Interaction of *Xenopus* Cdc2-Cyclin A1 with the Origin Recognition Complex*

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The initiation of DNA replication in eukaryotes is regulated in a minimum of at least two ways. First, several proteins, including origin recognition complex (ORC), Cdc6 protein, and the minichromosome maintenance (MCM) protein complex, need to be assembled on chromatin before initiation. Second, cyclin-dependent kinases regulate DNA replication in both a positive and a negative way by inducing the initiation of DNA replication at G1/S transition and preventing further rounds of origin firing within the same cell cycle. Here we characterize a link between the two levels. Immunoprecipitation of *Xenopus* origin recognition complex with anti-XOrc1 or anti-XOrc2 antibodies specifically co-immunoprecipitates a histone H1 kinase activity. The kinase activity is sensitive to several inhibitors of cyclin-dependent kinases including 6-dimethylaminopurine (6-DMPA), olomoucine, and p21cip1. This kinase activity also copurifies with ORC over several fractionation steps and was identified as a complex of the Cdc2 catalytic subunit and cyclin A1. Neither Cdk2 nor cyclin E could be detected in ORC immunoprecipitations. Reciprocal immunoprecipitations with anti-Xenopus Cdc2 or anti-Xenopus cyclin A1 antibodies specifically co-precipitate XOrc1 and XOrc2. Our results indicate that *Xenopus* ORC and Cdc2-cyclin A1 physically interact and demonstrate a physical link between an active cyclin-dependent kinase and proteins involved in the initiation of DNA replication.

The initiation of DNA replication in eukaryotic cell cycles is tightly regulated so that every fragment of genomic DNA is replicated exactly once (reviewed in Refs. 1 and 2). Many experiments have indicated that the initiation of eukaryotic DNA replication requires at least two components: replication-competent chromatin and protein kinases that exert both positive and negative effects (1–3). Recent studies in yeast and *Xenopus* have characterized several protein assemblies that are components of replication competent chromatin. The origin recognition complex (ORC) was initially identified in budding yeast as a protein complex which binds to origins of replication and is essential for the initiation of replication (4–8). ORC homologues are present throughout eukaryotes. In *Xenopus*, Dro sophila, and fission yeast they have been shown to be essential for DNA replication (9–16). Additional proteins: the minichromosome maintenance (MCM) protein complex (reviewed in Ref. 17) and Cdc6p, bind to ORC, or next to ORC, during the pre-replicative period of the cell cycle. Both are essential for the initiation of DNA replication; they are displaced from chromatin as replication proceeds and are absent from post-replicative chromatin (reviewed in Refs. 1 and 2). In *Xenopus*, ORC is bound to origins during both the pre-replicative and post-replicative period and the binding of Cdc6 and MCMs to chromatin is dependent on the presence of ORC (14, 15, 18). Indeed, a hierarchy of binding exists; ORC binds first, followed by ORC-dependent Cdc6 binding and Cdc6-dependent MCM binding.

A wealth of experimental data points to the involvement of protein kinases in the initiation of replication. In yeast, genetic studies have identified the cdc2/CDC28 genes encoding protein kinases necessary for entry into S phase and M phase (19, 20). In *Xenopus*, both Cdc2-cyclin A and Cdk2-cyclin E complexes have been implicated in triggering initiation of DNA replication (21–27). It is important to note that protein kinases of the Cdk family appear to exert both positive and negative effects on DNA replication; kinase activities present during S and G2 phases of the cell cycle prevent a second round of replication (reviewed in Ref. 28) and specifically inhibit rebinding of MCM proteins (27–31). Thus, a complex network of protein phosphorylation is likely to converge at the origins of replication to induce a single round of replication during S phase and prevent further initiations within the same cell cycle.

Although the nature of replication competent chromatin and cyclin-dependent kinases have been elucidated separately, a crucial gap in our understanding lies between them. In fission yeast, Orp2, a subunit of fission yeast ORC has been demonstrated to interact with Cdc2 kinase, which serves two functions performed by both Cdc2 and Cdk2 in higher eukaryotes (32). However, the kinase was not shown to be active and no cyclin partner was reported. Here, we show that *Xenopus* ORC co-purifies and co-immunoprecipitates with an active cyclin-dependent kinase, which we identify as Cdc2-cyclin A1. This is particularly interesting, as Cdc2 is more likely to be involved in the negative regulation discussed above, rather than in positive regulation of initiation at the onset of S phase. Therefore, the Cdc2-cyclin A1 complex becomes a strong candidate for the

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* The abbreviations used are: ORC, origin recognition complex; MCM, minichromosome maintenance; 6-DMPA, 6-dimethylaminopurine.

