The ORC1 Cycle in Human Cells

The origin recognition complex (ORC) plays a central role in regulating the initiation of DNA replication in eukaryotes. The level of the ORC1 subunit oscillates throughout the cell cycle, defining an ORC1 cycle. ORC1 accumulates in G1 and is degraded in S phase, although other ORC subunits (ORCs 2–5) remain at almost constant levels. The behavior of ORC components in human cell nuclei with respect to the ORC1 cycle demonstrates that ORCs 2–5 form a complex that is present throughout the cell cycle and that associates with ORC1 when it accumulates in G1 nuclei. ORCs 2–5 are found in both nuclease-insoluble and -soluble fractions. The appearance of nuclease-insoluble ORCs 2–5 parallels the increase in the level of ORC1 associating with nuclease-insoluble, non-chromatin nuclear structures. Thus, ORCs 2–5 are temporally recruited to nuclease-insoluble structures by formation of the ORC1–5 complex. An artificial reduction in the level of ORC1 in human cells by RNA interference results in a shift of ORC2 to the nuclease-soluble fraction, and the association of MCM proteins with chromatin fractions is also blocked by this treatment. These results indicate that ORC1 regulates the status of the ORC complex in human nuclei by tethering ORCs 2–5 to nuclear structures. This dynamic shift is further required for the loading of MCM proteins onto chromatin. Thus, the pre-replication complex in human cells may be regulated by the temporal accumulation of ORC1 in G1 nuclei.

The replication of eukaryotic chromosomes occurs in a highly regulated manner during S phase. In the budding yeast Saccharomyces cerevisiae, the ORC is the origin recognition complex (ORC), which is composed of six polypeptides, is essential for initiation, and it remains bound to replication origins throughout the cell cycle (1–3). Prior to the initiation of DNA replication, ORC forms a large protein complex, called the pre-replicative complex (pre-RC), in association with other initiation factors, including Cdc6 and the mini-chromosome maintenance (MCM) proteins. The pre-RC then changes to a post-replicative form with the dissociation of these factors, in parallel with alterations in the ORC-DNA interaction (2–6). Thus, dynamic changes in DNA-protein complexes at replication origins appear to be closely connected with the initiation of replication (2–7).

Counterparts of yeast pre-RC components have been identified in higher eukaryotes, indicating that basic mechanisms for the initiation of replication are highly conserved (7). Putative ORC subunits that constitute multi-protein complexes similar to those seen in S. cerevisiae have been identified in Drosophila (8, 9) and Xenopus (10–12). In the latter, the depletion of ORC from egg extracts inhibits the initiation of replication (10, 11). Similarly, in Drosophila, conditional mutations in the DmORC2, -3, and -5 genes cause strong defects in replication and cellular proliferation (13, 14). Putative human ORC genes have also been identified (12, 14, 15–20), and their products have been shown to interact with each other (21, 22), suggesting that a similar protein complex functions in replication in human cells.

Recent studies have demonstrated that several nuclear events, including replication, take place in specialized compartments where specific factors assemble in highly organized structures (23). Therefore, to understand how replication is initiated and regulated in eukaryotic cells, it is necessary to determine how replication proteins are assembled into higher order complexes and how they are distributed among subnuclear compartments. Previous studies have demonstrated that MCMs associate with pre-replicative chromatin and subsequently dissociate upon the initiation of replication (2, 5, 6, 24–26). Similarly, mammalian CDC6 proteins appear to accumulate in G1 phase nuclei and to move to the cytoplasm in early S phase (27–29). These observations suggest that the dynamic behavior of these proteins in S phase nuclei may be linked to the mechanism that restricts replication to only once per cell cycle. In budding yeast, however, ORC behaves as a static nuclear component and associates with chromatin DNA throughout the cell cycle (2, 3, 6). Despite the overall high conservation of ORC proteins among species, animal ORCs exhibit greater regulatory flexibility than their yeast counterparts. For example, expression of the mammalian ORC1 gene is regulated by the E2F protein (16, 30), and Xenopus ORC is known to dissociate from metaphase chromatin, as observed in an egg extract system (11). In addition, the Drosophila ORC1 protein changes in abundance depending on developmental
stage and on the cell cycle, and ectopic expression of DmORC1 alters the program of proliferation. This finding suggests that ORC, and especially ORC1, regulates the growth of animal cells by controlling S phase progression (30). Indeed, the accompanying paper (31) describes an ORC1 cycle in human cells, which is characterized by ORC1 accumulation during G1 and reduction during the S phase.

