Ciz1 cooperates with cyclin-A–CDK2 to activate mammalian DNA replication in vitro

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Summary

Initiation of mammalian DNA replication can be reconstituted from isolated G1-phase nuclei and cell extracts, supplemented with cyclin-dependent protein kinases (CDKs). Under these conditions, cyclin E supports pre-replication complex assembly, whereas cyclin-A-associated kinase acts later to terminate assembly and activate DNA replication. The mechanism by which these events are coordinated is unknown. Here, we show that the replication factor Ciz1 interacts with cyclins E and A sequentially through distinct cyclin-binding motifs. Cyclin A displaces cyclin E from Ciz1 in a manner that is dependent on functional domains that are essential for its role in DNA replication. Furthermore, in cell-free assays, recombinant cyclin-A–CDK2 complexes and recombinant Ciz1 cooperate to promote initiation of DNA replication in late G1-phase nuclei. In addition, Ciz1 supports immobilization of cyclin A in isolated nuclei and depletion of Ciz1 by RNAi impairs immobilization, suggesting that Ciz1 promotes initiation by helping to target the kinase to a specific subnuclear compartment. We propose that Ciz1 acts to coordinate the functions of cyclins E and A in the nucleus, by delivering cyclin-A-associated kinase to sites that are specified by cyclin E, helping to ensure that they execute their functions in the same place and in the correct order.

Key words: Cell-free system, Ciz1, Cyclin, Mammalian DNA replication

Introduction

Initiation of eukaryotic DNA replication is a multistep process that involves the assembly of pre-replication complexes (pre-RCs) at selected origins, followed by recruitment and activation of the DNA replication machinery. The initiation process can be reconstituted in vitro using nuclei isolated from G1-phase mammalian cells as a template, combined with extracts from S-phase cells (Krude et al., 1997; Stoeber et al., 1998). Furthermore, by using G1-phase extract supplemented with recombinant cyclin-dependent kinases (CDKs), it is possible to reconstitute the role of CDKs in the initiation process (Coverley et al., 2002). Under these conditions, cyclin E and cyclin A contribute to distinct and sequential steps. This and other evidence implicates cyclin E in pre-RC assembly, acting in concert with the assembly factors CDC6 and Cdt1 to support the recruitment of the minichromosome maintenance (MCM) protein complex (Su and O'Farrell, 1998; Furstenthal et al., 2001; Cook et al., 2002; Geng et al., 2007). In vitro, the cyclin-A–CDK2 complex appears to play multiple roles, first terminating the assembly stage by phosphorylating and inactivating CDC6 and then, as its levels rise in late G1, activating the DNA replication machinery. Reversal of the natural order in which nuclei are exposed to cyclins E and A blocks initiation in vitro (Coverley et al., 2002), highlighting the rigid requirement for their sequential activity and their specialized roles in the temporally separated assembly and activation steps in DNA replication of mammalian cells.

The initiation process is not only controlled temporally by CDKs, but also spatially constrained within the confines of the eukaryotic cell nucleus. This level of organization appears to be achieved by association with an insoluble nuclear framework and involves aggregation of multiple replication forks into immobilized electron-dense bodies, or replication factories (Hozak et al., 1993). Compelling evidence indicates that replication origins, newly synthesized DNA, replication intermediates (Berezney and Coffey, 1975; Dijkwel et al., 1979) and even the machinery that supports cell-cycle-regulated initiation (Radichev et al., 2005) all associate with nuclease-resistant structures referred to as the nuclear matrix. In mammalian cells, the pre-RC protein ORC1 (part of the six-subunit origin recognition complex or ORC) appears to undergo transient association with the nuclear matrix (Fujita, 1999) and its appearance in the cell cycle correlates with recruitment of the rest of the ORC complex (Tatsumi et al., 2003). This suggests that origins become associated during pre-RC assembly. Despite this and other evidence (Djeliova et al., 2001), the recruitment process and the factors involved are not well understood.