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kinase that prevents MCM rebinding (27–31) and thus reinitiation of DNA replication within a single cell cycle.

EXPERIMENTAL PROCEDURES

Antibodies—The following antibodies were used in this study: anti-XOrc1 (15), anti-XOrc2 (15), anti-XMcm3 (33), anti-XMcm7 (34); mouse anti-XCdc2 (Santa Cruz Biotechnologies, Santa Cruz, CA), mouse anti-XCdk2 (clone 2H2H15.3, a kind gift from Drs. T. Hunt and J. Gannon); rabbit anti-XCdc2 (a kind gift from Dr. M. Doree), mouse anti-PSTAIR (37), sheep anti-cyclin E (Ref. 38; kind gift from Dr. J. Maller), mouse anti-cyclin E (a kind gift from Drs. T. Hunt and J. Gannon), rabbit anti-cyclin A1 (a gift from Dr. A. Philpott), mouse anti-cyclin A1 (Ref. 39; a kind gift from Drs. T. Hunt and J. Gannon), rabbit anti-cyclin A1 and anti-cyclin A2 (kind gifts from Dr. U. Strausfeld), mouse anti-cyclin B1 and anti-cyclin B2 (kind gifts from Drs. T. Hunt and J. Gannon), and sheep anti-cyclin B1 and anti-cyclin B2 (kind gifts from Dr. J. Maller). Immunoprecipitations and immunoblotting were performed as described (33, 34).

ORC Purification—Xenopus ORC was purified from egg extract high speed supernatant (40) using a modified purification procedure broadly based on published protocols (4, 11, 14, 41). Egg extract was diluted 5-fold in buffer B/50 (buffer B is 50 mM HEPES, pH 7.5, 2 mM MgCl$_2$, 2 mM dithiothreitol, 10% glycerol, 1 μg/ml each of aprotinin, leupeptin, and pepstatin; B/50 is 50 mM KCl in buffer B) and ammonium sulfate was added to 20% saturation. Precipitated material was pelleted by centrifugation and the resulting supernatant was transferred to a new tube. Ammonium sulfate was added to the supernatant up to 35% saturation, and precipitated material was pelleted by centrifugation. The pellet was dissolved in buffer B/0 and loaded on heparin-Sepharose CL6B column (Amersham Pharmacia Biotech). The column was washed in buffer B/200 (200 mM KCl) and eluted in a single step with buffer B/500. The eluate was diluted 4-fold in buffer B to bring the concentration of KCl down to 125 mM and applied to a 1-ml MonoQ column equilibrated in buffer B/125 (Amersham Pharmacia Biotech). The column was washed with buffer B/125 and eluted with a 10-ml linear gradient of KCl (125–600 mM) in buffer B. XOrc1 and XOrc2-containing fractions (210–320 mM KCl) were pooled, diluted with buffer B to achieve KCl concentration of 200 mM, and applied to a 1-ml MonoS column (Amersham Pharmacia Biotech). The column was eluted in exactly the same way as MonoQ except that ORC eluted at approximately 230–320 mM KCl. ORC-containing fractions were pooled, dialyzed against the same buffer, and concentrated to 500 μl in a Vivaspin concentrator (approximately 10-fold). The concentrated material was applied to Superose 6 column equilibrated in buffer B/200.

H1 Kinase Assays—Reactions for co-immunoprecipitation and copurification kinase activities were performed in 50 mM Tris, pH 7.5, 150 mM NaCl, 12 mM MgCl$_2$, 2 mM dithiothreitol, 50 μM ATP, for 30 min at 37 °C in the presence of 1 μg/ml of histone H1 (Roche Molecular Biochemicals) and 10 μCi of [γ-32P]ATP as substrates. Samples were electrophoresed on a 12% denaturing gel, gels were dried and exposed to a Phosphoimager cassette (Molecular Dynamics). Recombinant Cdc2-cyclin B and Cdk2-cyclin E complexes were expressed in SF9 cells from viruses kindly supplied by W. Harper and purified as described (42); baculovirally expressed Cdc2-cyclin A and Cdk2-cyclin A were kind gifts from Drs. Mark Jackman and Jonathan Pines.

