Cdc7–Drf1 is a developmentally regulated protein kinase required for the initiation of vertebrate DNA replication

Tatsuro S. Takahashi and Johannes C. Walter1

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, USA

Cdc7, a protein kinase required for the initiation of eukaryotic DNA replication, is activated by a regulatory subunit, Dbf4. A second activator of Cdc7 called Drf1 exists in vertebrates, but its function is unknown. Here, we report that in Xenopus egg extracts, Cdc7–Drf1 is far more abundant than Cdc7–Dbf4, and removal of Drf1 but not Dbf4 severely inhibits phosphorylation of Mcm4 and DNA replication. After gasastern, when the cell cycle acquires somatic characteristics, Drf1 levels decline sharply and Cdc7–Dbf4 becomes the more abundant kinase. These results identify Drf1 as a developmentally regulated, essential activator of Cdc7 in Xenopus.

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Eukaryotic chromosomal DNA replication initiates via the ordered recruitment of replication proteins onto chromatin (for review, see Bell and Dutta 2002). In the G1 phase, ORC, Cdc6, and Cdt1 cooperate to load the Mcm2–7 complex onto chromatin, resulting in formation of the prereplication complex (pre-RC). The Mcm2–7 complex is believed to be the replicative DNA helicase (for review, see Takahashi et al. 2005). Activation of the Mcm2–7 helicase at the G1/S transition is a crucial step for initiation, which requires multiple factors, including Cdc7 protein kinase as well as Cdc45, a likely helicase cofactor (Pacek and Walter 2004).

Accumulating evidence suggests that a crucial substate of the Cdc7–kinase is the Mcm2–7 complex. Mcm2, Mcm3, Mcm4, and Mcm6 are in vitro substrates of the Cdc7–kinase in yeast and humans (Lei et al. 1997; Sato et al. 1997; Brown and Kelly 1998; Jares et al. 1999; Kumagai et al. 1999; Weinreich and Stillman 1999; Masaai et al. 2000; Lee et al. 2003). In Xenopus and yeast, phosphorylation of Mcm2 on chromatin during replication is dependent on Cdc7 (Lei et al. 1997; Jares and Blow 2000; Takeda et al. 2001). Finally, in budding yeast, a point mutation in Mcm5 renders Cdc7–Dbf4 dispensable for viability, further pointing to Mcm2–7 as a crucial Cdc7 target (Hardy et al. 1997). However, functionally important Cdc7 phosphorylation sites remain to be identified.

The activity of Cdc7-kinase is regulated by an activator subunit, Dbf4. Cdc7–Dbf4 has therefore been referred to as DDK (Dbf4-dependent kinase) (for review, see Johnston et al. 1999). Recently a Dbf4-related protein, Drf1, was found in humans and Xenopus (Montagnoli et al. 2002; Yanow et al. 2003; Yoshizawa-Sugata et al. 2005), and the question arises as to why vertebrates contain only two regulators of Cdc7. In Xenopus, Dbf4 activates the Cdc7-kinase in vitro, and recombinant Cdc7–Dbf4 can stimulate DNA replication when added to Xenopus egg extracts (Jares and Blow 2000; Furukohri et al. 2003, Shechter et al. 2004a). However, attempts to address the role of Dbf4 in DNA replication in egg extracts were confounded by nonspecific inhibition of DNA replication by the Dbf4 antibodies used (Jares et al. 2004). In turn, depletion of Drf1 showed no effect on DNA replication (Yanow et al. 2003). Therefore, the regulatory subunit that accounts for the essential role of Cdc7 (Jares and Blow 2000; Walter 2000) in Xenopus eggs remains elusive. In humans, both Dbf4 (also called ASK) and Drf1 (also called ASK1) activate Cdc7-kinase in vitro (Jiang et al. 1999; Kumagai et al. 1999; Montagnoli et al. 2002, Yoshizawa-Sugata et al. 2005). In human tissue culture cells, inhibition of Dbf4 via antibody interference blocks DNA replication, whereas inhibition of Drf1 via RNA interference (RNAi) slows S-phase progression (Kumagai et al. 1999; Yoshizawa-Sugata et al. 2005). Collectively, the available data suggest that Dbf4 may have a more important role for the initiation of DNA replication than Drf1, but the precise functions of Dbf4 and Drf1 have not been determined.

In this article, we compared directly the properties of Cdc7–Drf1 and Cdc7–Dbf4 in Xenopus egg extracts and during early Xenopus development. The data support the surprising conclusion that Cdc7–Drf1 plays a far more prominent role than Cdc7–Dbf4 in promoting DNA replication during the early embryonic cell cycles.