Ciz1 (Cip1-interacting zinc-finger protein 1) offers a molecular link between DNA replication and the nuclear matrix. Endogenous Ciz1 is localized within nuclease-resistant nuclear foci that overlap with newly synthesized DNA in a proportion of early S-phase nuclei, whereas recombinant Ciz1 becomes recruited to nuclease-resistant structures through C-terminal interaction domains in late G1 and early S phase (Ainscough et al., 2007). Furthermore, in support of an active role in DNA replication, recombinant Ciz1 promotes initiation in vitro, whereas RNAi-mediated depletion of newly synthesized Ciz1 inhibits S-phase entry (Coverley et al., 2005). Here, we show that Ciz1 associates specifically and sequentially with key regulators of the initiation process, cyclins E and A, and that it cooperates with cyclin-A–CDK2 to activate the DNA replication machinery in vitro. Taken together, the evidence suggests that Ciz1 might link the structures that organize DNA replication in nuclear space with the temporal regulators that control their activity within the cell cycle.

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Results

Ciz1 cooperates with cyclin-A–CDK2 to promote initiation of mammalian DNA replication in vitro

Previously, we reported that recombinant Ciz1 and an embryonic splice variant (designated ECiz1) promote initiation of mammalian DNA replication in cell-based and cell-free assays (Coverley et al., 2005). Typically, these cell-free analyses use 'replication-competent' nuclei that are derived from post-restriction point, late G1-phase cells in which pre-RCs have assembled in preparation for S phase. For the 3T3 cells used here, such populations are enriched 17 hours after release (Fig. 1A). Notably, the response of replication-competent nuclei to recombinant ECiz1 is strongly concentration dependent, peaking around 1 nM and disappearing by 10 nM (Coverley et al., 2005) (Fig. 1B). In a separate set of experiments, we also showed that recombinant cyclin-A–CDK2 is also capable of activating DNA synthesis in replication-competent nuclei (Coverley et al., 2002). When titrated into protein extracts with low endogenous cyclin A (prepared from cells 15 hours after release from quiescence), recombinant cyclin-A–CDK2 increases the number of nuclei that initiate DNA replication in vitro. As with Ciz1, this occurs within a narrowly defined range of concentrations, peaking around 0.1 ng/μl (13 nM) (Fig. 1C). Here, we demonstrate the combinatorial effects of Ciz1 and cyclin-A–CDK2 on replication-competent nuclei and propose a mechanism by which they could cooperate to initiate DNA synthesis in vitro.

Inclusion of 1 nM ECiz1 in cyclin-A–CDK2 titration experiments alters the response of replication-competent nuclei across the concentration gradient. Instead of peaking and falling as expected, replication-competent nuclei engage in DNA synthesis in the presence of much lower levels of cyclin-A–CDK2 and continue to respond even at tenfold higher concentrations (Fig. 1C). Thus, recombinant ECiz1 dramatically broadens the concentration range within which cyclin-A–CDK2 can activate DNA synthesis. We postulate that this reflects targeting of cyclin-A–CDK2. Perhaps Ciz1 helps to direct cyclin-A–CDK2 towards substrates that promote initiation and away from substrates whose phosphorylation inhibits initiation. To begin to address this possibility, we asked whether recombinant Ciz1 influences the subcellular localization of cyclin-A–CDK2, under conditions in which it promotes initiation. In fact, using 1nM ECiz1 (peak concentration), recruitment of recombinant cyclin-A–CDK2 (0.1 ng/μl) into the detergent-resistant nuclear compartment was significantly boosted, so that nearly twice as much cyclin A was immobilized than when ECiz1 was not present in the reaction (Fig. 1D). Notably, in the presence of tenfold more ECiz1, recruitment of cyclin A was not substantially affected and DNA synthesis was not stimulated (Fig. 1B,D). These results begin to suggest that Ciz1 cooperates with cyclin-A–CDK2 to support its functional assembly within nuclei and raise the question of whether they interact directly.

Ciz1 interacts directly with cyclin A and cyclin E

In immunoprecipitation experiments with anti-Ciz1 antibody 1793 (Coverley et al., 2005), cyclin A was successfully recovered from replication extracts through interaction with either recombinant ECiz1 or endogenous Ciz1 (Fig. 2A). Under identical conditions, Ciz1 also forms complexes with recombinant cyclin E (Fig. 2B). Thus, Ciz1 can bind to both cyclins in the context of protein extracts that support DNA replication in vitro. To determine whether they interact directly, we used purified recombinant ECiz1 or derived fragments that were immobilized, using a GST tag, on glutathione beads. In binding studies, ECiz1 stably interacted with purified

