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ATP-dependent Assembly of the Human Origin Recognition Complex*◆

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The origin recognition complex (ORC) was initially discovered in budding yeast extracts as a protein complex that binds with high affinity to autonomously replicating sequences in an ATP-dependent manner. We have cloned and expressed the human homologs of the ORC subunits as recombinant proteins. In contrast to other eukaryotic initiators examined thus far, assembly of human ORC *in vitro* is dependent on ATP binding. Mutations in the ATP-binding sites of Orc4 or Orc5 impair complex assembly, whereas Orc1 ATP binding is not required. Immunofluorescence staining of human cells with anti-Orc3 antibodies demonstrate cell cycle-dependent association with a nuclear structure. Immunoprecipitation experiments show that ORC disassembles as cells progress through S phase. The Orc6 protein binds directly to the Orc3 subunit and interacts as part of ORC *in vivo*. These data suggest that the assembly and disassembly of ORC in human cells is uniquely regulated and may contribute to restricting DNA replication to once in every cell division cycle.

Studies on the mechanism of initiation of DNA replication in budding yeast led to the identification of the six-subunit origin recognition complex (ORC)[3] that interacts with and licenses origins of replication prior to S phase (1, 2). The human homologs of the six ORC subunits have been identified and have been shown to interact with each other to form various complexes, suggesting that the licensing function of ORC may be conserved in humans (3–13). Budding yeast ORC binds with moderate affinity and sequence specificity to the autonomously replicating sequences consensus sequence at origins of DNA replication in yeast (14). The identification of specific DNA sequences that define origins of replication in the human genome has not been possible so far, and we sought to generate a biochemical system that would allow us to isolate and test putative DNA sequences that may function as the replicator sequences in human cells. As a first step toward this goal, we set out to establish protocols for the reconstitution of human ORC using a completely recombinant system, using baculovirus expression vectors.

The baculovirus expression system has been used successfully to generate recombinant six-subunit assemblies in insect cells, expressing ORC proteins native to *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Drosophila melanogaster* (15–17). Work from several groups has shown that human Orc1–Orc5 proteins interact with each other *in vivo* and can also form a complex in insect cells (6, 8, 9, 11); however, in these experiments, human Orc6 did not associate stoichiometrically. The interactions between human ORC subunits have been mapped, and complex architecture was proposed based on binary interactions between subsets of proteins co-expressed in insect cells (8, 10). A recent report showed that ATP plays a role in human ORC assembly, and an Orc4 protein with a mutation in the Walker A motif forms unstable complexes (8). This result was inconsistent with another report that performed a similar analysis and showed no effect on complex assembly when Orc1, Orc4, and Orc5 subunits had mutations that should have impaired ATP binding (9). We have performed similar experiments independently and have examined the nucleotide requirements for ORC assembly.

Three of the six ORC subunits (Orc1, Orc4, and Orc5) have been classified as typical AAA+ ATPases based on consensus motifs found in their primary sequence (12, 13, 18–20). Moreover, although they do not possess consensus ATP-binding motifs, Orc2 and Orc3 are also proposed to have an AAA+ structure (21, 22). ATP binding has been primarily implicated in the DNA binding activity of ORC in different systems. It is essential in budding yeast ORC (2, 22–24), and mutations in ScOrc1 that impair ATP binding result in loss of DNA binding (22, 23). The ScOrc4 protein does not have a typical Walker A motif. Corresponding mutations in ScOrc5 result in a temperature-sensitive growth phenotype (23, 25, 26) without a significant effect on the initiation of DNA replication, and it was thus proposed that ATP binding by yeast Orc5 has a role in chromosome integrity (26). *Drosophila* ORC requires ATP binding by Orc1 for its DNA binding activity, whereas mutations in the ATP-binding sites of DmOrc4 or DmOrc5 have no effect on the formation of ORC/DNA complexes (27). Human Orc1/Orc2/Orc3/Orc4/Orc5 complexes containing Walker A mutants of Orc1, Orc4, or Orc5 were compared with wild type in a recent study (9). These mutant complexes were found to have reduced DNA binding activity *in vitro* and were also unable to support

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[3] The abbreviations used are: ORC, origin recognition complex; HA, hemagglutinin; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; ATPγS, adenosine 5′-*O*-(thiotriphosphate); MCM, minichromosome maintenance.

sperm DNA replication in XlORC-depleted *Xenopus* extracts (9). Thus, ATP is essential for ORC function in all systems examined so far.

The stable association of human Orc6 with the ORC complex has been difficult to demonstrate so far, and only trace amounts are observed within complexes after overexpression (6–11). Interactions have been shown between Orc6 and ORC subunits by yeast two-hybrid analysis (28). The human Orc6 protein was identified on the basis of sequence homology to the *Drosophila* Orc6 protein, but the human and fly homologs share little sequence similarity with the budding yeast protein (7, 17). The reconstitution of *Drosophila* ORC showed that Orc6 interacts with the complex and promotes its DNA binding activity *in vitro* (17, 27). Overexpression of human Orc6 with Orc2 or Orc3 in the insect cell system results in a very weak interaction (11); however, no data have been presented to date showing a stable interaction between human ORC and Orc6 *in vivo*. Small interfering RNA-mediated reduction of Orc6 protein levels in human cells surprisingly resulted in a primary defect in cytokinesis, whereas a defect in DNA replication was only observed when all of the detectable Orc6 was lost from the cells (29). Moreover, Orc6 in human cells binds to kinetochores during mitosis (29). These observations raise the question whether human Orc6 has evolved to have different roles during cell cycle progression. As a first step toward addressing these questions, we have raised monoclonal antibodies against the Orc6 protein and demonstrated the association of Orc6 with ORC *in vivo* and *in vitro*.

In the present study, we have reconstituted the human ORC using a baculovirus expression system and present evidence that ATP is essential for human ORC assembly *in vitro*. We have performed pulldown assays to address complex formation and examined the role of ATP binding by Orc1, Orc4, and Orc5 in complex assembly. We have raised new antibodies against different ORC subunits and examined their association *in vivo* across the cell cycle. Based on these results, we present a model for ORC assembly and disassembly that is consistent with published data from different groups and that suggests a unique mode of regulation for ORC in human cells when compared with ORC from other species.

EXPERIMENTAL PROCEDURES

Expression Vectors and Mutagenesis—The following baculoviruses expressing recombinant proteins were gifts from Helena Yang and Sujit Dike: Orc1, His_6-Orc2, HA-Orc3, Orc4, Orc5, and Orc6. All constructs had been sequenced to ensure that no errors had been introduced during amplification.

The different ORC subunits were cloned into pCITE-2a (Novagen) for *in vitro* transcription and translation under the control of a T7 promoter. Vectors expressing wild type Orc2, Orc3, and Orc4 were gifts from Helena Yang. His_6-Orc2 was PCR-amplified out of pFastBac-Orc2 using Herculase polymerase (Stratagene), digested with BamHI and SpeI, and then cloned into the BamHI/XbaI sites of pCITE2a. Orc4 mutant vectors were subcloned after mutagenesis into the BamHI/XhoI sites of pCITE2a. pLPC-T7 Orc5 was used as template for mutagenesis, and then wild type and mutants were subcloned into BamHI/XhoI sites of pCITE2a. The vectors used were pCITE2a-hOrc1, pCITE2a-hOrc2, pCITE2a-His_6-hOrc2, pCITE2a-hOrc3, pCITE2a-hOrc4, pCITE2a-hOrc5, and pCITE2a-hOrc6.

Site-directed mutagenesis was performed on Orc1, Orc4, and Orc5, and sequences of oligonucleotides used to generate mutants are available on request. 50 ng of cDNA was used as a template with 125 ng each of the two different oligonucleotides and then PCR-amplified in a 50-μl reaction using *Pfu* Turbo polymerase (Stratagene). The resulting PCR reaction was digested for 1 h at 37 °C with restriction enzyme DpnI. 5 μl of the digested product was then transformed into XL10 Gold Ultracompetent *Escherichia coli* cells (Stratagene). The next day, colonies were picked, and miniprep DNA was prepared and sequenced to verify that the PCR had introduced only the desired changes.

Complex Assembly in Insect Cells—Insect cells (High-5 cells, Invitrogen) were plated in T-175 flasks and infected with a mixture of baculoviruses expressing the different ORC subunits at a multiplicity of infection = 5–10. After incubation for 60–72 h at 28 °C, cells were harvested, washed twice with PBS, and then washed and incubated in hypotonic lysis buffer (20 mM KPO_4, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 5 mM β-mercaptoethanol) followed by Dounce homogenization with a B pestle (Kontes) for 25–30 strokes to release nuclei. The nuclei were collected by centrifugation at 9600 rpm in an SLA-600TC rotor. Proteins were then extracted in nuclear extraction Buffer PK/50 (20 mM KPO_4, pH 7.5, 50 mM KCl, 0.02% Nonidet P-40, 10% glycerol, 5 mM β-mercaptoethanol, protease inhibitors (Complete EDTA-free from Roche Applied Science), and 5 mM magnesium acetate and 2 mM ATP where indicated). Solid ammonium sulfate powder was added to 10% saturation to aid release of chromatin-bound proteins. Human ORC subunits were precipitated next by saturation at 45% ammonium sulfate. For the complex assembly experiments, 300-μl aliquots of protein were incubated with 100 μl of Talon resin (Clontech) that was pre-equilibrated in Buffer PK/50. The resin was incubated with the extract for at least 2 h at 4 °C. The resin was then washed with a series of buffers: 2× Buffer PK/100 (containing 100 mM KCl) and then 2× Buffer PK/300, 2× Buffer PK/100. All wash buffers were supplemented with 10 mM imidazole. The proteins were eluted in Buffer PK/100 containing 100 mM imidazole. Aliquots of each step of the purification were separated on a 10% denaturing polyacrylamide gel by SDS-PAGE.

Complex Assembly in Rabbit Reticulocyte Lysates—To generate [^{35}S]methionine-labeled proteins, a 50-μl reaction mixture was set up as described by the TnT-coupled reticulocyte lysate system (Promega). 1.5 μg of plasmid DNA was used as a template for transcription. [^{35}S]methionine-Redivue was purchased from Amersham Biosciences. The reaction was incubated at 30 °C for 90 min to generate radioactively labeled protein. After translation was complete, it was diluted in binding Buffer HN/150 (20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 0.02% Nonidet P-40, 5 mM magnesium acetate, 5 mM β-mercaptoethanol, 10% glycerol, 10 mM imidazole, 1 mM ATP), and incubated for 2 h at 4 °C with Talon resin (Clontech). After binding, the resin was washed in Buffer HN/250 (*i.e.* binding buffer but with 250 mM NaCl) and eluted with Buffer HN/150 + 100 mM imidazole. The eluates were separated by SDS-PAGE,

and the gels were dried and exposed to a phosphorimaging screen (Fuji). The screen was then processed in a FLA-5100 imaging system (Fuji) to visualize the proteins.