Results and Discussion

To study the mechanism of DNA replication in Xenopus egg extracts, we use two related systems which differ in their requirement for nuclear envelope assembly (Supplementary Fig. S1). In the nucleus-dependent system, sperm chromatin is added to a low speed supernatant (CSS) of egg cytoplasm that is depleted of membrane precursors stimulates pre-RC formation, nuclear envelope assembly, and DNA replication. In the nucleus-independent system, a high-speed supernatant (HSS) of egg cytoplasm that is depleted of membrane precursors stimulates pre-RC formation on added DNA templates (plasmid or sperm chromatin). Subsequent addition of a nucleoplasmic extract (NPE) stimulates DNA replication in the absence of nuclear envelope assembly.

We raised antibodies against Xenopus Dbf4 and Drf1 and examined their abundance in egg extracts. Both antibodies recognized phosphorylated and unphosphorylated forms of their respective antigen in LSS and NPE (Fig. 1A). Densitometric quantification of Western blots demonstrated that the concentration of Drf1 in LSS is 22 nM, whereas the concentration of Dbf4 is only 1.2 nM (Supplementary Fig. S2A,B). Furthermore, Drf1 is en-
Figure 1. Cdc7–Drf1 is far more abundant than Cdc7–Dbf4 in *Xenopus* egg extracts. [A] Characterization of anti-Drf1 and Dbf4 antibodies: 0.2 µL LSS (lane 1), 0.2 µL λ-protein phosphatase-treated LSS (lane 2), 0.04 µL NPE (lane 3), and 0.04 µL λ-protein phosphatase-treated NPE (lane 4) were separated by SDS-PAGE and probed with anti-Dbf4 (top panel) or Dbf4 (bottom panel) antibodies alongside 500 pg of recombinant Drf1 (6XHis and StrepII-tagged) (lane 5, top panel) or 20 pg recombinant Dbf4 (6XHis-tagged) (lane 5, bottom panel). Asterisks indicate cross-reacting bands. (B, C) Cdc7 complexes were immunoprecipitated from LSS by control (lane 1), anti-Cdc7 (lane 2), anti-Dbf4 (lane 3), or anti-Drf1 (lane 4) antibodies, and the supernatant (B) and immunoprecipitate (C) fractions were probed with antibodies against Drf1 (top panels), Dbf4 (middle panels), and Cdc7 (bottom panels). Cross-reacting bands [*] serve as loading controls. In B and later figures involving Dbf4 blotting of LSS, extracts were treated with phosphatase due to a cross-reacting band migrating with phosphorylated Dbf4.

riched ~12-fold in NPE relative to LSS, whereas Dbf4 is enriched only threefold [Supplementary Fig. S2C]. Consistent with this different degree of nuclear enrichment, the concentrations of Drf1 and Dbf4 in NPE were estimated as 250 and 3.6 nM, respectively [Supplementary Fig. S2B]. Thus, Drf1 is ~18-fold more abundant in LSS and ~70-fold more abundant in NPE than Dbf4 [results summarized in Supplementary Table S1].

We next examined the relative abundance of Cdc7–Dbf4 and Cdc7–Drf1 complexes in egg extracts using reciprocal immunoprecipitation (IP). IP of Cdc7 removed all detectable Drf1 and Dbf4 from extracts, showing that all the Drf1 and Dbf4 associate with Cdc7 [Fig. 1B,C, lane 2]. Conversely, IP of Dbf4 coprecipitated only a small amount of Cdc7 [Fig. 1B,C, lane 3], whereas IP of Drf1 removed ~70% of Cdc7 from LSS [Fig. 1B,C, lane 4]. Therefore, the majority of Cdc7 is in a complex with Drf1. The data indicate that the concentration of Cdc7–Drf1 and Cdc7–Dbf4 are essentially the same as that of each regulatory subunit, 22 nM and 1.2 nM, respectively. About 30% of Cdc7 remained in the supernatant after Dbf4–Drf1 double depletion, indicating that 30% of Cdc7 (~9 nM) binds neither Drf1 nor Dbf4 in LSS [see Fig. 4D, below]. We obtained essentially the same results in NPE, except that the ratio was further biased in favor of Cdc7–Drf1 [Supplementary Fig. S3]. In summary, Cdc7–Drf1 is by far the predominant form of DDK in *Xenopus* egg extracts, especially in nuclei, where DNA replication takes place.