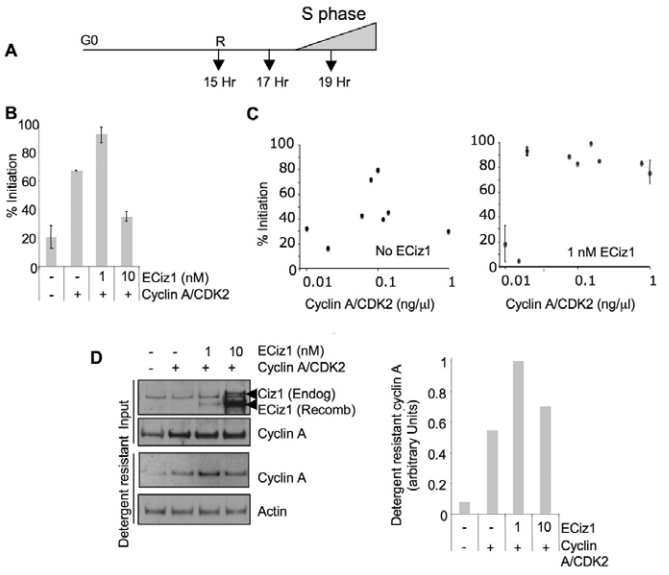

Fig. 1. Ciz1 supports immobilization of cyclin A within G1 nuclei and promotes initiation of DNA replication in vitro. (**A**) Experimental overview of cell synchrony, illustrating time points after release from quiescence (G0) when nuclei and extracts were harvested. R is restriction point. The triangle represents cells entering S phase. (**B**) Cell-free replication experiments showing the effect of 0.1 ng/μl cyclin-A–CDK2 on initiation of DNA replication in late G1 nuclei (17 hours) incubated in mid G1 extracts (15 hours) in the absence and presence of 1 nM and 10 nM ECiz1. (**C**) Cell-free replication in the presence of varying cyclin-A–CDK2 concentrations without (left panel) or with (right panel) ECiz1 (1 nM). Data are presented with standard error for key data points and show that Ciz1 dramatically broadens the concentration range over which cyclin-A–CDK2 can promote DNA synthesis in vitro. (**D**) Late G1 nuclei were incubated in mid G1 extracts, supplemented with recombinant cyclin-A–CDK2 and ECiz1 as in B. Immobilised cyclin A recovered in the detergent-resistant nuclear fraction was detected by western blot. Actin levels are included to show equivalent recovery of nuclei. The histogram shows relative band intensity for cyclin A normalized to actin.

recombinant cyclin A (Fig. 2C) and purified recombinant cyclin E (Fig. 2D), regardless of whether they were precomplexed with CDK2. Human Ciz1 has previously been shown to interact with CDK2 (Mitsui et al., 1999; den Hollander and Kumar, 2006); however, our data show clearly that Ciz1 can also interact directly with cyclins. In fact, the C-terminal Ciz1 sequences that were reported to support interaction with CDK2 are not present in the truncated forms of ECiz1 (fragments N471 and N391) that bind to recombinant cyclins in our assays (Fig. 2C-E and below), supporting the conclusion that Ciz1 interacts with cyclins E and A directly. Recovery of CDK2 by truncated ECiz1 appears to reflect indirect interaction mediated through cyclins.

Truncation of ECiz1 revealed important differences in the way that cyclins E and A interact with Ciz1. For cyclin A, binding was weakened by deletion of C-terminal sequences to generate Ciz1 fragment N471 (Fig. 2C) and completely lost by removal of a further 80 amino acids (fragment 80 in Fig. 2E). By contrast, interaction with cyclin E was not affected by truncation or loss of fragment 80, and only required sequences within fragment N391 (Fig. 2D). Therefore, the data indicate that cyclins E and A interact with Ciz1 through distinct sets of contacts.