RESULTS

Histone H1 Kinase Activity Co-immunoprecipitates with Xenopus ORC—A histone H1 kinase activity co-immunoprecipitates with Xenopus origin recognition complex from the egg extract (Fig. IA). This activity is co-immunoprecipitated with two different antibodies against XOrc1, as well as with two different antibodies against XOrc2. H1 kinase activity is not immunodepleted when egg extract has been immunodepleted of ORC before immunoprecipitation (compare —ORC and complete). This suggests that the presence of H1 kinase activity in the immunoprecipitates is due to its interaction with ORC rather than nonspecific binding to the antibodies or protein A-Sepharose beads. To address the specificity of interaction further, we performed immunoprecipitations with several control antibodies: anti-XMcm3 (M3), anti-XMcm7 (M7), anti-goat IgG (GlgG), and anti-sheep IgG (SlgG). None of these antibodies co-immunoprecipitated substantial amounts of H1 kinase activity from Xenopus egg extract. Thus, we conclude that an H1 kinase activity is specifically associated with the Xenopus origin recognition complex.

To characterize the coimmunoprecipitating kinase further, we have assayed several Cdk protein kinase inhibitors for their ability to inhibit the kinase activity (Fig. 1B). Both 6-dimethylaminopurine (6-DMAP) and olomoucine inhibited the activity at approximately the same concentrations as those required for inhibition of the initiation of DNA replication in Xenopus egg extracts (43, 44). Both 6-DMAP and olomoucine inhibit a wide range of kinases, while showing only a relative preference for Cdkks (44). Therefore, we asked whether the ORC-associated kinase activity was inhibited by p21$^{Cip1}$, a specific inhibitor of Cdk kinases (45–47). As shown in Fig. 1B, the effect of p21$^{Cip1}$ was evident at concentrations equal to or lower than those reported to inhibit replication in egg extracts (23, 24). Therefore, the sensitivity profile of the ORC-associated kinase to a range of inhibitors strongly suggests a Cdk family kinase.

**Xenopus Cdc2 Kinase and Cyclin A1 Copurify and Co-immunoprecipitate with ORC—Xenopus, like yeast, have several Cdk-cyclin complexes which have been implicated in the regulation of DNA replication (21–27). Immunodepletion of Cdc2 and Cdk2 from Xenopus egg extract using p13$^{Cdc2}$ beads or immunodepletion of Cdk2 using anti-Cdk2 antibodies inhibits DNA replication (21, 22). Addition of the Cdk2 kinase inhibitor p21$^{Cip1}$ also has a similar effect (27–30). The replication capacity of the extract can be rescued by re-addition of Cdk2/cyclin E, Cdk2/cyclin A, or Cdk2/cyclin A, as well as cyclin A or cyclin E alone (26). In the Xenopus egg, cyclin A is predominantly associated with p4Cl$^{Cdc2}$ rather than p33$^{Cdc2}$ (48). Furthermore, ablation of cyclin A mRNA (49) or immunodepletion of Cdc2 (22) do not block DNA synthesis in Xenopus egg extract. These results argue that Cdk2/cyclin E appears to be a better candidate for the Cdk-cyclin complex required for the initiation of DNA replication. However, as Cdc2/cyclin A or Cdk2/cyclin A can rescue replication capacity of egg extracts immunodepleted of Cdk2 (21, 26), other Cdk-cyclin complexes can clearly substitute for Cdk2/cyclin E. In addition to inducing initiation, Cdk2/cyclin complexes are likely mediators of other regulatory
functions in DNA replication. Ablation of cyclin A mRNA from *Xenopus* egg extracts has been demonstrated to abolish the dependence of mitosis on completion of DNA replication (49) and cyclin A has been shown to inhibit the activity of the replication licensing factor (43). Furthermore, addition of Cdc2-cyclin a to *Xenopus* egg extracts selectively induces the release of ORC but not MCMs from pre-replicative chromatin (27). Kinases that induce initiation of DNA replication or prevent further initiation events within the same cell cycle would be expected to interact with proteins present at replication origins before initiation. ORC, the eukaryotic initiator protein, is a prime candidate for such an interaction. Therefore, we have asked if the kinase that co-immunoprecipitates with ORC is any of those implicated in the regulation of DNA replication in *Xenopus* egg extracts.

