylation does not affect interactions between subunits. Furthermore, we identified a number of proteins that interact with ORC, which may be involved in functions of ORC.

Materials and methods

Strains and medium

Saccharomyces cerevisiae strains used in this study are W303-1A (MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1), EGY48 (MATα trp1 ura3 his3 6lexAop-LEU2) and EGY188 (MATα trp1 ura3 his3 2lexAop-LEU2) (Thomas & Rothstein, 1989; Makise *et al.*, 2007). Cells were cultured at 30 °C in synthetic complete (SC) medium with appropriate amino acid depletion.

Yeast two-hybrid analysis

Plasmids pSH18-34 (a reporter plasmid, in which the *lacZ* gene is downstream of an eightfold repeated operator of the *lexA* gene), pEG202 (a plasmid to express BD fusions under control of the *ADH* promoter) and pJG4-5 (a plasmid to express AD fusions under control of the *GAL1* promoter) were purchased from OriGene Technologies, Inc. To construct pJG4-5Cm^R, the ampicillin resistance gene in pJG4-5 was replaced by the chloramphenicol resistance gene (Makise *et al.*, 2007). DNA fragments encoding the ORF for each ORC subunit were prepared by PCR, and inserted into pEG202 or pJG4-5Cm^R.

Mutant *orc2* genes with substitution of Ala or Asp for Ser (16, 24, 188 and 206) and Thr (70 and 174), were constructed using a QuikChange Site-Directed Mutagenesis Kit (Stratagene), and named *orc2-All-A* or *orc2-All-D*, respectively. Mutant *orc6* genes with substitution of Ala or Asp for Ser (106, 116 and 123) and Thr (146) were constructed in a similar way and named *orc6-All-A* or *orc6-All-D*, respectively.

β-Galactosidase assay

EGY48 cells were transformed with pSH18-34, each pEG202 derivative and each pJG4-5Cm^R derivative. Cells were cultured in medium with 2% glucose for 12 h, diluted appropriately and further cultured for 4 h in medium with 2% galactose. Cells were collected by centrifugation, resuspended in 1 mL of Z buffer (100 mM phosphate buffer pH 7.0, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM β-mercaptoethanol) to which were added three drops of $CHCl_3$ 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 2-nitrophenyl β-D-galactopyranoside (ONPG), and stopped by addition of 0.5 mL of 1 M Na_2CO_3. After centrifugation, the $A_{420\,nm}$ of the supernatant was determined. One unit of β-galactosidase hydrolyzes 1 μmol ONPG min^{-1}.

Leucine-requirement assay

EGY188 cells were transformed with each pEG202 derivative and each pJG4-5Cm^R derivative. Cells were cultured in medium with 2% galactose, but without leucine when the $A_{600\,nm}$ reached 0.1. Cell suspensions were diluted and dropped onto agar plates with 2% galactose, but without leucine.

Preparation of yeast genomic libraries and screening for ORC-interacting proteins

Total chromosomal DNA from yeast W303-1A cells was partially digested by AfaI, AluI or HaeIII. DNA fragments (about 1 kb) were fractionated with 5–30% sucrose gradient and ligated into the EcoRI site of pJG4-5Cm^R using EcoRI linkers. The resultant yeast genomic libraries were amplified in *E. coli*.

EGY48 cells were transformed with pSH18-34, the pEG202 derivatives with ORF for each ORC subunit, and each pJG4-5Cm^R yeast genomic library. Transformants (2×10^6) for each ORC subunit were first selected on agar plates without leucine and second on agar plates with

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0.008% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Plasmid DNA was extracted from positive clones and the pJG4-5Cm^R inserts were sequenced. Using the yeast genome database, we identified nuclear proteins and reperformed the leucine-requirement assay and X-gal assay to select positive clones.

Full length *CAC1* and *MIH1* genes were amplified by PCR and cloned into pJG4-5Cm^R.

Results

Overall interaction between subunits of ORC

The ORF for each subunit of ORC was fused to a *lexA* DNA-binding domain or to a B42-HA transcriptional activation domain to construct binding domain (BD) fusion genes or activation domain (AD) fusion genes, respectively. Plasmids containing each AD fusion gene and each BD fusion gene were introduced into cells. If the fusion proteins interact with each other, they activate expression of reporter genes, *LacZ* or *LEU2*, resulting in β-galactosidase activity, or in growth on agar plates without leucine, respectively (Fields & Song, 1989).