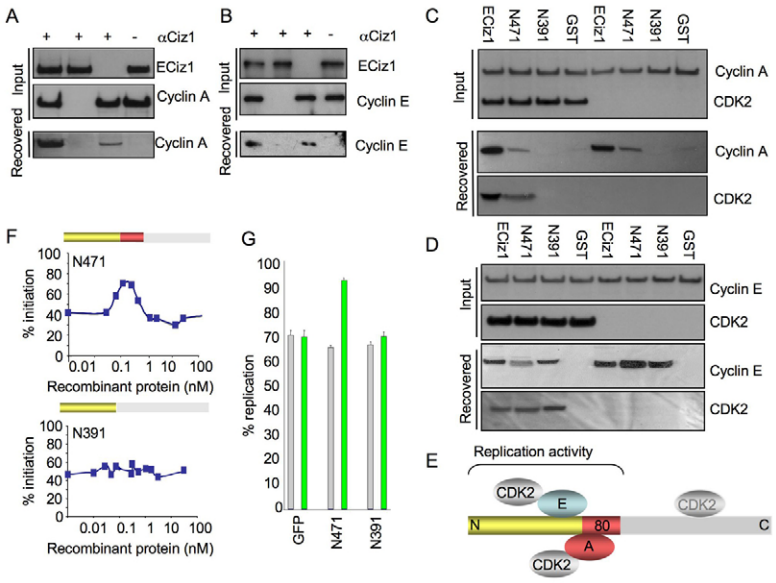

Fig. 2. Interaction with cyclin A and initiation of DNA replication in vitro depend on Ciz1 fragment 80. Coimmunoprecipitation reactions using recombinant ECiz1 and (**A**) cyclin-A–CDK2 or (**B**) cyclin-E–CDK2 in replication-type extracts from S-phase HeLa cells, with and without Ciz1 polyclonal antibody as indicated. After immunoprecipitation, both recombinant ECiz1 (lane 1) and endogenous Ciz1 (lane 3) recovered recombinant cyclin-A–CDK2 or cyclin-E–CDK2 from extracts, as detected by western blot. (**C**) Purified recombinant GST-tagged ECiz1, derived N-terminal fragments N471 and N391, and GST control were incubated with recombinant cyclin A or cyclin-A–CDK2; complexes were recovered and analysed by western blot. ECiz1 and N471 but not N391 recovered cyclin A; this occurred independently of CDK2. (**D**) Reactions were performed as for C, except recombinant cyclin E or cyclin-E–CDK2 complex was used. Cyclin E was recovered by both ECiz1 fragments and was independent of CDK2. (**E**) Schematic representation of cyclin E and A interactions with ECiz1. Fragments N471 (yellow and red domains), and N391 (yellow domain) are shown. Fragment 80, required for cyclin-A binding, is shown in red. The site of reported direct interaction with CDK2 is also shown (Mitsui et al., 1999; den Hollander and Kumar, 2006). (**F**) Cell-free replication experiments with late G1-phase nuclei in S-phase extracts, supplemented with increasing concentrations of ECiz1 fragments as indicated. The number of nuclei that initiated DNA replication was determined as described (Coverley et al., 2005). Fragment N471 but not N391 stimulates initiation of DNA replication. (**G**) Cycling murine 3T3 cells were transfected with the indicated GFP-tagged constructs and the number of replicating nuclei was determined by incorporation of BrdU as described (Coverley et al., 2005). Green bars show the number of GFP-ECiz1-transfected cells that engaged in DNA synthesis within 17 hours of transfection and grey bars show untransfected cells in the same population. The histogram shows the results of three separate transfections with standard errors.

When truncated fragments N471 (interacts with cyclins E and A) and N391 (interacts with cyclin E only) were tested for their ability to promote DNA synthesis in replication-competent nuclei under conditions described previously (Coverley et al., 2005), results showed clearly that Ciz1 activity is dependent on fragment 80; N471 increased the number of nuclei that initiate DNA replication in vitro, whereas N391 did not (Fig. 2F). Similarly, in cell-based replication assays also described previously (Coverley et al., 2005), N471 stimulated DNA replication, whereas N391 did not (Fig. 2G). Therefore, fragment 80 is essential not only for interaction with cyclin A, but also for Ciz1 activity, consistent with the idea that Ciz1 and cyclin A cooperate to promote initiation of DNA replication.

Ciz1 promotes initiation of DNA replication via Cy-motif-mediated interactions

Ciz1 contains five putative cyclin-binding motifs (K/RXL) (Harper and Adams, 2001), three of which are in active fragment N471 [Cy motifs i, ii and iii (see Fig. 3A)]. Mutations in two of these sites (to AXA) influence both cyclin binding and Ciz1 function in replication assays. Specifically, loss of Cy-ii impaired recovery of both cyclins E and A in GST 'pull-down' experiments (Fig. 3B). In addition, use of the much more quantitative approach of surface plasmon resonance revealed a reduction in binding affinity of approximately 60-fold in the case of cyclin A (Fig. 3C) and 2.5-fold in the case of cyclin E, which would appear to be sufficient to impinge on binding. Consistent with reduced cyclin binding, functional assays demonstrated that mutation of Cy-ii renders fragment N471 unable to promote DNA replication (Fig. 3D). Therefore, this site is essential for DNA replication function and the data suggest that cyclin binding at this site is involved.