In this study, we demonstrate that chromatin-bound ORC2–5 subunits are recruited to nucleosome-insoluble, non-chromatin structures in a manner that parallels the ORC1 cycle. Furthermore, this shift in the distribution of ORCs 2–5 tightly correlates with the partitioning of MCMs to chromatin fractions. These results strongly suggest that the accumulation of ORC1 precedes replication complex assembly in human nuclei prior to the S phase.

EXPERIMENTAL PROCEDURES

Cell Culture and Synchronization—Cell culture, synchronization, and monitoring of the cell cycle were done as described (31, 32). Construction of the 293 cell line expressing FLAG-ORC1 (1C2) was also described (31). Fractionation of Cellular Proteins—Whole cell extract preparation and fractionation of proteins from HeLa S3 cells with Triton X-100 (Triton), NaCl, or DNase I were done as described (31).

RNA Interference Experiments—RNA interference (RNAi) experiments were done essentially as described by the siRNA User’s Guide (www.miphc.gwdg.de/abteilungen/100/105/sirna.html). Annealed siRNAs specific for ORC1 (ORC1 plus 73a, 5'-UCUGCUAUAATTTG-3'), ORC1 plus 73b, 5'-UAUAGGUUUUGGAGUGCAGTT-3'), CDC6 (CDC6 plus 82, 5'-CUCAGAGGCAAGCCAAACU-3'), 5'-UAGUUGGCATACUGGAUAGTT-3'), and pGL (luciferase, 33) were transfected into HeLa S3 cells with Oligofectamine (Invitrogen) following the manufacturer’s instructions. Cells were harvested and fractionated 2 days after transfection.

Immunoprecipitation and Immunoblotting—All antibodies used in this study have been described (31). #209 (anti-ORC1 antibody) beads were prepared by immobilization of the purified antibody to protein A-Sepharose (Amersham Biosciences, UK), at a ratio of 2 μg of IgG to 10 μl of beads, with dimethyl pimelimidate (Sigma, St. Louis, MO). ORC1 was precipitated from 0.1% TX-100mcSK extracts (31, 32) prepared from 1 × 10⁷ HeLa S3 cells with 10 μl of #209-beaded links after incubation at 4 °C for 3 h. For ORC2, the same lysate was incubated with 1 μg of 3B7 (MBL) at 4 °C for 2 h, followed by addition of 10 μl of protein A-Sepharose and incubation at 4 °C for 1 h. The supernatants were recovered as unbound fractions. The beads were washed twice with 0.1% TX-300mcSK, and elutes were obtained with 0.1 M glycine (pH 2.0) and neutralized with 1/10 volume of 1 M Tris-HCl (pH 8.8). All immunoblotting procedures were described (32). Mass Spectrometry—All procedures were essentially done as described previously (34). Immunoprecipitated samples were separated by 12.5% SDS-PAGE, and the region of the gel-containing proteins from about 200 to 30,000 Da (Fig. 2) was cut at intervals of ~1 mm. Proteins in the gel slices were subjected to reduction, alkylation, and trypsin digestion with modified trypsin (Roche Applied Science, Germany) at 37 °C for 14 h. After in-gel digestion, the product peptides were extracted with 5% formic acid and acetonitrile, dried under vacuum, and dissolved in 0.1% formic acid and 5% formic acid. Multiply digested peptides from each gel slice were separated by micro-capillary C18-reverse phase chromatography (200 μM × 5-cm capillary, Microm BioResources) and directly applied to an LCQ Advantage quadrupole ion trap mass spectrometer (Finnigan) with a nanoelectrospray needle (New Objective) mounted on a 3D-stage (AMR, Japan). The primary ion spectrum data generated by LC/MS/MS were screened against the NCBI non-redundant protein database with the Mascot program (Matrix Science, UK) to identify high scoring proteins.