Purification of Recombinant Orc1—Orc1$^{401-861}$ was amplified by PCR and cloned into pGEX-6P-1 (Amersham Biosciences) for expression with an N-terminal glutathione *S*-transferase (GST) tag. Competent *E. coli* BL21 (DE3) Codon Plus strain (Stratagene) were transformed with this plasmid and grown on selective medium. The cells were grown in liquid culture until they reached a density of OD = 0.5–0.8 and then induced to express protein by the addition of 0.4 mM isopropyl-β-D-thiogalactoside (Roche Applied Science). Flasks were transferred to 16 °C and grown for an additional 4–6 h. Cells were collected by centrifugation, washed with PBS, and lysed in Buffer HN/300 (20 mM HEPES-NaOH, pH 7.5, 300 mM NaCl, 0.02% Nonidet P-40, 5 mM magnesium acetate, 10% glycerol). Lysis was aided by the addition of 200 mg/liter lysozyme (Sigma) for 30 min on ice. Lysates were sonicated to shear DNA, centrifuged at 14,000 rpm, and passed through a 0.45-μm filter to remove particulate material. The clarified supernatant was then incubated with glutathione-Sepharose (Amersham Biosciences) for 2 h at 4 °C to allow binding. The resin was washed three times with 10 volumes each of lysis buffer, and protein was eluted in HN/150 + 10 mM reduced glutathione (Sigma). Protein was concentrated, snap-frozen in aliquots in liquid nitrogen, and stored at −80 °C. For purification of the different GST-Orc1$^{401-861}$ mutants, the buffer used was optimized for maximal protein solubility and yield (20 mM KH_2PO_4, pH 7.5, 300 mM KCl, 0.02% Nonidet P-40, 10% glycerol, 5 mM magnesium acetate, 2 mM ATP, and 5 mM β-mercaptoethanol). All other steps were identical.

GST Pulldown Assays—4 μg of purified protein (GST or GST-hOrc1$^{401-861}$) was diluted in pulldown assay buffer (25 mM Tris-Cl, pH 7.5, 50 mM KCl, 10% glycerol, 0.02% Nonidet P-40. 0.1 mM EDTA, 5 mM magnesium acetate, 5 mM β-mercaptoethanol). Radioactively labeled ORC subunits were generated using the TNT-coupled reticulocyte lysate system (Promega) and added to the reaction. The pulldown was done at 4 °C for 2 h, and resin with bound proteins was washed three times in buffer containing 125 mM KCl, boiled, and analyzed by SDS-PAGE followed by phosphorimaging analysis. GST-Orc6 pulldowns were performed identically, except that the buffer used 200 mM KCl throughout the experiment.

Cell Culture and Synchronization—Sf9 cells (ATCC) were used to amplify and titrate baculovirus stocks. High-5 cells (Invitrogen) were used to express recombinant protein. All insect cells were cultured at 28 °C. Transient transfection experiments were done in human embryonic kidney (HEK293) cells cultured at 37 °C in a 5% CO_2 environment.

For the cell cycle experiments, HeLa cells were grown in suspension in spinner flasks at 37 °C. When cell density reached 1.0×10^5/ml, the culture medium was supplemented with 2.5 mM thymidine. Cells were incubated in thymidine for 14–16 h. They were then washed with sterile PBS to remove excess thymidine, resuspended into fresh warm medium, and incubated at 37 °C to initiate the first release. 10–12 h later, the culture medium was supplemented with 2.5 mM thymidine once again to set up the second block. Cells were synchronized at the G_1/S transition 14–16 h later. For time point $t = 0$ h, aliquots of cells were removed in medium containing thymidine. For $t = 1$ h and beyond, the cells were washed with sterile PBS to remove excess thymidine, resuspended into fresh warm medium, and incubated at 37 °C. For the experiment described in Fig. 6*a*, nocodazole was added at a final concentration of 0.1 ng/ml to the cells at 4 h after releasing from the second thymidine block. 2×10^6 cells were removed from synchronously cycling cells at the time points indicated for immunoprecipitation.

For flow cytometry, 5×10^5 cells were collected for each sample, washed with PBS, fixed in chilled (−20 °C) methanol, and stored overnight at 4 °C. Cells were then washed with PBS and stained in PBS containing 25 μg/ml propidium iodide (Sigma) and 10 μg/ml RNase A (Sigma) at 37 °C for 30 min. Cells were analyzed for DNA content in an LSR-II cell analyzer (BD Biosciences) using a 488-nm argon laser for excitation. Data were captured and analyzed using the FACSCalibur software (BD Biosciences).

Antibodies—Polyclonal antibodies were raised in New Zealand White Rabbits (Covance) by injecting peptide antigens coupled to activated KLH (Pierce). Antibodies used were: Orc1 (CS2117, antigen, VSQDDVLYALKDE), Orc3 (CS1890, antigen, KKRKISLPIEDYFNKGK), Orc4 (CS1568, antigen, KHLNDIYEEEPFNFQ), Orc4 (CS2100, antigen, GST-Orc4 protein), and Orc5 (CS1562, CS1569, antigen, KHHGKIKKTNFLKKHEKT). Antibodies were affinity-purified out of serum using peptide coupled to a Sulfolink column (Pierce). Antibodies against Orc2 and Orc6 have been described previously (4, 30).

Monoclonal antibodies were raised according to protocols described previously (31). Antibodies were raised against Orc3 using the peptide antigen (KKRKISLPIEDYFNKGK) and against Orc6 using recombinant GST-Orc6. Hybridomas were screened by dot blot and enzyme-linked immunosorbent assay analysis. Clones used in this study are PKS16-11 (Orc3) and monoclonal antibody 6-30 (Orc6).

Cell Manipulations and Immunoprecipitation—For transient transfections, 10 μg of plasmid DNA was transfected into HEK293 cells in a 10-cm plate using Lipofectamine 2000 (Invitrogen). Cells were harvested 24 h later, washed in PBS, and extracted in lysis buffer (20 mM Tris-Cl, pH 7.5, 500 mM KCl, 5 mM magnesium acetate, 0.1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol) supplemented with Complete EDTA-free protease inhibitor mixture (Roche Applied Science), phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM sodium β-glycerophosphate) and 25 μM MG-132 (Calbiochem). After incubation at 4 °C for 30 min, the extract was spun down, and the supernatant was diluted to decrease the salt concentration to 250 mm NaCl. Antibodies used for immunoprecipitation were anti-FLAG polyclonal (Sigma), anti-FLAG monoclonal M2 (Sigma), anti-Orc3 monoclonal (PKS16-11), and anti-Orc3 polyclonal (CS1890). Binding was done at 4 °C for 2 h, and immunoprecipitates were captured on Gamma Bind G-Sepharose resin (Amersham Biosciences) and washed three times in wash buffer (20 mM Tris-Cl, pH 7.5, 250 mM KCl, 5 mM magnesium acetate, 0.1 mM EDTA, 0.02% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol) supplemented with Complete EDTA-free prote-

ase inhibitor mixture (Roche Applied Science), phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM sodium β-glycerophosphate). The resin was dried, resuspended in 100 μl of Laemmli buffer, boiled, and analyzed by SDS-PAGE followed by Western blotting. Immunoprecipitation of Orc3 across the cell cycle was performed similarly with the following modifications. MG-132 was omitted from the lysis buffer, and in some experiments, the wash buffer used contained 350 mM KCl.

Immunofluorescence—HeLa cells were grown on coverslips, washed 3× in PBS, and fixed with 2% paraformaldehyde for 15 min at room temperature. After washing extensively with PBS, membranes were permeabilized with cold methanol. Cells were blocked in PBS + 3% normal goat serum, and all staining and washes were done in PBS + 1% normal goat serum. Antibody incubations were done for 1 h at room temperature in a humidified chamber. Primary antibodies used were anti-Orc3 polyclonal antibody (CS1890 1:400) and anti-p150CAF-1 monoclonal antibody (SS1 1:400). Secondary antibodies were Alexa Fluor 488-coupled anti-mouse or Cy5-conjugated anti-rabbit (Molecular Probes). These were both used at 1:1000 dilution. DNA was stained with 10 μg/ml propidium iodide. Cells were mounted in Vectashield (Vector Laboratories) and stored at −80 °C until ready for analysis.

To detect a fraction of protein that is tightly bound to the nucleus, a previously described method was adapted (32). Cells were washed in PBS and CSK buffer (10 mM PIPES, pH 7, 100 mM NaCl, 300 mM Sucrose, 3 mM $MgCl_2$) and then treated with CSK + 0.5% Triton X-100 to "pre-extract" the soluble non-chromatin-bound pool of protein. Cells were then fixed in 2% paraformaldehyde for 15 min at room temperature, treated with cold methanol for 5 min, washed extensively with PBS, and then blocked in PBS + 3% normal goat serum. All following steps were as described above.

Images were captured using a LSM 510 Meta confocal microscope (Carl Zeiss MicroImaging Inc.) using the following objectives: ×20/NA 0.75 and ×63/NA 1.4. Images were processed using MetaMorph Software (version 6.1) and assembled using Adobe PhotoShop (version 7.0) and Adobe Illustrator (version 10).

RESULTS

ATP Supports the Association of Orc4 with Orc2, Orc3, and Orc5—We chose to employ a baculovirus expression system similar to those previously used to generate and characterize the budding yeast and fly ORC complexes (15–17). Insect cells were infected with a mixture of baculoviruses, each expressing a different ORC subunit. The Orc2 virus was cloned with an N-terminal histidine affinity tag, and the Orc3 virus was cloned with an N-terminal hemagglutinin (HA) epitope tag for ease of purification. Initial trials to generate a six-subunit ORC complex showed inconsistent results, and there was varied expression and association of the Orc1 subunit, which was underrepresented relative to the Orc2, Orc3, Orc4, and Orc5 subunits (data not shown). The Orc6 protein was expressed in significant quantities but did not associate in a stoichiometric fashion under the conditions tested, similar to the results seen by other groups (data not shown). We hypothesized that mere co-expression of the different subunits was not enough to guarantee their assembly into stoichiometric complexes and that association of the subunits may follow an ordered pathway. To test this idea, we set out to analyze the assembly of smaller complexes and to ask whether there was a specific pathway for the addition of subunits to form higher order complexes.

High-5 cells were infected with baculoviruses expressing His_6-Orc2, HA-Orc3, Orc4, and Orc5. Nuclear extracts were prepared from these cells and incubated with Talon resin (Clontech) to capture His_6-Orc2 and associated proteins. The extracts were incubated either with or without 2 mM ATP, and this condition was maintained in each sample over the entire purification. The Talon resin was washed extensively to remove any proteins that were bound non-specifically. After eluting, the proteins were separated by SDS-PAGE, stained with silver, and developed to near equivalent intensity without saturating the silver stain. As shown in Fig. 1*a*, the association of Orc4 was significantly enhanced when the proteins were purified in the presence of ATP (lanes *E1* and *E2*) than in its absence (*lane E′*). The association between the Orc2, Orc3, and Orc5 subunits was not visibly affected in the presence or absence of ATP, suggesting that these three proteins form a complex independent of nucleotide. Gel filtration analysis of purified His_6-Orc2/HA-Orc3/Orc5 showed that the proteins had a peak elution volume consistent with the formation of a heterotrimeric complex with one subunit each (data not shown).