Figure 1a shows all results from the β-galactosidase assay. Coexpression of a BD fusion of *ORC5* (BD–*ORC5*) and an AD fusion of *ORC4* (AD–*ORC4*) resulted in higher β-galactosidase activity than the vector-alone control (Fig. 1a). Expression of the opposite combination (BD–*ORC4* and AD–*ORC5*) also resulted in higher β-galactosidase activity (Fig. 1a). These results suggest that Orc4p and Orc5p interact, and are consistent with our previous findings (Makise *et al*., 2007). Furthermore, we identified two other pairs of subunits (2p–5p and 2p–6p) that showed similar results (Fig. 1a). Another two pairs of subunits (2p–3p and 3p–6p) showed higher β-galactosidase activity in one combination but not in the opposite one (Fig. 1a). We confirmed, by immunoblotting, that all AD and BD fusion proteins were expressed approximately equally (data not shown). Thus, results in Fig. 1a revealed five pairs of interacting subunits (4p–5p, 2p–5p, 2p–6p, 2p–3p and 3p–6p). Orc5p also interacts with itself, as does Orc6p. Orc1p did not interact significantly with other subunits.

For further confirmation of these interactions between subunits, we repeated the experiments using a leucine-requirement assay as a reporter system. Cells expressing a BD fusion and an AD fusion of each ORC subunit were diluted appropriately and incubated on agar plates with galactose, but without leucine. Of the five pairs of interacting subunits suggested by the β-galactosidase assay, three pairs (4p–5p, 3p–6p and 2p–3p) showed positive result in both AD/BD combinations (Fig. 1b). Two other pairs (2p–5p and 2p–6p) showed a positive result in one combination but not in the opposite one (Fig. 1b). The leucine-requirement assay identified one new pair (1p–4p), which showed the positive result in both AD/BD combinations and three other pairs (3p–4p, 3p–5p and 5p–6p), which showed positive results in one combination but not in the opposite one (Fig. 1b). Figure 1c summarizes interactions between subunits of ORC according to the results of both assays.

Effect of mutations in phosphorylated amino acid residues on interactions between subunits

As described in the 'Introduction', phosphorylation of Orc2p and Orc6p regulates initiation of DNA replication. The phosphorylated amino acid residues have been identified; Ser (16, 24, 188 and 206) and Thr (70 and 174) in Orc2p; and Ser (106, 116 and 123) and Thr (146) in Orc6p (Nguyen *et al*., 2001). We examined here how replacement of these amino acid residues by others affected interaction between subunits. We mutated Orc2p by replacing these six amino acid residues with Ala (inert for phosphorylation) or Asp (phosphomimic) (Orc2p-All-A or Orc2p-All-D, respectively). We also mutated Orc6p by replacing its four amino acid residues with Ala or Asp (Orc6p-All-A or Orc6p-All-D, respectively). Figure 2a shows that BD-*orc2*-All-D and BD-*orc2*-All-A showed approximately the same β-galactosidase activity as BD–*ORC2* when coexpressed with another AD fused ORC subunit. Similar results were obtained with BD-*orc6-All-D* and BD-*orc6-All-A* (Fig. 2b). These results suggest that phosphorylation of ORC does not affect the interaction between subunits.

Screening for ORC-interacting proteins

We prepared BD fusions of yeast genomic libraries and searched for genes whose coexpression with an AD-fused ORC subunit causes a positive result in yeast two-hybrid analysis. The screening was performed in four steps: selection of colonies on agar plates without leucine; selection of positive colonies on agar plates with X-gal; DNA sequencing and identification of nuclear proteins; reassessment by β-galactosidase assay and leucine-requirement assay. We measured the β-galactosidase activity in the finally selected clones (Fig. 3). No clone was selected for Orc1p and Orc4p. As shown in Fig. 3a–d, some clones showed identical β-galactosidase activity to the vector control. Thus, we finally selected Sir4p and Mad1p as Orc2p-interacting proteins, Cac1p and Ykr077wp as Orc3p-interacting proteins, Rrm3p and Swi6p as Orc5p-interacting proteins, and Mih1p as an Orc6p-interacting protein. We examined interaction between full length Cac1p and Mih1p and each of the ORC subunits. Yeast two-hybrid analysis confirmed that full length Cac1p interacts with Orc3p (Fig. 4a). Cac1p can also interact with Orc5p and Orc6p. We confirmed that full length Mih1p interacts with Orc6p. Mih1p can also interact with Orc2p, Orc3p and Orc5p.