Due to its high abundance, we revisited the role of Cdc7–Drf1 in DNA replication. As shown in Figure 2, depletion of Drf1 reduced the initial rate of DNA replication approximately sevenfold compared with the mock depletion. Addition of recombinant Cdc7–Drf1 [rCdc7–Drf1] to the depleted extract at 40 nM final concentration restored replication to nearly control levels [Fig. 2, filled circles], whereas rCdc7–Drf1 did not significantly enhance DNA replication of the mock-depleted extract [Fig. 2, open circles]. Furthermore, we found that recombinant Drf1 alone could rescue DNA replication in Drf1-depleted LSS [Supplementary Fig. S4]. This result is consistent with data presented in Figure 1, which indicate that a significant fraction of Cdc7 (30% in LSS) is not bound to any known regulatory subunits. Our data show that Cdc7–Drf1 is specifically required for efficient DNA replication in *Xenopus* egg extracts. Previous attempts to functionally deplete Drf1 from LSS [Yanow et al. 2003] were likely unsuccessful due to the high concentration of this protein present in the extract.

Previous results showed that immunodepletion of Cdc7 impairs replication initiation, since origin unwinding, chromatin loading of Cdc45, and Mcm2 phosphorylation are reduced [Jares and Blow 2000; Walter 2000]. To determine whether Drf1 is required for replication initiation, we performed an origin unwinding assay in the nucleus-free replication system [Walter and Newport 2000]. Thus, when DNA replication is initiated on a circular plasmid in the presence of the DNA polymerase inhibitor aphidicolin, an Mcm2–7-dependent helicase is uncoupled from the replication fork and a highly unwound form of the plasmid called U-form DNA is generated [Pacek and Walter 2004; Shechter et al. 2004b]. While mock-depleted HSS and NPE supported hyper-unwinding, extracts depleted of Drf1 did not [Fig. 3A, cf. lanes 3–5 and 9–11]. The complete absence of U-form DNA is consistent with the fact that Drf1 depletion completely blocked replication in NPE [data not shown]. Importantly, rCdc7–Drf1 restored generation of U-form DNA [Fig. 3A, lanes 12–14]. These data indicate that Cdc7–Drf1 is required for activation of the replicative DNA helicase.

We next asked whether Cdc7–Drf1 is required for chromatin loading of Cdc45. Compared with the mock-depleted control, Drf1 depletion significantly impaired Cdc45 loading onto sperm chromatin in LSS, and rCdc7–
Drf1 is essential for DNA replication

Figure 3. Cdc7–Drf1 is required for initiation of DNA replication. (A) Drf1 is required for origin unwinding. pBluescriptII was incubated in mock-depleted (lane 1) or Drf1-depleted (lane 2) HSS at 40 ng/µL concentration for 30 min and isolated, or further incubated in mock-depleted [lanes 3–8] or Drf1-depleted [lanes 9–14] NPE containing 50 µg/mL aphidicolin and buffer (lanes 3–5,9–11) or 500 nM rCdc7–Drf1 [lanes 6–8,12–14]. At the indicated times, the plasmid topology was analyzed by agarose gel electrophoresis in the presence of 1.8 µM chloroquine. (B) Requirement of Drf1 for Mcm4 phosphorylation and Cdc45 loading. Sperm chromatin was incubated in mock-depleted (lane 1) Drf1 is required for origin unwinding. pBluescriptII was incubated in mock-depleted (lane 2) or Drf1-depleted (lanes 3–8,9–14) or 50 nM rCdc7–Drf1 [lanes 3,4,7,8] and recovered through a sucrose cushion at the indicated time points. Bound proteins were eluted and analyzed by Western blotting using the indicated antibodies. (*) Cross-reacting band.

Drf1 reversed the loading defect [Fig. 3B, panel II, lanes 3,4]. Mcm4 phosphorylation [Fig. 3B, panel II, lanes 3,4]. Finally, we show that Mcm4 phosphorylation correlates closely with Cdc7–Drf1 chromatin loading, and that it immediately precedes Cdc45 recruitment [Supplementary Fig. S5B]. The results are consistent with the idea that Cdc7–Drf1-dependent phosphorylation of Mcm4 is an important step in Cdc45 recruitment to chromatin.

As shown previously [Yanow et al. 2003], Drf1 depletion also inhibited chromatin association of Cdc7 [Fig. 3B, panels III,V,V, lanes 5,6]. However, chromatin association of Dbf4 was unaffected by Drf1 depletion, indicating that chromatin binding of Cdc7–Dbf4 is independent of Cdc7–Drf1 [Fig. 3B, panel IV, cf. lanes 1,2 and 5,6]. Consistent with this, we detected weak binding of Cdc7 but not Drf1 in a longer exposure of the same membrane [Fig. 3B, panels VI, VII, and this residual Cdc7 binding was Dbf4 dependent, since it was absent in extracts depleted of Drf1 and Dbf4 (data not shown).