The effect of nucleotides other than ATP on complex assembly was tested by performing parallel purifications in the presence of ATP, ADP, or ATPγS (which is a non-hydrolyzable triphosphate analog). Fig. 1*b* shows that millimolar concentrations of ADP were unable to support the association of Orc4 with the Orc2/Orc3/Orc5 subcomplex (lane E″), whereas ATPγS was efficient to a similar extent as ATP (lanes E′ and E2 respectively). These results suggest that a nucleoside triphosphate is required for the formation of a stable Orc2/Orc3/Orc4/Orc5 complex.

To verify that the ATP-dependent assembly was not restricted to proteins generated in insect cells, we set up the following *in vitro* assembly assay for Orc2/Orc3/Orc4/Orc5 complex formation. The subunits were cloned into pCITE2a (Novagen) for *in vitro* transcription and translation. Orc2, Orc3, Orc4, and Orc5 were co-expressed in a single reaction using a rabbit reticulocyte lysate system (Promega) and radioactively labeled using [^{35}S]methionine (Amersham Biosciences). Orc2 was expressed either as an untagged protein in control reactions or as a His_6-tagged protein in the test reaction. Orc5 was expressed as a T7-tagged construct for efficient incorporation of radioactive methionine label.

After co-expressing the Orc2–Orc5 proteins, they were diluted in buffer containing 100 mM NaCl and incubated with Talon resin, either with or without ATP, to capture His_6-Orc2 and associated protein complexes. The Talon resin was then washed extensively in parallel reactions with buffer containing 100, 250, or 500 mM NaCl to remove non-specifically bound proteins. After elution with imidazole, proteins were separated by SDS-PAGE and visualized by using a phosphorimaging system (Fuji). Orc4 associated with Orc2/Orc3/Orc5 in an ATP-dependent manner similar to that seen in the insect cell system

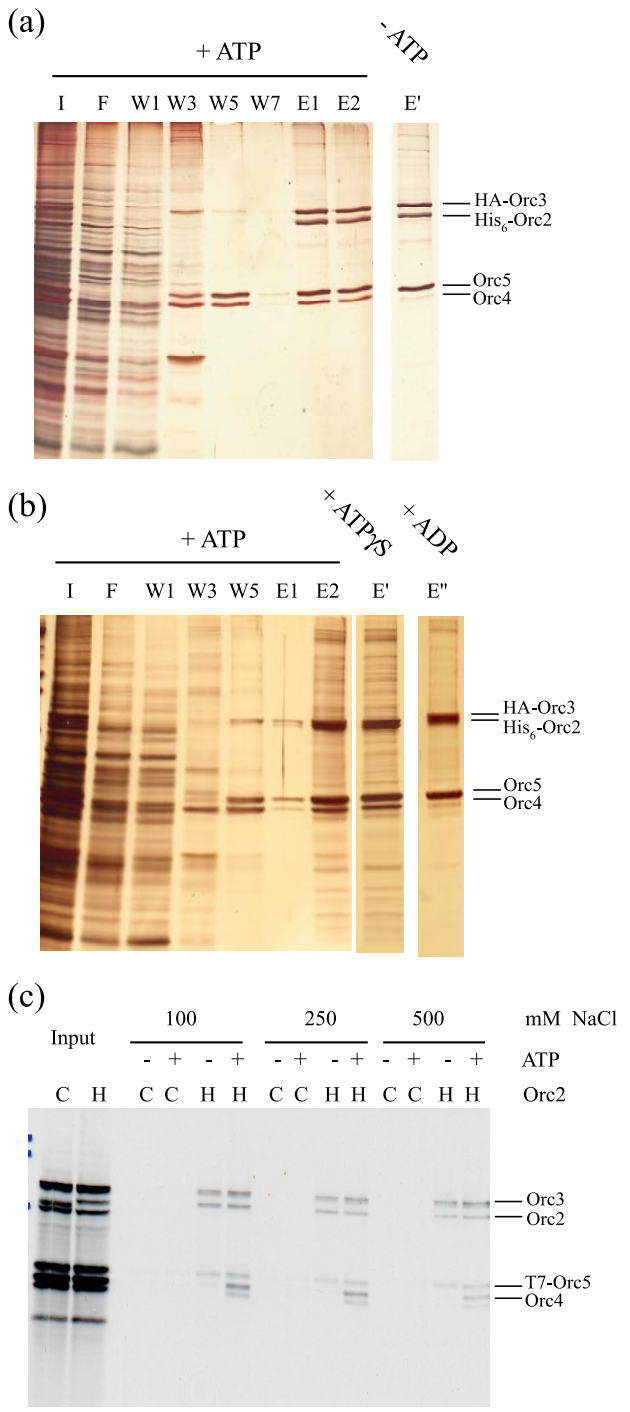

FIGURE 1. **ATP-dependent assembly of Orc2-Orc5.** *a*, ATP supports the association of Orc4 with Orc2/Orc3/Orc5 in insect cell extracts. Baculoviruses expressing His_6-Orc2, HA-Orc3, Orc4, and Orc5 were co-infected into insect cells, and nuclear extracts from these cells were incubated with a Talon resin to purify His_6-Orc2 and associated complexes, either in the presence or in the absence of ATP. Aliquots removed at various steps of the purification were analyzed by SDS-PAGE followed by silver staining. *I* = input; *F* = Flow through; *W* = Wash fractions; *E* = elution fractions. *E1* and *E2* are samples purified in the presence of ATP, and *E′* is the elution fraction of an equivalent sample purified in the absence of ATP. *b*, ATPγS supports the assembly of Orc2–Orc5 similar to ATP, but ADP does not. Baculoviruses expressing His_6-Orc2, HA-Orc3, Orc4, and Orc5 were co-infected into insect cells, and nuclear extracts from these cells were incubated with a Talon resin to purify His_6-Orc2 and associated complexes, in the presence of ATP, ATPγS, or ADP. Samples were processed as in *panel a*. *E2* is sample purified in the presence of ATP, *E′* is an equivalent sample purified in the presence of ATPγS, and *E″* is an equivalent sample purified in the presence of ADP. *c*, the ATP-dependent association of Orc2–Orc5 is recapitulated using *in vitro* transcribed and translated [^{35}S]methionine-labeled proteins. Untagged Orc2 (*C*) or His_6-tagged Orc2 (*H*) were co-expressed with Orc3, Orc4, and T7-Orc5 in a rabbit reticulocyte lysate, and resulting complexes were purified using a Talon resin either in the presence (+) or in the absence (−) of ATP. Three separate reactions were performed, with wash buffer containing 100, 250, or 500 mM NaCl. Equivalent amounts of control untagged Orc2 and His_6-tagged Orc2 containing reactions were analyzed by SDS-PAGE followed by phosphorimaging analysis.

(Fig. 1*c*). The interaction between Orc2, Orc3, and Orc5 was stable up to 500 mM NaCl.

ATP Binding by Orc4 and Orc5 are Essential for Formation of Orc2/Orc3/Orc4/Orc5 Complex—In the Orc2/Orc3/Orc4/Orc5 complex, there are two subunits, namely Orc4 and Orc5, that have the potential to bind ATP due to conserved Walker A and B motifs. To test whether ATP binding by the Orc4 or Orc5 subunits was essential for complex formation, we generated a series of mutants based on existing data on the corresponding yeast and fly mutants as well as on structural data on AAA+ proteins (5, 19, 23, 25, 27, 33). These mutants are indicated in Fig. 2*a*. Mutations in the Walker A motif or the first half of the Walker B motif of these proteins were predicted to disrupt ATP binding, whereas mutations in the second half of the Walker B motif were predicted to allow ATP binding but block ATP hydrolysis (23). The association of Orc4 with Orc2/Orc3/Orc5 was tested in the presence of 1 mM ATP using such mutants of Orc4 (Fig. 2*b*) or Orc5 (Fig. 2*c*) using the *in vitro* assembly assay described above. Mutants designed to block ATP binding in Orc4 (Fig. 2*b*, compare *lanes 3–6* with *lanes 13–16*) and Orc5 (Fig. 2*c*, compare *lanes 3–5* with *lanes 11–13*) show impaired Orc4 association with Orc2/Orc3/Orc5. The Orc5 subunit associated with Orc2 and Orc3 independent of the Orc5 mutation status (Fig. 2*c*). Mutants designed to block ATP hydrolysis (Fig. 2, *b*, *lanes 7* and *17*, and *c*, *lanes 6* and *14*) or in conserved arginine residues predicted to make contact with the γ-phosphate (the sensor 1 motif; Fig. 2, *b*, *lanes 8* and *9* and *18* and *19*, *c*, *lanes 7* and *15*) showed results similar to wild type. Therefore, we conclude that ATP binding by Orc4 and Orc5 are essential for Orc2/Orc3/Orc4/Orc5 complex assembly *in vitro*. This assay also reveals that Orc4 association in the complex is ordered and is not favored in the absence of Orc5 even in the presence of ATP (Fig. 2*c* compare *lanes 2* and *10*).

ATP Supports the Association of Orc1 with Orc2–Orc5—We next tested the requirements for the formation of a stable Orc1/Orc2/Orc3/Orc4/Orc5 complex. Recombinant baculoviruses expressing Orc1, His_6-Orc2, HA-Orc3, Orc4, and Orc5 were co-infected into insect cells. Nuclear extracts were prepared and incubated with Talon resin, either with or without ATP to capture His_6-Orc2 and associated protein complexes. In the presence of ATP, a five-subunit Orc1/Orc2/Orc3/Orc4/Orc5 complex was purified (Fig. 3*a*). In the absence of ATP, levels of Orc4 and Orc1 associated with the complex were significantly reduced. Therefore, we conclude that Orc1 association with the complex is ATP-dependent and/or Orc4-dependent.