Our attempts to identify the ORC-associated kinase by Western blotting of the immunoprecipitated material failed, presumably due to insufficient amounts of protein obtained by this method. Therefore, we resorted to larger scale biochemical purification of ORC from the egg extract and followed XOrc1, XOrc2, candidate cyclins, and Cdks throughout the purification by Western blotting. ORC was partially purified by ammonium sulfate precipitation followed by heparin-Sepharose, MonoQ, and MonoS chromatography columns as described under “Experimental Procedures.” The partially purified ORC was concentrated and loaded on a Superose 6 gel filtration column. Fig. 2 shows immunoblots across the ORC-containing fractions from this column. Examination of XOrc1 and XOrc2 elution profiles suggests that only a part of XOrc2 is present in a complex with XOrc1, whereas significant amounts of XOrc2 appear to be present in a slightly smaller complex lacking XOrc1. This observation agrees with previously published data demonstrating that immunodepletion of ORC with anti-XOrc1 antibodies failed to immunodeplete XOrc2 completely (15). However, as XOrc1 immunodepletion abolished the ability of egg extract to support DNA replication, the complex lacking XOrc1 cannot perform all the functions required for initiation (14, 15).

We have used anti-PSTAIR antibody to detect both Cdc2 and Cdk2 with similar affinity and to allow quantitation of their relative abundance (35). A 34-kDa PSTAIR-reactive band could be detected in the ORC-containing fractions. In addition, small amounts of a 33-kDa PSTAIR-reactive protein could also be detected. Independent immunoblotting with antibodies specific to p34<sup>Cdc2</sup> or p33<sup>Cdk2</sup> confirmed that the 34-kDa band corresponds to the Cdc2 kinase catalytic subunit (data not shown). Careful examination of the immunoblots indicates that, whereas Cdc2 elutes identically to XOrc1, Cdk2 elutes later, overlapping with the broad XOrc2 peak but not the narrow XOrc1 peak. As shown in Fig. 2, Cdc2 elutes at exactly the same position as XOrc1, whereas very little Cdk2 can be detected in XOrc1-containing fractions. Using an antibody that is specific for *Xenopus* cyclin A1, an immunoblot revealed a peak of cyclin A1 overlapping the peak of XOrc1 and Cdc2. No cyclin B could be detected in XOrc1- or XOrc2-containing fractions. An anti-cyclin E immunoblot revealed a weak peak of cyclin E overlapping the peaks of Cdk2 kinase and XOrc2 but not coin-
cident with Cdc2 or XOrc1. All fractions were independently assayed for H1 kinase activity. The elution profile of histone H1 kinase activity corresponded well with the presence of Cdc2 and Cdk2 kinases detected by Western blotting. We conclude that two kinase complexes, Cdc2-cyclin A1 and Cdk2-cyclin E, cofractionate with XOrc2. However, only one of these, Cdc2-cyclin A1, exactly co-elutes with fractions containing both XOrc1 and XOrc2, whereas the other, Cdk2-cyclin E, is absent from fractions containing XOrc1. Cyclin A2 is not expressed in Xenopus stage 2 embryos until stage 10 (50). The absence of cyclin A2 from egg extracts and ORC-containing fractions was confirmed by immunoblotting with cyclin A2-specific antibodies (kind gift of Dr. Uli Strausfeld; data not shown).

To obtain further evidence for interaction between ORC and Cdc2 or Cdk2, we performed immunoprecipitations from the individual Superose 6 fractions with anti-XOrc1 and anti-XOrc2 antibodies and assayed the immunoprecipitated material for the presence of H1 kinase activity, Cdc2 and Cdk2 (Fig. 3). Both antibodies co-immunoprecipitated XOrc1, XOrc2, and Cdc2 kinase but not detectable Cdk2 kinase. The pattern of bands obtained with anti-PSTAIR antibody was identical to that on anti-Cdc2 immunoblots, ruling out the possibility that failure to detect Cdk2 was due to different sensitivities of anti-Cdc2 and anti-Cdk2 antibodies. In agreement with immunoblottting results, the peak of H1 kinase activity overlapped exactly with the presence of Cdc2 kinase (Fig. 3). Thus, Cdc2 kinase rather than Cdk2 kinase is tightly associated with Xenopus origin recognition complex in the egg extract.