We next addressed whether Cdc7–Dbf4 is required for DNA replication in Xenopus egg extracts. Our Dbf4 antibodies removed at least 99% of Dbf4 from LSS [Fig. 4A], reducing the Dbf4 concentration to at most ~12 pM. However, there was no significant effect on DNA replication [Fig. 4B], Mcm4 phosphorylation [Fig. 4C], or the chromatin loading of Cdc45, Drf1, or Cdc7 [Fig. 4C]. Assuming that replication initiation occurs approximately every 10 kb on sperm chromatin (3 × 109 bp), in our experimental conditions (3000 sperm/µL), there are 9 × 109 origins per microliter extract. At a concentration of 12 pM, there is only one Cdc7–Dbf4 complex available for approximately every 125 origins that are normally activated. The effective concentration of Cdc7–Dbf4 is probably even lower, since only ~10% of Dbf4 normally localizes to nuclei [see Supplementary Table S1]. Since it is unlikely that a single Cdc7–Dbf4 complex could rapidly activate >100 origins, the data suggest that Cdc7–Dbf4 is not normally required for DNA replication in Xenopus egg extracts.

To test whether Dbf4 might account for the residual DNA replication observed in Drf1-depleted extracts, we depleted Drf1 and Dbf4 simultaneously and compared replication with Drf1-depleted extracts. Western blotting showed that all detectable Dbf4 and Drf1 were removed by the double depletion [Fig. 4D]. As shown above, Drf1 depletion significantly reduced DNA replication [Fig. 4E, filled squares], but depletion of Dbf4 caused no further decrease [Fig. 4E, filled triangles], suggesting that the residual replication activity seen in Drf1-depleted extracts is not due to Dbf4. It is possible that the remaining DNA replication seen in Drf1/Dbf4-depleted extracts is due to a third Cdc7 regulatory subunit that remains to be identified, or to a low level of Cdc7-independent origin firing. Interestingly, as is the case for Drf1, depletion of Cdc7 resulted in extracts that supported residual DNA replication (data not shown). However, as for Drf1 depletion, we are unable to determine whether this residual replication is due to trace amounts of Cdc7 remaining in the extract, or due to a low level of Cdc7-independent origin firing.

Since Dbf4 depletion had no effect on DNA replication in any context, the question arose whether Xenopus Cdc7–Dbf4 has the capacity to stimulate DNA replication. Previous results showed that addition of Cdc7–Dbf4 to mock-depleted or Cdc7-depleted egg extracts could boost DNA replication [Jares et al. 2004; Shechter et al. 2004a]. Consistent with this, Figure 4F shows that
the defect in DNA replication caused by Drf1 deletion was completely reversed by recombinant Cdc7–Dbf4 complex. Therefore, while Cdc7–Dbf4 has the ability to promote DNA replication, endogenous Cdc7–Dbf4 in Xenopus egg extracts does not appear to contribute to DNA replication, probably due to its very low concentration, and possibly due to inefficient nuclear transport [Supplementary Table S1] and/or chromatin binding [Fig. 3B].

To extend the comparison between Dbf4 and Drf1, we examined how they are recruited to chromatin. Like Drf1 [Yanow et al. 2003], and in contrast to previous reports [Furukohri et al. 2003; Jares et al. 2004], Dbf4 bound to chromatin in a manner that was pre-RC-dependent [Supplementary Fig. S6]. Moreover, like Dbf4 [Jares et al. 2004], Drf1 was essential to recruit Cdc7 to chromatin [Supplementary Fig. S7]. Therefore, both Drf1 and Dbf4 not only function as Cdc7 activating proteins, but also recruit Cdc7 to its likely site of action, the pre-RC.

Our data indicate that in Xenopus egg extracts, Cdc7–Drf1 is critical for DNA replication while Cdc7–Dbf4 is dispensable, yet antibody injection experiments suggest that Dbf4 is essential for DNA replication in somatic cells [Kumagai et al. 1999]. One way to reconcile these findings would be if the contributions to DNA replication of Drf1 and Dbf4 were developmentally regulated, being inverted between embryonic and somatic cells. To test this possibility, we asked whether levels of Cdc7–Drf1 and Cdc7–Dbf4 change during development.