To address Orc1/Orc2/Orc3/Orc4/Orc5 complex assembly *in vitro*, we set up a GST pulldown assay as follows. Orc2–Orc5 proteins were co-expressed in a rabbit reticulocyte lysate to generate [^{35}S]methionine-labeled proteins. These were diluted

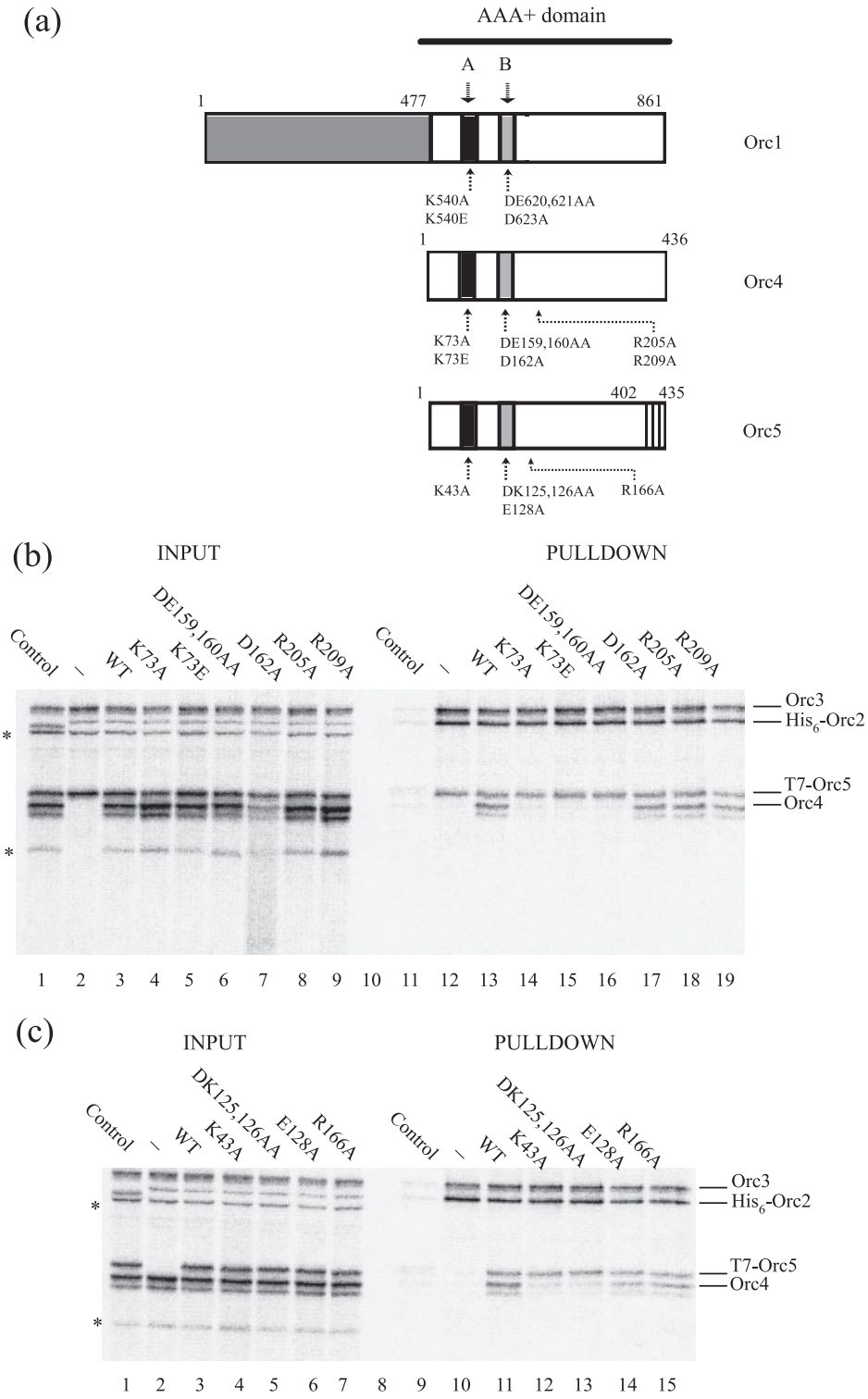

FIGURE 2. **Analysis of Orc2–5 complex assembly *in vitro*.** *a*, schematic representation of Orc1, Orc4, and Orc5 showing the AAA+ motifs and mutations used in this study. The AAA+ domains of Orc1, Orc4, and Orc5 are shown as described (5). Positions of the different mutations analyzed in this study are indicated by *arrows*. The *small black box* in all three constructs represents the Walker A motif, and the *small gray box* represents the Walker B motif. The large N-terminal non AAA+ region of Orc1 (amino acids 1–477) is shaded in *gray*. *b*, mutants of Orc4 that are predicted to disrupt ATP binding are impaired for Orc2–Orc5 assembly in rabbit reticulocyte lysates. His_6-Orc2, Orc3, and T7-Orc5 were co-expressed in rabbit reticulocyte lysate with either wild type (*WT*) or mutant Orc4 constructs, and resulting complexes were isolated in the presence of 1 mM ATP using Talon resin. A reaction with untagged Orc2 was used to control for nonspecific binding to the resin (*lanes 1* and *11*) and one lacking Orc4 to reveal the bands specific to the Orc4 reaction (*lane 2*). Input proteins are shown in *lanes 1–9*. *Lanes 11–19* show the His_6-Orc2 pulldown. *Asterisks* indicate bands that represent nonspecific or truncated products from the *in vitro* translation reaction. *c*, mutants of Orc5 that are predicted to disrupt ATP binding are impaired for Orc2–Orc5 assembly in rabbit reticulocyte lysates. His_6-Orc2, Orc3, and Orc4 were co-expressed in rabbit reticulocyte lysate with either wild type or mutant T7-Orc5 constructs, and resulting complexes were isolated in the presence of 1 mM ATP using Talon resin. A reaction with untagged Orc2 was used to control for nonspecific binding to the resin (*lanes 1* and *9*), and one lacking Orc5 was used to reveal the bands specific to the Orc5 reaction (*lane 2*). Input proteins are shown in *lanes 1–7*, and those bound by His_6-Orc2 pulldown are shown in *lanes 9–15*. *Asterisks* indicate bands that represent nonspecific or truncated products from the *in vitro* translation reaction.

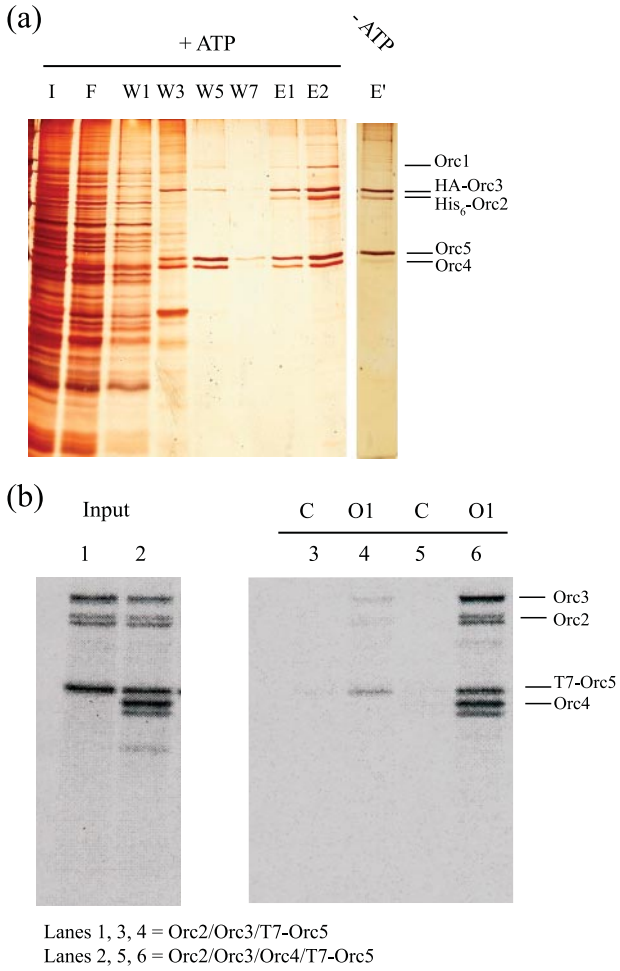

FIGURE 3. **Assembly of Orc1 into ORC *in vitro*.** *a*, association of Orc1 with Orc2/Orc3/Orc5 is ATP- and/or Orc4-dependent in insect cells. Baculoviruses expressing Orc1, His_6-Orc2, HA-Orc3, Orc4, and Orc5 were co-infected into insect cells, and nuclear extracts from these cells were incubated with a Talon resin to purify His_6-Orc2 and associated complexes, either in the presence or in the absence of ATP. Aliquots removed at various steps of the purification were analyzed by SDS-PAGE followed by silver staining. *I* = input; *F* = Flow through; *W* = Wash fractions; *E* = elution fractions. *E1* and *E2* are samples purified in the presence of ATP, and *E′* is the elution fraction of an equivalent sample purified in the absence of ATP. *b*, association of Orc1 with Orc2/Orc3/Orc5 is Orc4-dependent. Orc2, Orc3, and T7-Orc5 were co-expressed in rabbit reticulocyte lysates, either without (*lane 1*) or with (*lane 2*) Orc4. These were mixed either with purified GST (*C*) or with GST-$Orc1^{401-861}$ (*O1*) to test for specific association with $Orc1^{401-861}$ in the presence of 1 mM ATP.

in buffer containing ATP, and the reaction was incubated with glutathione-Sepharose resin containing either GST or a soluble fragment of Orc1 (amino acids 401–861) expressed as a GST-fusion protein. Preliminary experiments showed that a fragment containing the AAA+ domain of Orc1 was sufficient for complex assembly (data not shown). After a 2-h incubation to allow complex formation, the resin was washed extensively to remove non-specifically bound proteins, boiled in Laemmli buffer, and analyzed by SDS-PAGE followed by phosphorimaging analysis. The results in Fig. 3*b* show that GST-$Orc1^{401-861}$ interacts stably with Orc2/Orc3/Orc5 only when Orc4 is included in the reaction and very weakly in its absence. Therefore, we conclude that Orc1 interacts weakly with the Orc2/Orc3/Orc5 complex and that the formation of a stable Orc1/Orc2/Orc3/Orc4/Orc5 complex is Orc4-dependent.

Contribution of ATP Binding by the AAA+ Subunits in Orc1/Orc2/Orc3/Orc4/Orc5 Complex Assembly in Vitro—We tested the requirement for ATP binding by Orc1, Orc4, and Orc5 for Orc1/Orc2/Orc3/Orc4/Orc5 complex formation, introducing mutants of only one subunit at a time in the GST pulldown assay described above. Mutants of Orc1 were expressed as GST-$Orc1^{401-861}$ fusion proteins and tested for Orc2–Orc5 association in the presence of ATP. Mutations in Orc1 predicted to disrupt ATP binding or hydrolysis have no significant impairment in complex formation *in vitro* (Fig. 4*a*). Thus, we conclude that ATP binding by Orc1 is not essential for complex formation and may have other roles in DNA replication.

Orc1/Orc2/Orc3/Orc4/Orc5 complex formation was then assayed in the presence of wild type or mutant Orc4 subunits using the GST pulldown assay (Fig. 4*b*). The data show that Orc4 mutants that are impaired for ATP binding do not support the formation of Orc1/Orc2/Orc3/Orc4/Orc5 complexes. Also, Orc1 does not interact well with Orc4 alone even in the presence of ATP (*lanes 1*, *10*, and *11*). Therefore, Orc1 association with Orc2–Orc5 requires ATP binding by Orc4 and suggests that stable Orc1 interaction is dependent on the prior formation of an Orc2/Orc3/Orc4/Orc5 complex.

Fig. 4*c* shows the results of Orc1/Orc2/Orc3/Orc4/Orc5 complex formation in the presence of mutant Orc5 subunits using the GST pulldown assay. The data show that Orc5 mutants that are impaired for ATP binding form Orc1/Orc2/Orc3/Orc4/Orc5 complexes with similar efficiency as the wild type, in contrast to what was observed for Orc2–Orc5 formation (Fig. 2*c*). This result suggests that Orc5 ATP binding may be essential for interaction of Orc5 with Orc4; however, the presence of wild type Orc1 may bridge this interaction in the mutants, stabilizing higher order complexes.