RESULTS

Subcellular Localization of ORC Subunits in HeLa S3 Cells—To study the behavior of ORC subunits in human cells, proteins extracted from HeLa S3 cells growing asynchronously were fractionated into soluble and insoluble fractions by treatment with 0.1% TX-100mcSK. ORC1, 2, –3, –4, and –5 were predominantly found in the insoluble fraction (Fig. 1A), suggesting that they localize to chromatin. When the washed nuclei were further treated with DNase I to extract bulk chromatin-associated DNA (32), most ORC1 remained associated with nucleosome-insoluble nuclear structures (non-chromatin nuclear structures) (Fig. 1C). In contrast, about half of the ORC2, -3, -4, and -5 subunits (ORCs 2–5) were solubilized by this treatment, indicating that half of them are associated with nuclear structures (Fig. 1C). When the Triton-insoluble fraction was treated with buffers containing increasing concentrations of NaCl, ORC1 was solubilized by 0.3 M NaCl but not by 0.2 M NaCl, whereas ORCs 2–5 were gradually solubilized by 0.2 and 0.3 M NaCl, as shown in Fig. 1B.

Composition of the ORC Complex in HeLa S3 Cells—To determine whether human ORC subunits are in a stable complex similar to yeast ORC, we studied co-immunoprecipitates with ORC1 from the Triton-insoluble fraction of HeLa S3 cells extracted with 0.3 M NaCl. Several specific bands were detectable on SDS-polyacrylamide gels of proteins precipitated with the #209 antibody (Fig. 2). To identify the proteins in these bands, slices from the gel were subjected to mass spectrometric analysis. Peptides with ORC1 amino acid sequences were found in gel slice #10, which contained proteins of 100-kDa molecular mass. Peptides with ORC2 and ORC3 amino acid sequences were identified in gel slice #16 (67 kDa), and peptides with ORC4 and ORC5 amino acid sequences were found in gel slice #25 (45 kDa). To confirm this result, immunoprecipitated samples were subjected to immunoblot analysis with specific antibodies against ORCs 2–5 (Fig. 3A). Under conditions in which most ORC1 in the lysate was precipitated, roughly half of the ORC2–5 proteins in the extract were co-immunoprecipitated with ORC1. This result was further confirmed by co-immunoprecipitation of FLAG-tagged ORC1 with an anti-FLAG antibody from the 293-cell line, 1C2, which expresses the tagged protein at the same level as endogenous ORC1 (31). ORCs 2–5 were specifically precipitated with FLAG-ORC1 (Fig. 3B). These results indicate that a protein complex consisting of the ORC1–5 subunits is present in the chromatin-bound fraction of human cell extracts.

Cell Cycle-dependent Formation of the ORC1–5 Complex—Next, we studied whether ORC1–5 persist as a tight complex throughout the cell cycle, as observed for yeast ORC. We prepared 0.3 M NaCl lysates from synchronously growing HeLa S3 cells after release from M phase arrest mediated by the drug TN16. Most cellular ORC1–5 subunits were extracted with 0.3 M NaCl (Fig. 1B). In this experiment, the level of ORC1 was low.
in metaphase and early G1 (0–3 h), rose between 6 and 12 h (middle G1), reached a peak at 12 h (G1/S), and then decreased from S to G2 phase, slightly in advance of the decrease in the level of cyclin E, as described in a previous study (31) (Fig. 4A).

The levels of the ORC2–5 subunits, however, remained essentially constant over time. The #209 antibody precipitated ORC1 at 9 and 12 h, times at which it accumulates, and it co-precipitated ORC2–4 subunits in the same period (Fig. 4, A and B). In a converse experiment with the anti-ORC2 antibody, constant amounts of both ORC2 and ORC3 were precipitated throughout the cell cycle (Fig. 4C), and ORC1 appeared in precipitates when it accumulated in cells. Interestingly, although the levels of ORC4 and 5 are constant throughout the cell cycle (31) (Fig. 4A), the amounts of these proteins that co-immunoprecipitated with ORC2 increased when ORC1 was present (Fig. 4C). These results indicate that ORC2 and ORC3 form a stable complex throughout the cell cycle and that their association with ORC4 and ORC5 is stabilized transiently when ORC1 accumulates on chromatin and associates with the ORC2–5 complex from late G1 to early S phase.

**Recruitment of ORCs 2–5 to a Non-chromatin Nuclear Structure through Interaction with ORC1**—Although most ORC1 protein was found in a non-chromatin nuclear fraction in extracts from asynchronously growing cells, ORCs 2–5 partitioned into both DNase I-soluble and -insoluble fractions (Fig. 1C). Because accumulated ORC1 appeared to readily associate with ORCs 2–5 (Fig. 4), we speculated that ORCs 2–5 are recruited to DNase I-insoluble structures through their association with ORC1. To determine whether the re-distribution of ORCs 2–5 to the DNase I-insoluble fraction is cell cycle-dependent and occurs in G1, we fractionated ORC1 and ORC2 from synchronously growing cells after release from TN16-mediated arrest by DNase I digestion sensitivity (Fig. 5). From 0 (M phase) to 6 h (early G1 phase), most cellular ORC2 was soluble with DNase I. Subsequently, ORC2 started to appear in the insoluble fraction at 9 h and peaked at 12 h. This temporal association of ORC2 with the nuclease-insoluble fraction correlates well with the abundance of ORC1 in this fraction.