Upon fertilization, Xenopus eggs undergo 12 rapid, ~25-min cell divisions which lack gap phases. Following the 12th division, at a time known as the mid-blastula transition (MBT), the cell cycle is extended and becomes asynchronous, and zygotic transcription starts [Newport and Kirschner 1982a,b]. Three divisions after the MBT, at the early-gastrula transition (EGT), maternal mRNAs are degraded and zygotic transcription assumes control of the cell cycle [Newport and Dasso 1989; Howe et al. 1995]. As a result, the cell cycle is dramatically expanded and it takes on somatic characteristics.

To examine Cdc7–Drf1 and Cdc7–Dbf4 levels during development, we fertilized eggs in vitro. Embryos were collected at the indicated time points and staged [Nieuwkoop and Faber 1967], and Cdc7 was immunoprecipitated. Cdc7 recovery was similar at all developmental stages [Fig. 5A]. Cdc7, Drf1, and Dbf4 were all hypermodified in meiotically arrested eggs [Fig. 5A, lane 1]. Intriguingly, Cdc7-associated Drf1 levels began to drop just before the onset of gastrulation [stage 9], when the cell cycle becomes somatic in nature, and they became undetectable at the neurula stage [stage 20]. Conversely, levels of Dbf4 associated with Cdc7 gradually went up during the first 12 h of development, and they further increased significantly between 12 and 24 h, during which time all detectable Cdc7–Drf1 disappeared.

For a more quantitative comparison of Cdc7–Dbf4 and Cdc7–Drf1 levels during development, we performed reciprocal IPs using Cdc7, Dbf4, and Drf1 antibodies at stages 3 and 11. As seen above, IP of Cdc7 at stage 3 coprecipitated significant amounts of Drf1 [Fig. 5B, lane 2]. Conversely, IP of Drf1 precipitated most of the Cdc7, as seen by comparison with the amount of Cdc7 recovered in the Cdc7-IP [Fig. 5B, cf. lanes 4 and 2]. In contrast, IP of Dbf4 precipitated only a small amount of Cdc7 at this stage [Fig. 5B, lane 3]. Therefore, Cdc7–Drf1 is still the predominant DDK at stage 3. At stage 11, IP of Cdc7 coprecipitated far less Drf1 and a significantly greater amount of Dbf4 than at stage 3 [Fig. 5B, cf. lanes 6 and 2]. Moreover, at this stage, Dbf4 coprecipitated slightly more Cdc7 than did Drf1, indicating that the concentration of Cdc7–Dbf4 is now greater than Cdc7–Drf1 [Fig. 5B, lanes 7, 8]. Western blotting of the total extracts revealed that the IPs removed the vast majority of each target protein, and that the total level of Drf1 severely decreased, while that of Dbf4 significantly increased between stage 3 and stage 11 [Supplementary Fig. S8]. Together, these results argue that the ratio of Cdc7–Drf1 and Cdc7–Dbf4 undergoes a reversal after gastrulation.

In summary, our data indicate that Cdc7–Drf1 plays an essential role in DNA replication in the early embryonic cell cycles. Given the ability of Drf1 to support DNA replication and Cdc7–Dbf4 complex, these data suggest that they contribute differentially to DNA replication in somatic and embryonic cells.
Drf1 is essential for DNA replication

Materials and methods

Extracts, DNA replication, and chromatin binding

Extract preparation, replication assays, and chromatin-binding assays in the nucleus-free replication system were carried out as described (Walter et al. 1998; Walter and Newport 2000). Sperm nuclei were incubated in HSS at 10,000/µL in all experiments. In LSS (Blow 1993), sperm nuclei were incubated at 3000/µL in all experiments, and chromatin isolation and replication were carried out as described previously (Takahashi et al. 1998; Walter and Newport 2000). Sperm nuclei were incubated in the nucleus-free replication system were carried out as described (Walter et al. 1998; Walter and Newport 2000). Sperm nuclei were incubated in HSS at 10,000/µL in all experiments. In LSS (Blow 1993), sperm nuclei were incubated at 3000/µL in all experiments, and chromatin isolation and replication were carried out as described previously (Takahashi et al. 2004). The amount of DNA synthesis was calculated using the method described by Blow and Laskey (1986), which takes into account the concentration of endogenous dNTPs. Endogenous dNTP concentration was measured for each fresh extract, and for immunodepleted extracts, this number was multiplied by 0.8, the experimentally determined dilution factor that results from the immunodepletion procedure. For phosphorylation, 1 µL of extract was incubated with 400 U of λ protein phosphatase (New England Biolabs) containing 2 mM MnCl₂ at 30°C for 30 min. Other experimental procedures are described in the Supplemental Material.

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Tatsuro S. Takahashi and Johannes C. Walter

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