The C Terminus of Orc1 Containing the AAA+ Domain Is Necessary and Sufficient for Complex Assembly in Vivo—The Orc1/Orc2/Orc3/Orc4/Orc5 complex was assembled *in vitro* using a truncated fragment of Orc1 lacking the N-terminal 400 amino acids (Fig. 3*b*). We decided to test what was the smallest region of Orc1 that was required for complex formation in human cells using the following assay. Full-length Orc1 or truncated fragments (either N-terminal or C-terminal) were cloned with a FLAG epitope tag in a plasmid vector under control of a cytomegalovirus promoter (pLPC). These were overexpressed in HEK293 cells by transient transfection. Fig. 5*a* shows a representation of the Orc1 constructs used in this experiment. The expressed Orc1 protein was stabilized by incubation of the transfected cells with proteasome inhibitor, MG-132 (34). Whole cell extracts were prepared from these cells and were subject to immunoprecipitation using monoclonal antibodies either against the FLAG epitope tag (M2-Sigma) or against Orc3 (PKS16-11). The resulting immunoprecipitates were analyzed for the presence of Orc2–Orc5 subunits by Western blotting using polyclonal antibodies to these proteins. Orc1-FLAG was detected using anti-FLAG polyclonal antibodies (Sigma). Fig. 5*b* shows that anti-FLAG antibodies immunoprecipitated all the different Orc1 constructs in similar quantities. However, only $Orc1^{401-861}$ and $Orc1^{501-861}$ showed association of Orc2–Orc5 subunits comparable with full-length Orc1 (*lanes 2–4*). Truncation of an additional 100 amino acids of Orc1

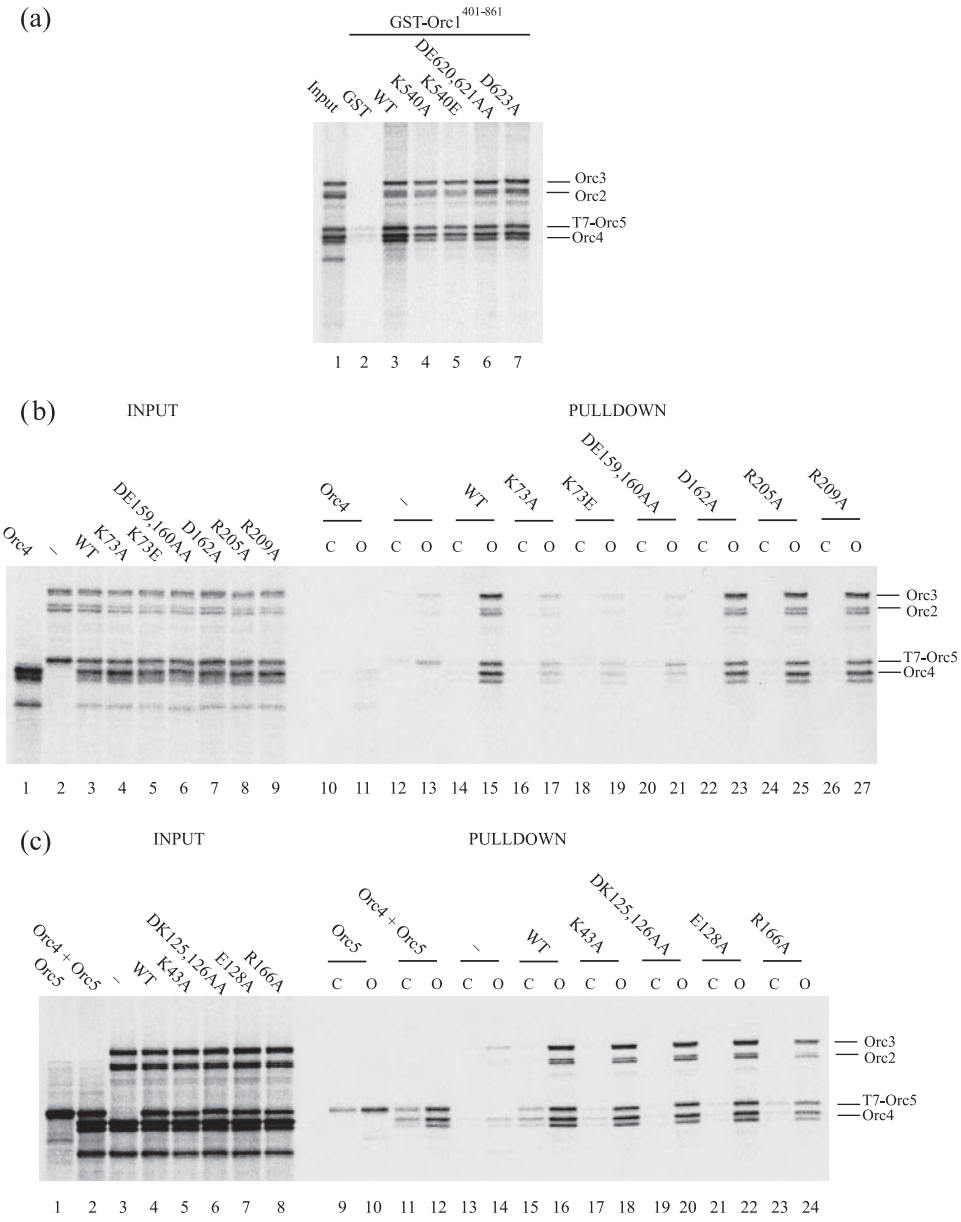

FIGURE 4. **Analysis of Orc1–Orc5 complex assembly *in vitro*.** *a*, association of Orc1 with Orc2/Orc3/Orc5 does not require ATP binding by Orc1. Orc2, Orc3, Orc4, and T7-Orc5 were co-expressed in rabbit reticulocyte lysates, and the resulting complexes were tested for association with GST-Orc1$^{401-861}$, either wild type (*WT*, *lane 3*) or mutants (*lanes 4–7*), in the presence of 1 mM ATP. GST was used a control for nonspecific protein association (*lane 2*). *b*, association of Orc1 with Orc2/Orc3/Orc5 is Orc4-dependent and requires a functional ATP-binding site in Orc4. Orc2, Orc3, and T7-Orc5 were co-expressed in rabbit reticulocyte lysate either with wild type or mutant Orc4 constructs. Input proteins are shown in *lanes 3–9*. The resulting complexes were mixed either with purified GST (*C*) or with GST-Orc1$^{401-861}$ (*O*) to test for specific association with Orc1$^{401-861}$ in the presence of 1 mM ATP (*lanes 14–27*). As controls, Orc4 (*lanes 1*, *10*, and *11*) and Orc2/Orc3/T7-Orc5 (*lanes 2*, *12*, and *13*) were tested separately for association with Orc1$^{401-861}$. *c*, association of Orc1 with Orc2/Orc3/Orc4/Orc5 does not require ATP binding by Orc5. Orc2, Orc3, and Orc4 were co-expressed in rabbit reticulocyte lysate either with wild type or mutant T7-Orc5 constructs. The input proteins are in *lanes 3–8*. The resulting complexes were mixed either with purified GST or with GST-Orc1$^{401-861}$ to test for specific association with Orc1$^{401-861}$ in the presence of 1 mM ATP (*lanes 13–24*). As controls, Orc5 or Orc4 and Orc4 were tested for binding Orc1 (*lanes 1*, *2*, and *9–12*).

(Orc1$^{601-861}$, *lane 5*) reduced the amount of co-precipitating Orc2–Orc5 significantly. The N-terminal fragments of Orc1 lacking any AAA+ motifs did not interact with Orc2–Orc5 in this assay (*lanes 6* and *7*). Supporting these results, it is seen in the Orc3 immunoprecipitates that the Orc1$^{401-861}$ and the Orc1$^{501-861}$ associate with Orc3 similar to the full-length protein (*lanes 10–12*); however, Orc1$^{601-861}$ as well as the N-terminal fragments are compromised for this interaction (*lanes 13–15*). This result shows that a fragment containing an intact AAA+ domain of Orc1 is necessary and sufficient for interaction with Orc2–Orc5 *in vivo* and also validates the results from the *in vitro* GST pulldown assay using this fragment.

Interestingly, it was observed that in the Orc3 immunoprecipitates, Orc4 was significantly enhanced in samples where Orc1, Orc1$^{401-861}$, and Orc1$^{501-861}$ were overexpressed (Fig. 5*b*, *lanes 10*, *11*, and *12*). The corresponding levels of Orc5 were only modestly increased, whereas Orc2 was unchanged. Total levels of the Orc2–Orc5 subunits were not increased as a result of Orc1-FLAG overexpression when compared with the untransfected or vector control transfections, and the input levels of Orc2–Orc5 in these immunoprecipitates were similar (data not shown). This result suggests that Orc1 promotes or stabilizes the association of Orc4 to form higher complexes *in vivo*.

The Human Origin Recognition Complex Disassembles during S Phase—Based on the above observations, we hypothesized that Orc4 association with ORC would be promoted in the presence of Orc1 subunit during the cell cycle. Therefore, during S phase, when Orc1 levels are decreased due to ubiquitin-mediated proteolysis (34, 35), we predicted that Orc4 association with Orc2–Orc5 would be decreased. This idea was tested in the following experiment. HeLa cells were synchronized at the G_1/S boundary using a double thymidine block and then released into fresh medium to allow synchronous passage into S phase. Samples were collected every hour over a period of 10 h. Extracts were prepared from these samples and subjected to immunoprecipitation using monoclonal antibodies directed either against the Myc epitope tag as control (data not shown) or against Orc3. The immunoprecipitates were examined for the different ORC subunits by Western blotting as shown in Fig. 6*a*. The blots show that total levels of Orc1 progressively decrease as the cells passage through S phase consistent with its ubiquitin-mediated proteolysis described previously (34). The total levels of Orc2,