To obtain more convincing evidence that ORC1 recruits ORCs 2–5 to DNase I-insoluble nuclear structures, we artificially eliminated ORC1 from cells by RNA interference (RNAi) and monitored the distribution of ORC2 in nuclear structures. As shown in Fig. 6A, within 2 days of transfection of ORC1
siRNA into HeLa S3 cells, ORC1 became undetectable. This reduction in ORC1 is specific to the ORC1 siRNA, because siRNAs specific for CDC6, lamin A/C, or luciferase (pGL2) did not affect the level of ORC1 but clearly caused their respective target proteins to disappear. When ORC1 was eliminated from cells, we observed an obvious shift of ORC2 from DNase I-insoluble fractions to DNase I-soluble fractions (Fig. 6B, ORC2). This result strongly supports the ORC1-dependent recruitment of ORC2 to DNase I-insoluble fractions as mentioned above.

The Loading of MCM onto Chromatin Requires the ORC1–5 Complex and CDC6 Protein—We observed the temporal formation of the ORC1–5 complex in non-chromatin nuclear structures in G1 phase human cells. What is the functional meaning of this assembly? In budding yeast, MCM is loaded onto origin DNA in an ORC- and CDC6-dependent manner to form the pre-RC. In mammalian cells, the loading of MCM onto chromatin in G1 is simultaneous with the accumulation of ORC1 (Fig. 4) (31, 35). This result implies that ORC1–5 complex formation may be required for loading MCM onto chromatin. To test this possibility, we analyzed the effect of the ORC1-specific siRNA on MCM loading. The level of MCM3 in the chromatin fraction (Triton ppt) was greatly diminished by the reduction of ORC1 in HeLa S3 cells (Fig. 6A), strongly suggesting that the loading of MCM onto chromatin is not mediated by ORCs 2–5 but by the complete ORC1–5 complex. Interestingly, the elimination of CDC6 from cells by RNAi inhibited MCM loading. Thus, both CDC6 and ORCs1–5 are necessary for MCM loading onto chromatin in human cells. It is noteworthy that CDC6-specific siRNA treatment did not interfere with the association of ORC2 with the DNase I-insoluble fraction. On the other hand, elimination of ORC1 also did not affect the association of CDC6.
with nuclease-insoluble fractions. Thus, the association of CDC6 with nuclear structures and the formation of the ORC1–5 complex on nuclear structures are independent.

**DISCUSSION**

ORC was originally identified in budding yeast as a complex of six different subunits. All six subunit components of ORC have been identified in various eukaryotes, including humans, indicating that the configuration of the complex is conserved among eukaryotes (7, 36). To date, stable ORC1–6 complexes have been isolated from yeast, flies, and frogs. However, although we detected the ORC1–5 complex in human cell lysates by immunoprecipitation with anti-ORC1 or ORC2 antibodies, we could detect ORC6 in these precipitates neither by the sensitive technique of mass spectrometry (Fig. 2) nor by immunoblot analysis with the anti-ORC6 antibody (data not shown). Thus, ORC6 in human cells may interact with other ORC subunits only weakly, if at all. The same limited affinity of ORC1 for the ORC1–5 complex has been reported for reconstitution experiments using a baculovirus expression system (21, 22). These findings do not necessarily mean that ORC6 is less important for ORC function, because it has been reported that *Drosophila* ORC6 is important for the specific binding of ORC to origin DNA (37). Therefore, ORC6 has a role in the regulation of ORC activity by its transient interaction with the ORC1–5 complex. Interestingly, it has been reported that human ORC6 has a role as a kinetochore complex component, in addition to its role in the initiation of replication (38). Further studies of its behavior during the cell cycle will be necessary to understand how human ORC6 functions in replication.