Unlike Cy-ii, mutation of the Cy motif within fragment 80 (Cy-iii) illustrates important differences in the way that Ciz1 interacts with cyclins E and A. Consistent with the observation that interaction with cyclin E does not require fragment 80 (Fig. 2D), loss of Cy-iii had little effect on cyclin E recovery or apparent dissociation constant (k_D). However, interaction with cyclin A was significantly impaired (Fig. 3B,C). Loss of Cy-iii also impaired N471 function in DNA replication assays, shifting the active concentration tenfold, but not abolishing it (Fig. 3D). This outcome is consistent with a reduction in cyclin A binding affinity that can be overcome at higher concentrations and suggests that Cy-iii might play a non-essential role in Ciz1 function.

Taken together, the data indicate that cyclin A interacts with two sites in Ciz1 (Cy-ii and Cy-iii), and that interaction with Cy-ii is dependent upon sequences within fragment 80 (possibly Cy-iii). This begins to suggest a two-step interaction in which fragment 80 contributes to recruitment of cyclin A to Cy-ii.

Cyclin A displaces cyclin E from Cy-ii

The data described above clearly demonstrate that cyclins E and A interact with Ciz1 via distinct mechanisms, raising the possibility

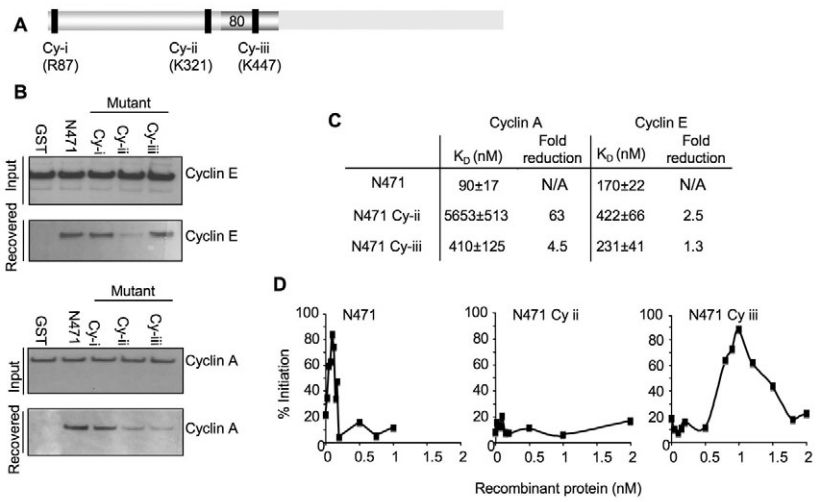

Fig. 3. Cyclin-binding motifs are essential for Ciz1 activity. (**A**) Schematic representation of ECiz1 N471, showing the location of the three Cy motifs that were individually mutated (R/KXL to AXA). (**B**) GST-N471 and derived Cy-motif mutants were immobilized on glutathione beads and incubated with either recombinant cyclin E or cyclin A (input); complexes were recovered and detected by western blot. Only mutation of Cy-ii weakened association with cyclin E, whereas cyclin-A binding was weakened by mutation of both Cy-ii and Cy-iii. (**C**) Quantification of the effect of Cy-motif mutants on cyclin binding, by surface plasmon resonance. Increasing concentrations of N471 or derived mutants were bound to immobilized cyclin A or cyclin E. A summary table is shown with apparent dissociation constants and standard error. (**D**) Cell-free DNA replication experiments in which N471 or derived Cy-motif mutants were titrated into cell-free replication experiments with late G1-phase nuclei and S-phase extracts. The number of replicating nuclei was determined and plotted as described (Coverley et al., 2005). N471 stimulated initiation of DNA replication in a concentration-dependent manner, peaking at 0.1 nM. Mutation of Cy-ii abolished Ciz1 activity, whereas mutation of Cy-iii shifted peak activity tenfold to 1 nM.