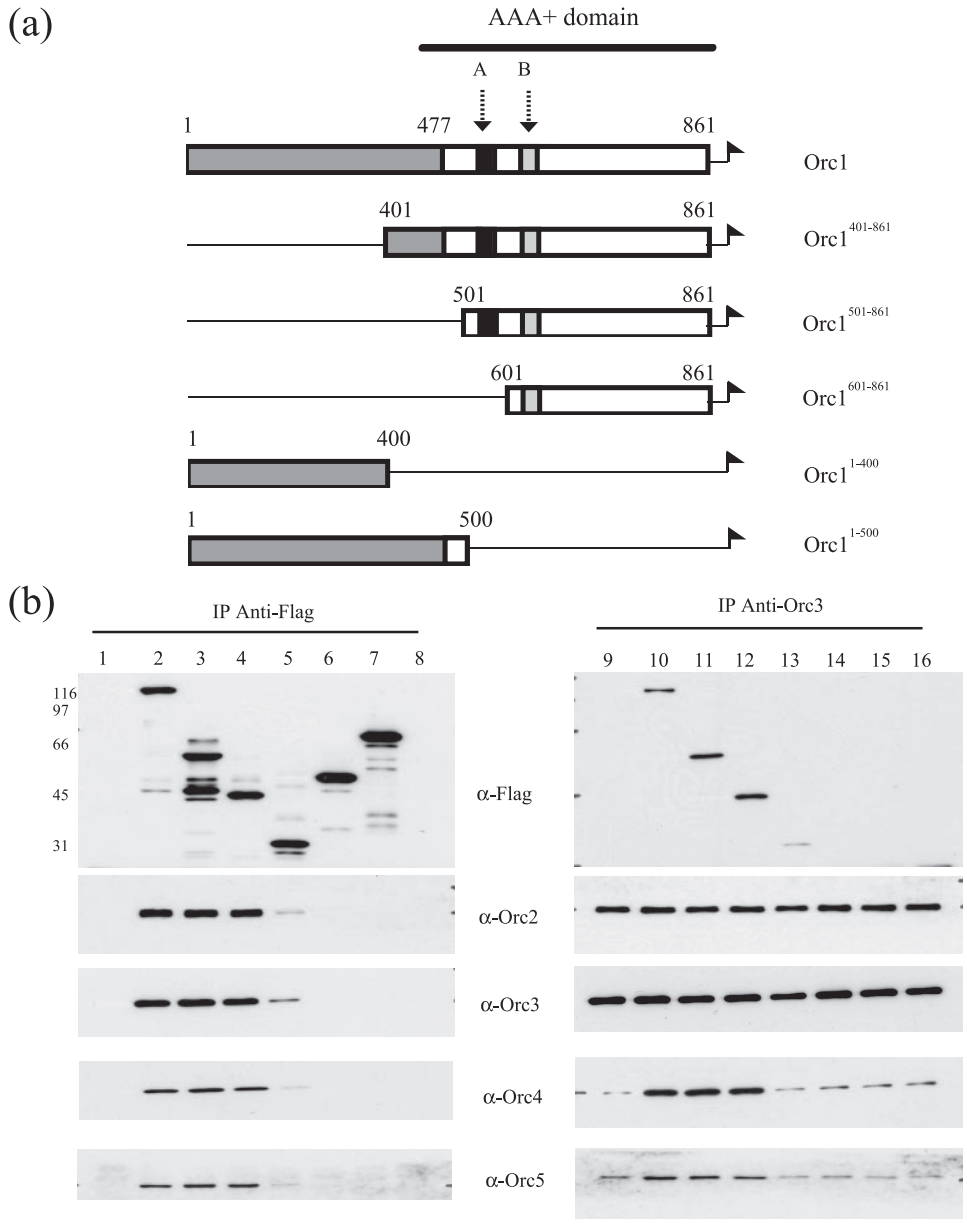

FIGURE 5. **Mapping of the Orc1 domain for ORC complex assembly *in vivo*.** *a*, schematic representation of all Orc1 (full-length and deletion) constructs used in the transient transfection experiments. Orc1 full-length and deletion constructs were cloned with a C-terminal FLAG epitope tag. The AAA+ motifs are indicated by the small boxes (*black* = Walker A motif; *gray* = Walker B motif). The large N-terminal non-AAA+ domain is shaded in *gray*. The amino acids included in the constructs are indicated on the *right* with *superscript*. *b*, the C terminus of Orc1 containing the AAA+ domain is necessary and sufficient for interaction with Orc2–Orc5 *in vivo*. The constructs described in *panel a* were transfected into HEK293 cells, and extracts were prepared from these for immunoprecipitation (*IP*), either using anti-FLAG (M2 Sigma) or using anti-Orc3 (PKS16 clone number 11) monoclonal antibodies. *Lanes 1–8* show the results of the immunoprecipitation using anti-FLAG antibodies, and *lanes 9–16* show the results using anti-Orc3 antibodies. The inputs for these immunoprecipitations show similar levels for all proteins examined (data not shown). *Lanes 1* and *9* = empty vector control; *lanes 2* and *10* = Orc1-FLAG; *lanes 3* and *11* = Orc1$^{401-861}$-FLAG; *lanes 4* and *12* = Orc1$^{501-861}$-FLAG; *lanes 5* and *13* = Orc1$^{601-861}$-FLAG; *lanes 6* and *14* = Orc1$^{1-400}$-FLAG; *lanes 7* and *15* = Orc1$^{1-500}$-FLAG; *lanes 8* and *16* = untransfected cells.

Orc3, Orc4, and Orc5 remain relatively unchanged during this period. In the Orc3 immunoprecipitates, the associated Orc2 and Orc5 remain constant throughout the cell cycle, in agreement with the observation that they can form an ATP-independent heterotrimer *in vitro*. However, levels of associated Orc1 and Orc4 consistently decreased in parallel. This result suggests that degradation of Orc1 is accompanied by a loss of Orc4 association with the complex. In the experiment described in Fig. 6*a*, nocodazole was added at 4 h after release from the second thymidine block to prevent cells from entering the G_1 phase of the next cell cycle; however, similar results were obtained in experiments when cells were released in the absence of the drug (data not shown).

Orc3 Levels Decrease in the Chromatin-enriched Fraction during S Phase—The Orc1 subunit has been implicated in the DNA binding activity of human ORC (10). The results above suggested that the ORC disassembles across S phase, concomitant with the degradation of Orc1. We hypothesized that as a consequence of Orc1 destruction, the association of the remaining ORC subunits with DNA may be compromised. Supporting this hypothesis, it had been previously demonstrated that Orc2 was progressively diminished during S phase when cells were extracted with detergent prior to immunofluorescence staining in HeLa cells (30). We tested whether this was true for the Orc3 subunit. Antibodies against Orc3 (CS1890) showed a uniform punctate nuclear signal in cultured tumor cells (Fig. 6*b*). HeLa cells were washed with PBS and then fixed with paraformaldehyde, or they were washed in parallel with CSK buffer containing Triton X-100 to remove soluble nuclear proteins and then fixed and stained with anti-Orc3 antibodies. This process of pre-extraction of the cells with detergent showed significant differences in the staining intensities of the cells (Fig. 6*b*), suggesting that Orc3 was more tightly bound to the chromatin fraction in some cells *versus* others. To test whether these staining differences were cell cycle-regulated, the cells were co-stained with p150 CAF-1, a protein known to localize to replication factories only visible during S phase in tumor cells (36–40). The staining patterns of p150CAF-1 would thus facilitate distinction of cells in different stages of S phase. In cells that did not score positive for CAF-1, the staining intensities of propidium iodide facilitated the identification of cells that were in G_1 or late S/G_2 phase of the cell cycle. The results in Fig. 6*c* show that following pre-extraction with Triton

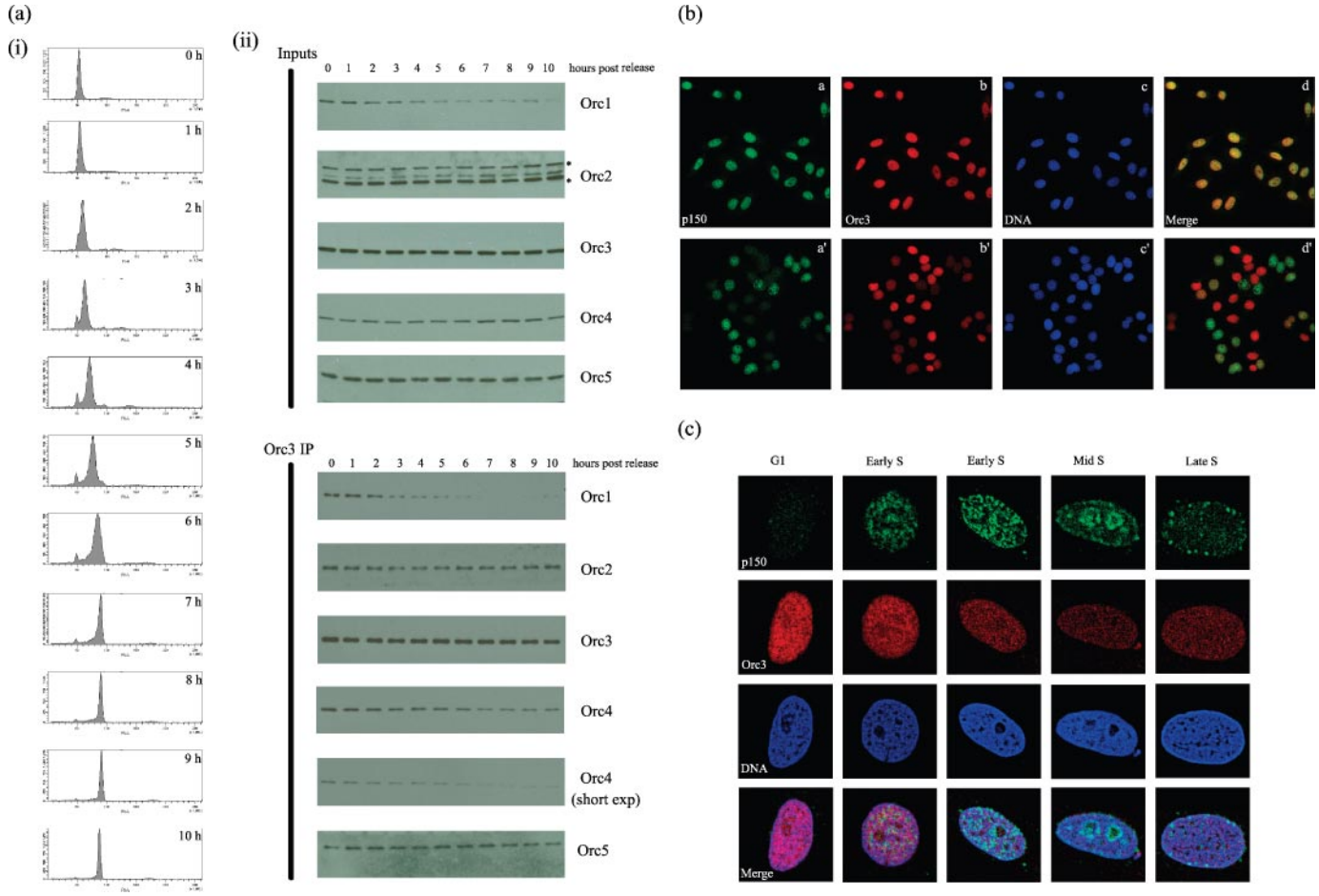

FIGURE 6. **Dissociation of ORC during S phase.** *a*, the human origin recognition complex disassembles in S phase in HeLa cells. HeLa cells were arrested at the G_1/S transition by a double thymidine block protocol and then released into S phase by incubation in fresh medium. Aliquots of cells were harvested at 1-h intervals and were examined for DNA content by staining with propidium iodide followed by flow cytometry (*panel i*) or used to prepare whole cell extracts followed by immunoprecipitation (*IP*) using anti-Orc3 antibodies (*panel ii*). The Orc3 immunoprecipitates were analyzed for the presence of Orc1–Orc5 subunits by Western blotting. Inputs are shown in the *top five panels*, and corresponding immunoprecipitates are shown in *bottom six panels*. Two different exposures for the Orc4 immunoprecipitates have been shown to highlight the decreasing signal in the later time points. The *asterisks* in the Orc2 immunoblot denote nonspecific bands as verified previously by RNA interference analysis (Prasanth *et al.* 30). *b*, Orc3 staining patterns in HeLa cells with or without treatment with Triton X-100 to reveal chromatin-bound protein. HeLa cells were stained with anti-p150 CAF1 monoclonal antibodies (SS1) and anti-Orc3 polyclonal antibodies (CS1890) either without pre-extraction (*panels a–d*) or following pre-extraction with CSK buffer + 0.5% Triton X-100 (*panels a′*, *b′*, *c′*, and *d′*) Cells were co-stained with propidium iodide (*panels c* and *c′*) to visualize DNA. *Panels d* and *d′* show a merge of *panels a* and *b* and *panels a′* and *b′*, respectively. All images have been captured using a ×20/NA 0.75 objective. *c*, chromatin-bound Orc3 shows higher signal in cells in early S phase than in cells in mid-to-late S phase. HeLa cells were pre-extracted with CSK buffer + 0.5% Triton X-100 and co-stained with anti-Orc3 polyclonal antibodies (*red*) and anti-p150CAF1 monoclonal antibodies (*green*). The p150 CAF1 staining patterns reveal that cells in mid-to-late S phase show lower intensities of Orc3 signal than cells in G_1 or early S phase. Cells were co-stained with propidium iodide (*blue*) to visualize DNA. The *last row* shows a merge of all three channels.