We have demonstrated that the appearance of the ORC1–5 complex is cell cycle-dependent and regulated by the ORC1 cycle. Furthermore, ORC2 and ORC3 are always in a stable complex, but ORC4 and ORC5 are separable from ORC2 and ORC3 in the absence of ORC1. Thus, human ORC subunits have a dynamic assembly mechanism that is regulated primarily by the level of ORC1. This result indicates that the subunit configuration of ORC in animal cells is apparently different from that in budding yeast and that there are multiple assembly states, each of which has a distinct role in regulating replication complex formation at origins (Fig. 7).

A prominent protein assembly in human cells is the ORC2–5 complex, which is always found in the chromatin fraction. It has been reported that ORC proteins specifically bind to replication origin sequences in budding yeast, fission yeast, and *Drosophila* (1, 37, 39). Furthermore, this association is maintained throughout the cell cycle in budding yeast. Recent studies have demonstrated that human ORC2 and ORC3 bind to the Epstein-Barr virus replication origin (oriP) and that ORC2 binds to a potential replication origin region in the human MCM4 locus throughout the cell cycle (40). In contrast, human ORC1 associates with the MCM4 origin only in G1 phase (40). The ORC2–5/chromatin complex that we have observed associates with ORC1 in G1. Due to the coincidence of the reported origin association and our observed chromatin binding of ORC subunits during the cell cycle, we suggest that the ORC2–5 complex associates with chromatin by binding to DNA, probably to potential origin sequences (Fig. 7A). If this is the case, human ORC2–5 are sufficiently active to recognize potential replication origin sequences and to bind them, like budding yeast ORC.

Subsequently, some replication origins will be chosen by ORC1 and recruited to nuclear structures where replication machineries assemble (Fig. 7B). Formation of the ORC1–5 complex in nuclear structures promotes binding of MCM to chromatin, which corresponds to pre-RC formation (Fig. 7C). Once MCM is loaded, the ORC1–5 complex disassembles by the degradation of ORC1 (Fig. 7D). Subsequently, further initiation steps occur, including the loading of the DNA synthesis apparatus (Fig. 7E). This proposed mechanism has two implications. The first concerns the selection of origins to be fired from among the many sites potentially bound by the ORC2–5 complex. Thus, ORC1 may determine the timing and order of origin firing by recruiting ORC2–5/chromatin complexes to nuclear structures or sites. The second is to ensure that origins are fired only once per cell cycle, by the inactivation of ORC1 immediately after use. This model is consistent with previous reports that ori-β at the dihydrofolate reductase locus of Chinese hamster ovary cells and that the β-globin locus origin of
HeLa S3 cells associate with similar nuclease-insoluble nuclear structures in late G1 and dissociate from them after the initiation of replication in S phase (41).

It is well known that in yeast and Xenopus, the loading of MCMs onto the ORC-origin complex corresponds to the formation of the pre-RC, a direct precursor of the complex that initiates replication. This step requires the essential components CDC6 and Cdt1, in addition to an active ORC complex (7, 36). We showed with RNAi experiments that both ORC1 and CDC6 are required for the loading of MCMs onto chromatin in human cells (Fig. 6). We also observed that the presence of the ORC2–5 complex in chromatin fractions is not sufficient for MCM loading onto chromatin. In yeast, ORCs 1–6 form a stable complex, and the transient accumulation of activator proteins primes a reaction to MCM loading. As discussed above, if the human ORC2–5 complex functions like the budding yeast ORC1–6 complex, human ORC1 and CDC6 may function as activators. In this respect, because the level of human ORC1 oscillates, so that it accumulates in concert with the loading of MCM, ORC1 may activate ORC functions as human ORC2 does in budding yeast. Indeed, ORC1 and CDC6 share significant amino acid sequence similarity and are derived from the same ancestor (42). Nonetheless, human CDC6 exhibits a pattern of oscillation opposite to that of ORC1, accumulating during S phase, peaking in M phase and decreasing in the following G1 phase (35).2 This observation indicates that, although both CDC6 and ORC1 are required for MCM loading in human cells, they have distinct temporal patterns of expression roles and different roles in pre-RC formation.

We have demonstrated that human ORC components exhibit a dynamic pattern of assembly during the cell cycle. Several lines of evidence strongly support the proposal that this assembly directly links temporal and spatial controls in the initiation of replication in human chromosomes. However, many questions remain to be answered: how does ORC recognize specific regions of replication in human chromosomes. However, many questions remain to be answered: how does ORC recognize specific regions of replication in human chromosomes. However, many questions remain to be answered: how does ORC recognize specific regions of replication in human chromosomes.

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