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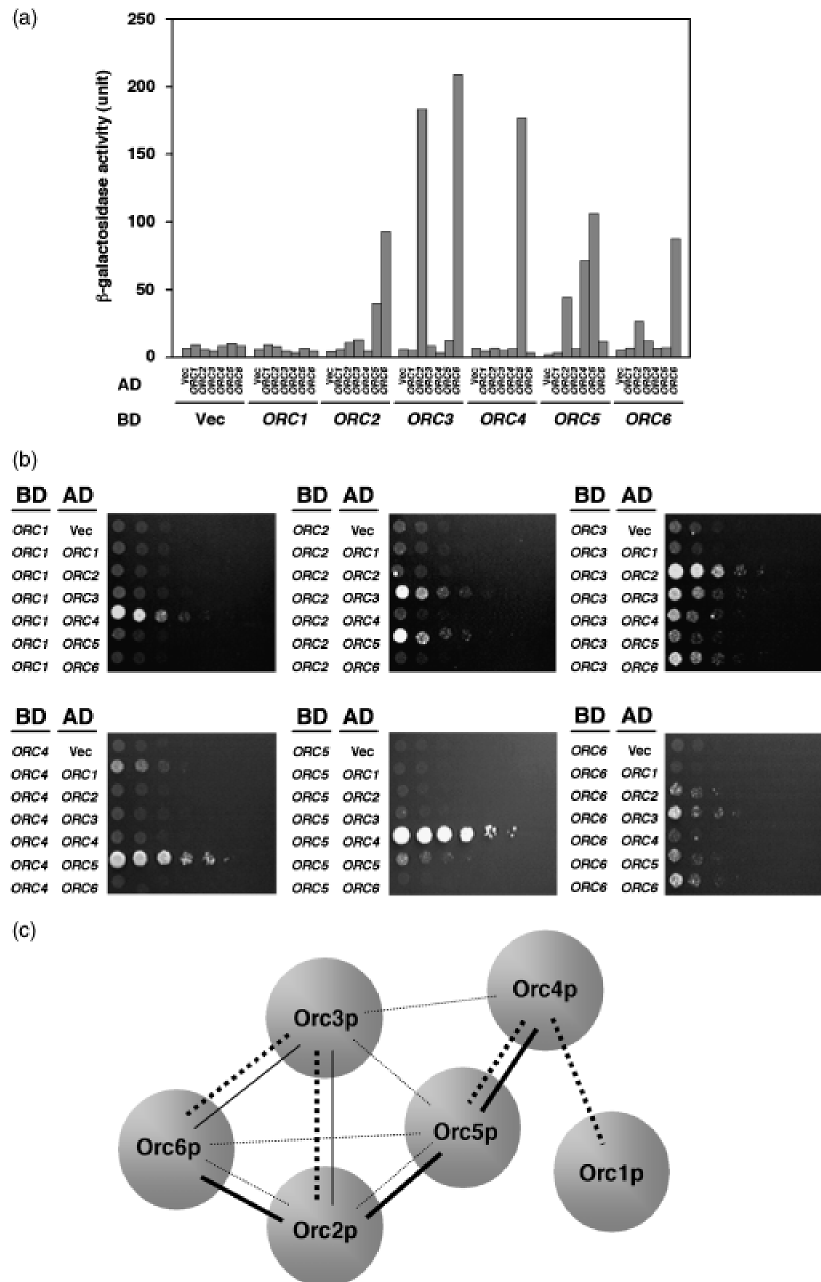

Fig. 1. Yeast two-hybrid analysis of the interaction between subunits of ORC. (a) EGY48 cells were transformed with pSH18-34, a pEG202 derivative (pEG202 alone or pEG202-*ORC1* to -*ORC6*; BD fusions), and a pJG4-5Cm^R derivative (pJG4-5Cm^R alone or pJG4-5Cm^R-*ORC1* to -*ORC6*; AD fusions). The activity of β-galactosidase in cells is expressed as total units in 0.8 mL yeast cell culture with $OD_{600\,nm}$ value as 1.0. (b) As (a), but cell suspensions of each strain were dropped onto agar plates without leucine and incubated for 3 days (four times serial dilution – from left to right – of a suspension with $OD_{600\,nm} = 0.1$). (c) Interaction map for *Saccharomyces cerevisiae* ORC. Lines or dotted lines show interactions revealed by β-galactosidase assay or leucine-requirement assay, respectively. Heavy or light lines show interactions confirmed by both combinations (BD and AD) or one combination only, respectively.