that their interactions might have distinct consequences. Furthermore, given their well-documented temporally distinct expression profiles (Sherr, 1996), cyclin E and cyclin A might be expected to interact with Ciz1 at different times during G1 phase. In soluble extracts prepared from synchronized HeLa cells, Ciz1 protein levels peak at the same time as cyclin E and before cyclin A, 9 hours after release from mitotic arrest (Fig. 4A,B). When the natural order in which cyclins E and A are expressed is taken into account, a narrow window of opportunity exists for Ciz1 to interact with cyclin E before cyclin A levels rise and displace it. Focussing on the essential functional site at Cy-ii (to which both cyclins bind), we tested the relative affinities of cyclins E and A in competition experiments that mimic the rising gradient of cyclin A that occurs in late G1 phase, against a background of prior cyclin E expression. Under these conditions, recombinant cyclin-A–CDK2 displaced recombinant cyclin E from active fragment N471 when present at equimolar concentrations (Fig. 4C). Although more cyclin E is recovered by GST-N471 or N391 fragments if CDK2 is left out of the reaction (Fig. 2D), absence of CDK2 had only a slight impact on the ability of cyclin A to displace cyclin E (Fig. 4D). Consistent with this, inclusion of roscovitine in displacement assays carried out in the presence of CDK2 did not significantly reduce displacement (data not shown). These data suggest that displacement of cyclin E by cyclin A is not strictly dependent upon kinase activity.

Cyclin-A-mediated displacement of cyclin E is absolutely dependent on fragment 80 because N391 does not support displacement (Fig. 4E); however, it is not dependent on the Cy-iii site (Fig. 4C). Thus, not only does cyclin A interact with Cy-ii in a manner dependent upon contacts in fragment 80, but it is also capable of displacing cyclin E in the process. Although this can occur in the absence of Cy-iii in vitro, a contribution from this site to a two-step interaction mechanism could account for the reduction in replication activity seen with the Cy-iii mutant (Fig. 3D).

The data described here suggest (i) that Ciz1 would first interact with cyclin E in G1 and then, as cyclin A levels rise, cyclin E would be displaced in favour of interaction with cyclin A and (ii) that Ciz1 and cyclin A functionally cooperate to activate DNA synthesis in licensed replication-competent nuclei. Taken together, these two lines of investigation are consistent with the idea that cyclin A could be localized to sites within the nucleus where cyclin E is present, through its interaction with Ciz1.

Ciz1 depletion disrupts immobilization of cyclin A but not cyclin E

If Ciz1 does indeed target the activity of cyclin-A–CDK2, then depletion of Ciz1 as cells pass through G1 phase should affect recruitment of cyclin A into the immobilized nuclear fraction (Fig. 5A). Using anti-Ciz1 small interfering (si)RNA (Coverley et al., 2005), which effectively suppresses production of Ciz1 protein (Fig. 5B) and completely abolishes recruitment of newly synthesized Ciz1 to detergent- or nuclease-resistant structures (Fig. 5C), we found that recruitment of cyclin A is significantly compromised. Under conditions in which Ciz1 transcript levels were reduced by ~70% (Fig. 5D), the number of cells that entered S phase was dramatically reduced compared to mock-depleted control cells (Fig. 5E) and recruitment of cyclin A to detergent-resistant nuclear structures was impaired (Fig. 5F). This was most notable at 15 and 20 hours after release. Later time points revealed a decrease in immobilized cyclin A even in control cells (Fig. 5F) that was not reflected in total cyclin A levels (data not shown). To confirm the Ciz1-dependent decrease in immobilized cyclin A at 20 hours, cells were also analysed by western blot. Despite comparable levels of cyclin A in the total protein fraction in Ciz1-depleted and -undepleted cells, the immobilized fraction was reduced by 50% (Fig. 5G). Unlike cyclin A, cyclin E immobilization was not significantly affected by Ciz1 depletion (Fig. 5H,I). Thus, cyclin E appears to be immobilized in