Independent evidence for this interaction was obtained from reciprocal immunoprecipitations with antibodies directed against Xenopus Cdc2 and cyclin A1. As shown in Fig. 4, both anti-Cdc2 and anti-cyclin A1 antibodies, but not control antibodies, co-immunoprecipitated significant amounts of XOrc1 and XOrc2 from the pool of ORC-containing fractions from the MonoS column (see “Experimental Procedures”). In summary, these results provide evidence that a soluble complex between ORC and Cdc2-cyclin A1 exists in Xenopus egg extracts.

Several recombinant Cdk-cyclin complexes were tested for their ability to phosphorylate recombinant XOrc2 in vitro. As shown in Fig. 5A, all Cdc2- and Cdk2-containing complexes tested were able to phosphorylate XOrc2. However, XOrc2 was phosphorylated by Cdc2-cyclin A with approximately 4-fold higher efficiency than by Cdk2-cyclin B or Cdk2-containing complexes (Fig. 5B), and of course, this is the same complex that interacts with ORC in Xenopus egg extracts (Figs. 2 and 4). The H1 kinase activity co-immunoprecipitating with ORC is sensitive to p21Cip1. p21Cip1 is known to inhibit efficiently Cdk4-cyclin D, Cdk2-cyclin A, and Cdk2-cyclin E complexes and weakly Cdc2-cyclin B, but its activity against Cdc2-cyclin A has not been reported so far (44–47). As shown in Fig. 5C, p21Cip1 efficiently inhibits the kinase activity of recombinant Cdc2-cyclin A toward histone H1 and XOrc2 used as substrates.

**DISCUSSION**

In this report, we have used three different approaches to identify a direct physical interaction between the Xenopus origin recognition complex and Cdc2-cyclin A1. First, immunoprecipitations of Xenopus ORC with highly specific antibodies against XOrc1 or XOrc2 co-precipitates Cdc2-cyclin A1. Likewise, anti-Xenopus Cdc2 or anti-Xenopus cyclin A1 antibodies specifically co-precipitate XOrc1 and XOrc2. In contrast, antibodies against XMcM3, XMcm7, sheep IgG, or goat IgG fail to co-precipitate Cdc2-cyclin A1. In the second approach, Cdc2-cyclin A1 co-fractionates with ORC over several fractionation steps including ion exchange and gel filtration. Finally, in the third approach, XOrc2 is shown to be phosphorylated most efficiently by recombinant Cdc2-cyclin A1 in comparison to recombinant Cdc2-cyclin B and Cdk2 complexes. These results argue that the interaction between Xenopus ORC and Cdc2-cyclin A1 is highly specific.
We also report that histone H1 kinase activity is associated with the ORC-Cdc2-cyclin A1 complex following both fractionation and co-immunoprecipitation. This kinase activity is sensitive to the cyclin-dependent kinase inhibitors 6-DMP, olomoucine, and p21Cip1. Interestingly, antibodies against Cdk2, cyclin E, and cyclin B show an absence of these proteins from the ORC-Cdc2-cyclin A1 complex. From these results it can be concluded Cdc2-cyclin A1 accounts for the majority of the H1 kinase activity that both co-immunoprecipitates and co-fractionates with ORC.

We have not directly identified the function of the ORC-associated kinase, but it is a very strong candidate for one specific role. As outlined in the Introduction, two major roles are known for protein kinases in the control of DNA replication. The first is S phase promoting activity. However, the fact that cyclin A ablation does not inhibit DNA replication in the extract (49) appears to point to the Cdk2-cyclin E complex as the prime candidate for an S phase promoting activity. It also appears to argue against the possibility that ORC-associated kinase identified in this paper triggers the initiation of DNA replication by phosphorylating proteins associated with ORC or ORC itself. The second known major role of a protein kinase in controlling DNA replication is the prevention of re-initiation of replication within a single cell cycle. Conditional mutations of cdc2 and deletion of cdc13, the B-type cyclin in Schizosaccharomyces pombe, promote over-replication (51, 52). Similarly, inhibition of protein kinases in G2 in mammalian cells makes them competent to replicate again (53, 54) and causes MCM cyclin-dependent kinase throughout S and G2 appears to be actively prevented by the accumulation of cyclin A or E in sperm

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