Discussion

In this study, we examined the overall interaction between subunits of ORC, and we searched for ORC-binding proteins using a yeast two-hybrid system. It is certain that the interaction between proteins cannot be proved using data from only the yeast two-hybrid system; in other words, other approaches (such as coprecipitation assay) are required to show that the interaction has biological significance. We consider that this paper is the first step to study these interactions.

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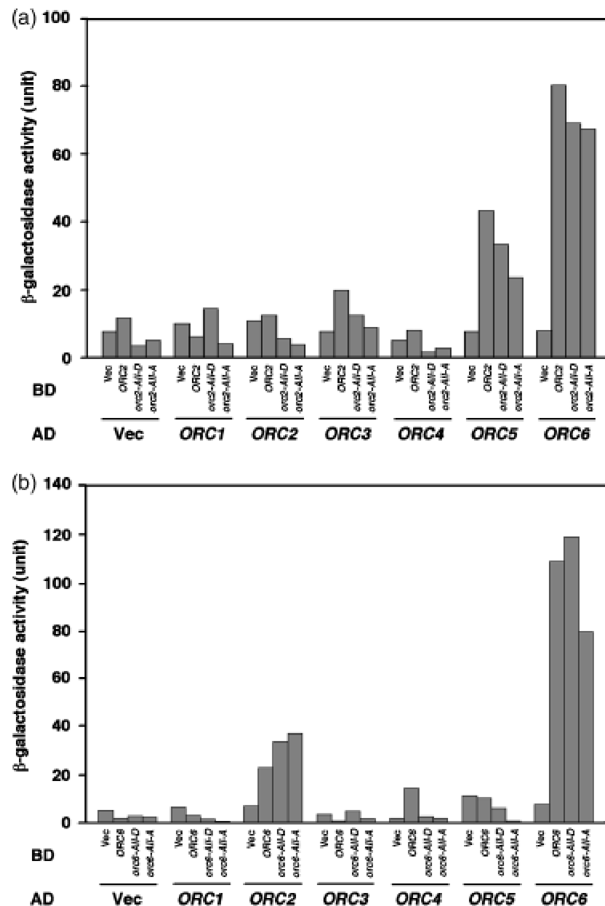

Fig. 2. Effect of mutations in phosphorylation sites of Orc2p and Orc6p on their interaction with other subunits of ORC. (a) EGY48 cells were transformed with pSH18-34, a pJG4-5Cm^R derivative (either empty vector or vector plus an ORC subunit), and a pEG202 derivative (pEG202 alone, pEG202-*ORC2*, pEG202-*orc2-All-D* or pEG202 – *orc2-All-A*). β-Galactosidase activity was determined as for Fig. 1. (b) As (a), but the pEG202 derivatives contained *orc6* sequences instead of *orc2* sequences.