X-100, the Orc3 signal was higher in the cells showing early S phase patterns but was reduced in cells showing mid-to-late S phase patterns. From these data, we concluded that, as shown previously for Orc2 (30), Orc3 was less tightly bound to nuclear structures in later stages of S phase and may reflect its dissociation from chromatin. Such an analysis was not possible for Orc4 and Orc5 because of a lack of suitable antibodies that work well for immunostaining.

Orc6 Interacts with ORC Subunits in Vivo and in Vitro—The human Orc6 protein was identified based on a high degree of sequence similarity with the *Drosophila* homolog (6). Since DmOrc6 participates in the formation of a six-subunit complex (17), we hypothesized that the human Orc6 protein also interacts with its cognate complex. Using polyclonal antibodies against Orc3, Orc4, and Orc5, native ORC was immunoprecipitated from HEK293 nuclear extracts. Rabbit IgG was used as a control to identify nonspecific interactions. All immunoprecipitates were examined for the presence of different ORC subunits by Western blotting. The results in Fig. 7*a* reveal that three independent antibodies against Orc3 and Orc5 were able to co-precipitate Orc6, but not antibodies directed against Orc4 or Rabbit IgG. The ratio of the amount of protein brought down in the immunoprecipitates when compared with the input level was considerably lower for Orc6 when compared with the ratios for Orc2, Orc3, and Orc4, suggesting that a large fraction of Orc6 was not associated with the ORC complex. An immunoprecipitation experiment was also performed using monoclonal antibodies, either against Orc3 or against the Myc epitope tag as a control. The different ORC subunits were analyzed by Western blotting with polyclonal antibodies (Fig. 7*b*). Again, a small but specific signal for Orc6 was observed in Orc3 but not in control immunoprecipitates. A nonspecific protein

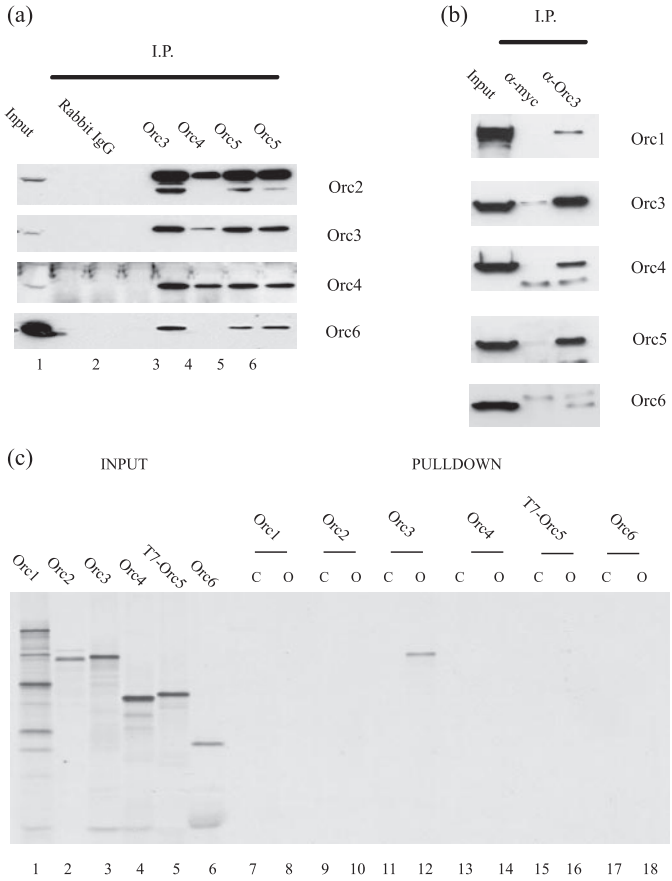

FIGURE 7. **Association of Orc6 with ORC *in vivo* and *in vitro*.** *a*, polyclonal antibodies against Orc3 and Orc5 co-precipitate with Orc6 from HEK293 nuclear extracts. Polyclonal antibodies against Orc3 (CS1890), Orc4 (CS2100), and Orc5 (CS1562, CS1569) were used to immunoprecipitate ORC from HEK293 nuclear extracts. Purified rabbit IgG was used as control. The immunoprecipitates (*I.P.*) were examined for the presence of ORC subunits by Western blotting. *b*, monoclonal antibodies against Orc3 co-precipitate with Orc6 from HEK293 nuclear extracts. Monoclonal antibodies against Orc3 (PKS16 clone number 11) or Myc epitope (9E10) were used to immunoprecipitate ORC from HEK293 nuclear extracts. The immunoprecipitates were examined for the presence of ORC subunits by Western blotting. *c*, Orc6 interacts with Orc3 *in vitro* using a GST pulldown assay. GST or GST-Orc6 was incubated with [^{35}S]methionine-labeled ORC subunits and washed extensively. The bound proteins were analyzed by SDS-PAGE followed by autoradiography and showed that Orc6 interacts specifically with Orc3 using this assay. *C*, purified GST; *O*, GST-Orc6.

was present in both precipitates and may be the light chain of IgG. Thus, we conclude that Orc6 interacts with the other ORC subunits in a complex in human nuclear extracts.

To test whether Orc6 interacts directly with any ORC subunit, a GST pulldown assay was performed as follows. Human Orc1–Orc6 were expressed separately in a rabbit reticulocyte lysate to generate [^{35}S]methionine-labeled proteins. The proteins were diluted in buffer and incubated either with purified GST or with GST-Orc6 bound to glutathione-Sepharose. After incubation for 2 h to allow stable protein interactions to form, the resin was washed extensively to remove non-specifically bound proteins. The bound proteins were then boiled and analyzed by SDS-PAGE followed by autoradiography. The results demonstrate that GST-Orc6 specifically pulls down Orc3 (Fig. 7*c*). Thus, we present evidence that Orc6 associates with ORC subunits both *in vitro* and *in vivo* and propose that Orc6 may be recruited to the complex by virtue of its interaction with Orc3.

DISCUSSION

Based on previous research on yeast and *Drosophila* ORC, it was expected that the human ORC proteins would similarly form a six-subunit complex (15–17). Human ORC, however, follows an ordered pathway for the sequential addition of the different subunits. Orc2 and Orc3 bind to each other and then recruit Orc5. The Orc2/Orc3/Orc5 complex recruits Orc4 and then Orc1 in an ATP-dependent process. Similar results for human ORC have been described recently in an independent study (8). In other eukaryotic systems examined thus far (15–17, 41), a nucleotide requirement for complex assembly has not been reported, although work on simian virus 40 (SV40) and papillomavirus DNA replication has revealed that ATP promotes the formation of T-antigen or E1 hexamers, respectively, at origins of replication (42–44). Such dynamic subunit interactions between the human ORC subunits suggest an additional mechanism for regulation at the level of complex assembly and disassembly and may have implications for the cell cycle control of human ORC function *in vivo*.

Analysis of mutants revealed that ATP binding by Orc1 was not essential for complex formation. ATP binding by human Orc4 is essential for both Orc2–Orc5 as well as Orc1–Orc5 association (8). ATP binding by Orc5 is necessary for Orc2–Orc5 assembly but is not essential for Orc1–Orc5 association *in vitro*. One possible explanation is that Orc4 may require nucleotide binding to form productive interactions with both Orc1 and Orc5, whereas Orc1 may form nucleotide-independent contacts with Orc5 that allow it to rescue complex formation with an ATP-binding mutant. The requirement for ATP binding by Orc5 and Orc4 suggests that these proteins may have flexible domains existing in multiple conformations, and the ATP-bound form is the "active" form for complex assembly. Two observations support this idea. Firstly, mutants of Orc4 and Orc5 that do not bind ATP are expressed very poorly in comparison with wild type Orc4 and Orc5 in cultured human cells, whereas the corresponding mutants of Orc1 are expressed similarly to wild type Orc1 (data not shown). Secondly, a three-dimensional structure of recombinant *Drosophila* ORC has been reported to assume different conformations when incubated with different nucleotides, which may be attributable to various nucleotide-dependent confirmations of the AAA+ proteins (21). This result is reminiscent of other AAA+ protein assemblies that exhibit different conformations in the presence of ATP *versus* ADP (33, 45–50).