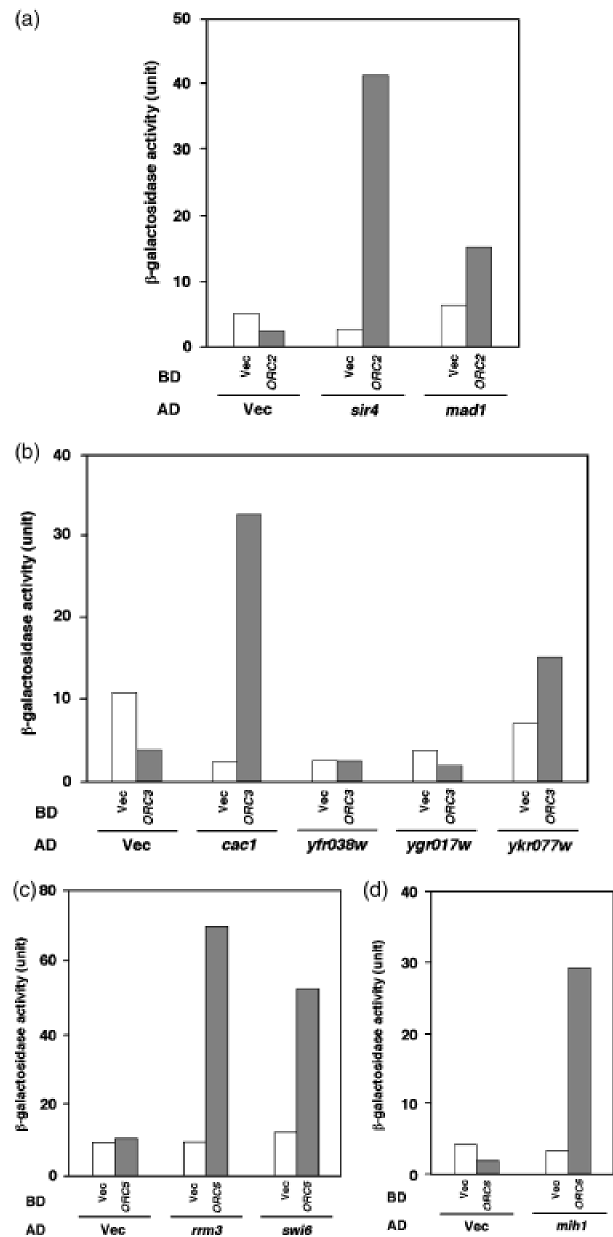

Fig. 3. Screening of yeast genomic libraries, to identify sequences of ORC-interacting proteins. EGY48 cells were transformed with two plasmids: either a pEG202 derivative containing an ORC subunit (BD fusions) or an empty vector (Vec), plus either a pJG4-5Cm^R derivative encoding a library-derived protein sequence believed to interact with ORC (AD fusions) or an empty vector (Vec). β-Galactosidase activity was determined as in Fig. 1. (a) pEG202-*ORC2* plus pJG4-5Cm^R-*sir4* or -*mad1*. (b) pEG202-*ORC3* plus pJG4-5Cm^R-*cac1, -yfr038w, -ygr017w* or -*ykr077w*. (c) pEG202-*ORC5* plus pJG4-5Cm^R-*rrm3* or -*swi6*. (d) pEG202-*ORC6* plus pJG4-5Cm^R-*mih1*.

As shown in Fig. 1c, we judged the significance of interaction based on two criteria: whether the interaction is observed in both combinations (BD and AD) and in two assays (β-galactosidase assay and leucine-requirement assay). The most potent interaction is Orc4p–5p, followed by interactions of 2p–3p, 2p–5p, 2p–6p and 3p–6p. Lee & Bell (1997) reported, using protein-DNA cross-linking, that 4p and 5p, or 2p and 3p, are located very close to each other on origins of replication. They also showed that when ORC was purified from overproducing cells, lack of 5p or 3p led to a loss of 4p or 2p, respectively, from the complex, confirming the interactions of 4p–5p and 2p–3p. The interaction of 4p–5p has been observed in ORC from other species, such as humans (Ranjan & Gossen, 2006) and mouse (Kneissl *et al.*, 2003). We previously reported that this interaction depends on ATP-binding to Orc5p, which is important for stability of ORC in cells (Makise *et al.*, 2007). These results suggest that ORC degradation could be regulated by ATP-binding to Orc5p, not only in budding yeast, but also in other species. Results also suggest that 2p–3p–6p may form a core complex

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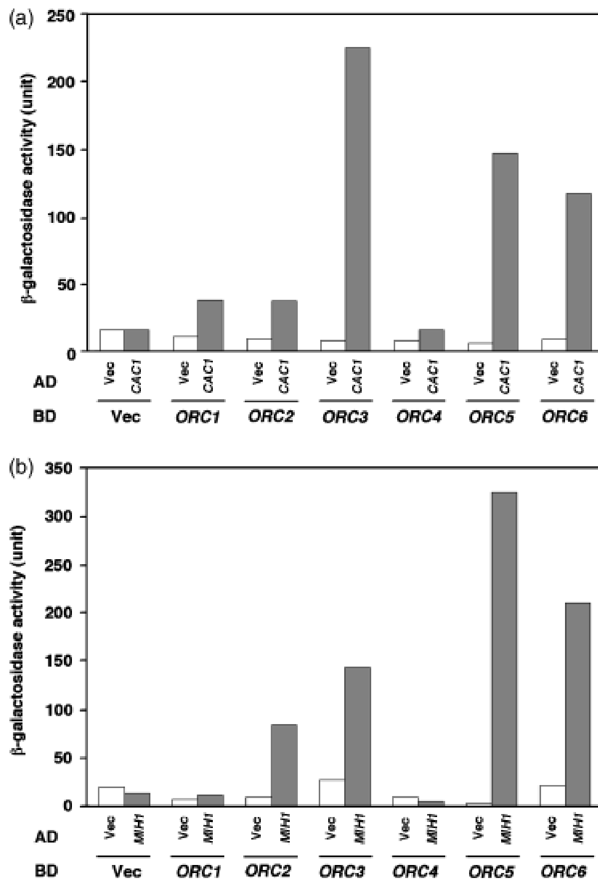

Fig. 4. Interaction of full length Cac1p or Mih1p with ORC subunits 1–6. (a) EGY48 cells were cotransfected with pSH18-34, a pEG202 derivative (pEG202 alone; or BD fusions pEG202-*ORC1* to pEG202-*ORC-6*) and a pJG4-5Cm^R derivative (pJG4-5Cm^R alone or AD fusion pJG4-5Cm^R-*CAC1*). β-Galactosidase activity was determined as in Fig. 1. (b) As (a), except the AD fusion was pJG4-5Cm^R-*MIH1*.

in yeast. Similar core complexes have been predicted for other species. For example, 2p–3p–4p–5p was suggested for humans and *Arabidopsis* (Dhar *et al.*, 2001; Vashee *et al.*, 2001; Diaz-Trivino *et al.*, 2005; Ranjan & Gossen, 2006), 2p–3p–5p for mouse (Kneissl *et al.*, 2003), and 2p–3p–4p for maize ORC (Witmer *et al.*, 2003). Thus, the 2p–3p interaction is usually important for the core complex formation and other subunits of ORC are involved, depending on species. Orc1p is not important.

We found here a number of candidate proteins that could bind ORC: Sir4p and Mad1p bind with Orc2p, Cac1p and Ykr077wp with Orc3p, Rrm3p and Swi6p with Orc5p, and Mih1p with Orc6p. Sir4p helps silence the HM mating-type loci (Laurenson & Rine, 1992). These loci contain an ORC-binding DNA sequence and ORC is required for this type of silencing (Bell *et al.*, 1993). ORC recruits Sir1p, which is also important for silencing the HM mating-type loci, and Sir4p stabilizes the interaction between ORC and Sir1p (Bose *et al.*, 2004) Thus, the interaction of Orc2p and Sir4p may be important for stable interaction between ORC, Sir1p and Sir4p on the HM mating-type loci.

Cac1p is the largest subunit of chromatin assembly factor I. It is involved in telomeric silencing and silencing of the HM mating-type loci (Enomoto & Berman, 1998). Thus, the interaction of Orc3p (Orc5p and Orc6p) and Cac1p may also be responsible for the silencing of the HM mating-type loci by ORC.

Mad1p is involved in the spindle assembly check point system and some temperature-sensitive *orc* mutants induce this system at nonpermissive temperatures (Hardwick & Murray, 1995; Gibson *et al.*, 2006). Thus, the interaction of Mad1p and Orc2p may be involved in the induction of the spindle assembly check point system in *orc* mutants.

Rrm3p is a DNA helicase which moves with the replication fork and is involved in chromosomal DNA replication (Azvolinsky *et al.*, 2006). Thus, Orc5p may recruit Rrm3p to origins of chromosomal DNA replication upon initiation of DNA replication.

DNA replication must be co-ordinated with other cell cycle-regulated events, such as transcription and mitosis. Swi6p is one of the proteins constituting the DSC/MBF complex (DNA synthesis control/MluI cell cycle box binding factor complex). This complex is the key factor for cell cycle-regulated transcription of DNA replication-related genes (Johnston & Lowndes, 1992; Koch *et al.*, 1993). On the other hand, Mih1p is the phosphatase for Cdc28p and helps regulate entry into mitosis (Russell *et al.*, 1989). Thus, interactions of Orc5p and Swi6p, or Orc6p (Orc2p, Orc3p and Orc5p) and Mih1p, could co-ordinate DNA replication with transcription, or mitosis, respectively. Furthermore, Cdc28p phosphorylates Orc2p and Orc6p, and Cdc28p is activated by Mih1p-dependent de-phosphorylation (Sorger & Murray, 1992; Nguyen *et al.*, 2001). Thus, interaction of Mih1p with Orc2p and Orc6p may be involved in ORC phosphorylation in S phase, which is important for inhibition of reinitiation of DNA replication in G2/M phase (Nguyen *et al.*, 2001).

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