Mutants of Orc1, Orc4, and Orc5 predicted to disrupt ATP hydrolysis formed complexes that were indistinguishable from complexes containing the wild type proteins. ATPγS also supported Orc2/Orc3/Orc4/Orc5 complex formation. This result suggests that ATP hydrolysis in the complex is not required for assembly and may perform a different function in DNA replication, such as binding Cdc6 proteins at the origins or loading MCM proteins (22, 51). ADP does not support Orc2/Orc3/Orc4/Orc5 complex assembly, and this result may have physiological relevance. ATP hydrolysis within human ORC, perhaps by the Orc4 subunit, may couple the ATPase activity with the disassembly of the complex since it would adopt a non-permissive ADP-bound conformation. If the bound ADP does

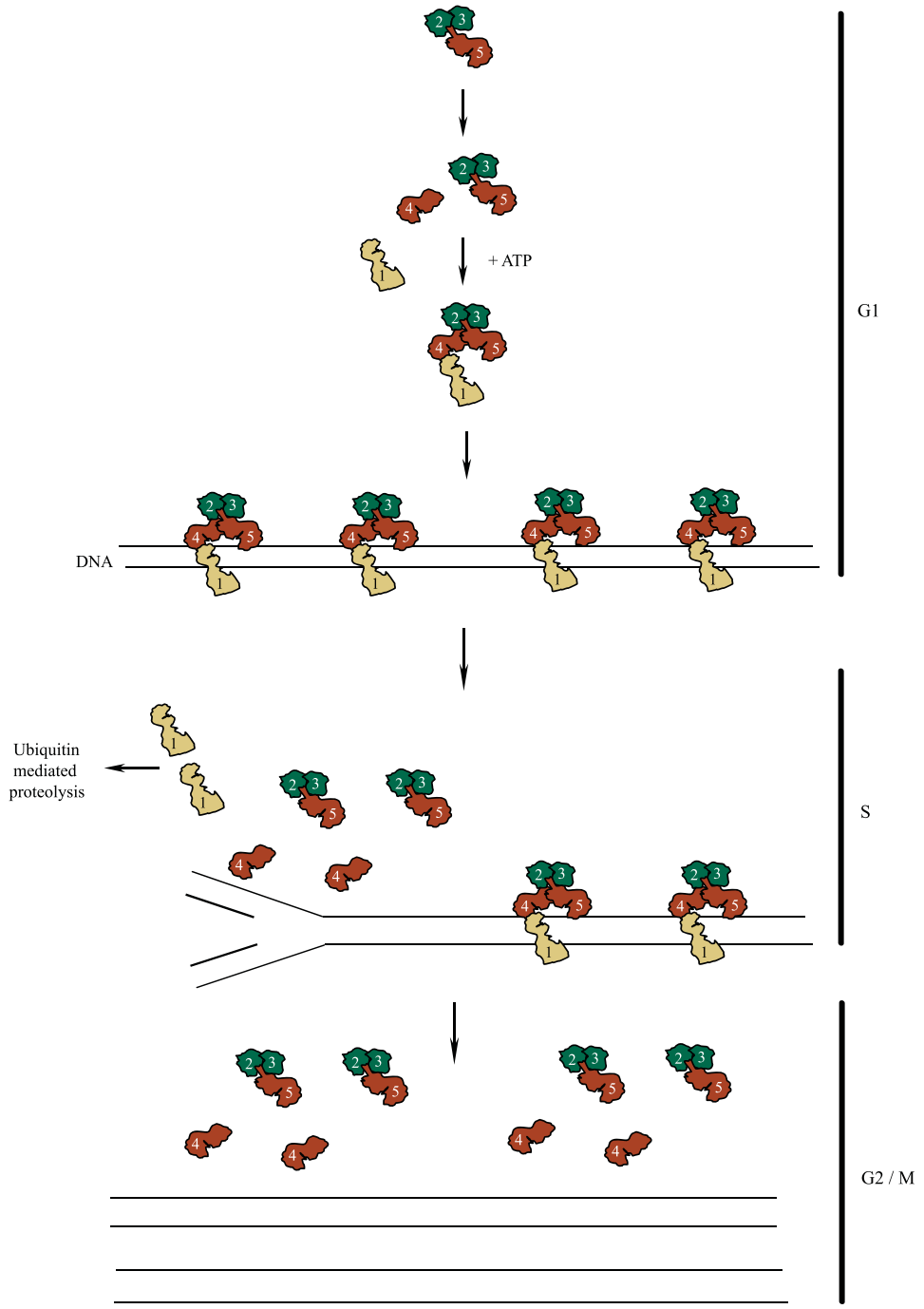

FIGURE 8. **Proposed model for the regulation of human ORC during the cell cycle in tumor cells.** This model has been generated based on data presented in this study as well as others (8, 30, 34, 35, 60, 61, 63, 64) and suggests that human ORC is a dynamic complex when compared with its budding yeast counterpart. During mitotic exit or in G_1, the complex is assembled in an ATP-dependent process and binds to origins of DNA replication in an Orc1-dependent manner. Entry into S phase results in degradation of Orc1 by ubiquitin-mediated proteolysis, and ORC is disassembled. Concomitant with Orc1 destruction, Orc4 dissociates from the core Orc2 and Orc3 complex.

not dissociate easily, it could inhibit the formation of active Orc2/Orc3/Orc4/Orc5 complexes during S and G_2 phases when Orc1 is absent. ATP hydrolysis-coupled disassembly has been reported in AAA+ molecular machines whereby engagement and the subsequent degradation of substrate results in disassembly of the 26 S proteasome as part of each catalytic cycle (52). Also, a "nucleotide-switch" mode of regulation is seen in prokaryotic DNA replication that limits DNA replication initiation to once per cell cycle (53, 54). ATP hydrolysis by the initiator DnaA results in the ADP-DnaA form that accumulates for the duration of the cell cycle, and the next cell cycle requires new protein synthesis to generate ATP-DnaA. It may be that the ADP dissociation rate controls when ORC subunits are competent to reform the ATP-dependent complex. Alternatively, events during early mitosis may reset the nucleotide-bound state of Orc4 and Orc5, and new synthesis of Orc1 would allow ATP-dependent assembly of ORC as cells exit mitosis before assembly of the pre-replicative complex.

The results of the immunoprecipitation analysis showed that overexpression of Orc1 in human cells promoted association of Orc4 with the complex. Furthermore, analysis of Orc3 immunoprecipitates across S phase revealed that association of both Orc1 and Orc4 with the core ORC (Orc2, Orc3, and Orc5) are lost in a parallel fashion, again supporting the view that Orc4 association is Orc1-dependent. A similar cell cycle-dependent enrichment of Orc4 with ORC has been observed previously (35). This result suggests that Orc1 is limiting for complex formation *in vivo* and restricts complex assembly to the G_1 phase of the cell cycle. Perhaps one consequence of the S phase-dependent degradation of Orc1 is to couple the initiation of DNA replication to complex disassembly, thus ensuring that pre-replicative complexes cannot be formed once cells have started DNA synthesis.

The immunofluorescence analysis of Orc2 (30) and Orc3 (this study) suggests a mechanism whereby ORC subunits are released from chromatin in a cell cycle-dependent manner. Evidence from at least three other reports supports a view for dynamic ORC-chromatin interactions in higher eukaryotes. ORC is progressively removed from chromatin as the replication proceeds across the cell cycle in *Xenopus* egg extracts (55), and secondly, the half-life of ORC on sperm chromatin is decreased in an MCM-dependent manner *in vitro* (56). In a third report, pre-replicative complexes were assembled onto a circular plasmid *in vitro*, and the analysis revealed that inhibiting MCM loading by the

addition of geminin allows the recovery of significantly greater quantities of ORC bound to plasmid (57). All three reports point to the fact that ORC is essential for MCM loading; however, once MCMs are loaded, they promote the removal of ORC from these DNA templates, most likely in a replication-dependent process. In addition, it has been reported for both *Xenopus* as well as budding yeast systems that upon pre-replicative complex formation on DNA, the MCM subunits are resistant to high salt washes; however, ORC can be easily extracted under the same conditions (58, 59). In support of these observations, in human cells, Orc1 and Orc2 subunits have been shown to dissociate from chromatin as cells enter S phase (34, 60), and the Orc1 subunit is subsequently degraded by a ubiquitin-mediated mechanism (34, 35, 61). More recently, it was also demonstrated that Orc2 localizes in a cell-cycle dependent manner at human chromosomal origins in HeLa cells (65).

It has been shown that ATP binding by Orc1 is necessary for its DNA binding activity in various systems (9, 17, 23). Recent work on human ORC has shown that Walker A mutants of Orc1, Orc4, and Orc5 have reduced DNA binding *in vitro* and are unable to support sperm DNA replication in a *Xenopus* cell-free system (8, 9). Surprisingly, in the study described by Giordano-Coltart *et al.* (9), recombinant human ORC complexes containing a mutant Orc4 subunit were purified from insect cells. This is contradictory to our observations that imply that it may not be possible to generate such a complex. There are two possible explanations for this. The overexpression of human ORC proteins in a heterologous system supports complex formation due to overexpression, or alternatively, the Orc4 K73A mutant has a reduced affinity for ATP, and complex formation is rescued by inclusion of millimolar concentrations of ATP during the purification. Our results are consistent with those reported by Ranjan and Gossen (8), and we were unable to obtain stable Orc1/Orc2/Orc3/Orc4/Orc5 complexes using several Orc4 mutants, either by using recombinant human ORC expressed in insect cells or by using *in vitro* transcribed and translated proteins. Our studies extend a role for ATP binding by Orc5 in complex formation, and further analysis of these mutants *in vivo* will be required to determine the extent of their contribution in human cells.

Finally, we asked whether Orc6 was an interacting partner of the human origin recognition complex and were able to demonstrate using a number of specific antibodies that the six ORC subunits can be co-precipitated from human nuclear extracts. The association between the Orc6 and Orc3 subunits *in vitro* may explain the binding of Orc6 with ORC *in vivo*, although this complex may require additional factors or post-translational modifications to generate stoichiometric complexes. The interaction between Orc6 and ORC is regulated;[4] however, these questions will be addressed in greater detail in a separate report.

Based on the above data and other published results (6, 8, 30, 34, 35, 60, 61, 63, 64), we suggest a model to integrate our *in vitro* biochemical results with those obtained from studying the native protein complex in cultured cells, and this is summarized in Fig. 8. We propose that Orc1/Orc2/Orc3/Orc4/Orc5 complexes are formed in an ATP-dependent manner as cells exit anaphase of mitosis, and this ATP-loaded complex binds to chromatin in an Orc1-dependent manner to direct the formation of pre-replicative complexes. As cells enter S phase, Orc1 is degraded, and as a result, the complexes are disassembled. An alternate possibility is that ATP hydrolysis in the complex converts it into the non-permissive ADP-bound form, resulting in ejection of the Orc1 subunit from the complex, whose degradation is a consequence, rather than a cause, of complex disassembly. In contrast to budding yeast, the human Orc4 protein contains an intact Walker B motif and may be competent to hydrolyze ATP, in addition to Orc1. Future experiments will be designed to address this possibility. Also, the availability of non-degradable Orc1 mutants would allow us, in the future, to ascertain whether either of these alternatives is the correct one. In the absence of Orc1, the Orc2 and Orc3 subunits are removed from chromatin, perhaps accompanied by Orc5 as a stable Orc2/Orc3/Orc5 subcomplex. The Orc4 subunit may be dissociated from chromatin or may remain bound to DNA by virtue of its DNA binding activity (62), and it may serve to mark origins that have already fired. Toward the end of the cell cycle, Orc1 is regenerated and cooperates with Orc4 to direct the formation of new complexes and the cycle of pre-RC formation is repeated. At present, it is not clear at what step Orc6 participates in complex assembly, and it may be recruited in an ATP-independent manner by Orc3. Future experiments will be designed to specifically address the chromatin binding of the different ORC subunits *in vivo* at known origins of replication across the cell cycle.

The role of ATP binding by the human origin recognition complex offers an additional mechanism to control pre-replicative complex assembly and may be intimately linked with another regulatory mechanism unique to human cells, *i.e.* the S phase-mediated degradation of the Orc1 subunit. Whether ATP hydrolysis by the ORC subunits is involved in the disassembly of the complex or in Orc1 degradation will be the focus of future studies. These studies will lead to a better understanding of the DNA binding activity of human ORC and will ultimately aid the identification of endogenous DNA substrates of this complex that may define origins of replication in human cells.

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[4] K. Siddiqui and B. Stillman, unpublished observations.

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