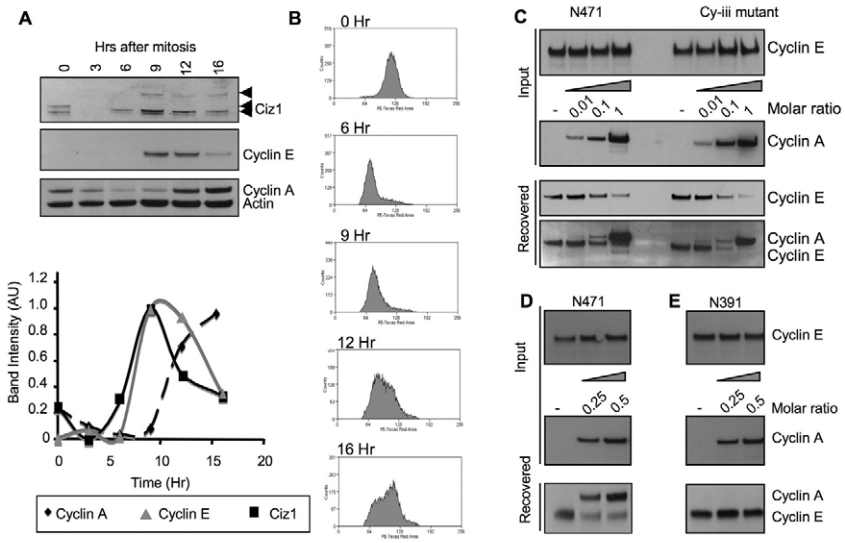

Fig. 4. Cyclin A displaces cyclin E from its interaction site at Cy-ii. (**A**) Western blots show relative levels of the indicated proteins in replication-type extracts prepared from HeLa cells synchronized by release from mitosis over a 16 hour time course. The graph shows quantification of band intensities for cyclin E, cyclin A and Ciz1 over the time course relative to actin; the maximum level for each protein is normalized to 1. Peak soluble Ciz1 levels coincide with peak cyclin E and precede the rise in cyclin A. (**B**) Flow cytometry profiles showing the DNA content of HeLa cell populations in A. (**C**) Immobilized N471 or Cy-iii mutants were pre-bound to cyclin-E–CDK2, followed by incubation with cyclin-A–CDK2 complex at the indicated molar ratios (cyclin A:cyclin E). Sequential western blotting of recovered proteins, cyclin E (upper panel) and cyclin A (lower panel), shows that cyclin-A–CDK2 efficiently displaced cyclin E. (**D**) Experiments were performed for N471 as for C, except that cyclin E and A were used in the absence of CDK2. (**E**) As for D, except that the N391 fragment was used to immobilize cyclin E. Under these conditions, displacement of cyclin E by cyclin A does not occur, demonstrating that fragment 80 is required for cyclin exchange.

a manner that is independent of Ciz1, probably before the point at which Ciz1 and cyclin-A–CDK2 promote initiation of DNA replication. This is consistent with the idea that Ciz1 might act to focus cyclin A to sites that contain cyclin E. Thus, Ciz1 appears to link cyclin E and cyclin-A-dependent kinase, and might help to coordinate their sequential functions in the replication initiation process in time and space.

Discussion

The data show that Ciz1 cooperates with cyclin-A–CDK2 to activate the DNA replication machinery in late G1-phase nuclei and suggest that this is achieved by focussing cyclin-A–CDK2 to appropriate sites in the nucleus. Based on the interaction and displacement studies described, we propose that Ciz1 might also play a role, coupling the activities of cyclin E and cyclin-A-associated kinase. A body of evidence supports a role for cyclin E in pre-RC assembly, although convincing experiments suggest this is only essential in specific circumstances that include exit from quiescence (Geng et al., 2003; Parisi et al., 2003). Several mechanisms have been proposed for this synergy, including stabilization of CDC6 against proteolysis by the anaphase-promoting complex/cyclosome (APC/C) (Mailand and Diffley, 2005). However, the data suggest that, in higher eukaryotes, cyclin E also interacts directly with CDC6 to support MCM assembly at replication origins (Cook et al., 2002; Coverley et al., 2002; Geng et al., 2003). Using *Xenopus* egg extracts, it was shown that cyclin E is immobilized during DNA replication and that its presence at replication origins contributes to the initiation process (Chevalier and Blow, 1996; Furstenthal et al., 2001); more recent evidence from analysis of murine cells suggests that at least part of this function of cyclin E might occur independently of CDK2 activity (Geng et al., 2003; Geng et al., 2007).

Cyclins interact with Cy motifs at sites distant from phosphorylation sites, imposing spatial constraints on CDKs that influence phosphorylation site selection (Brown et al., 1999; Takeda et al., 2001; Wohlschlegel et al., 2001). However, in the case of the CDK inhibitors p21 and p27, it has been shown that Cy-motif-mediated interactions support processes other than phosphorylation (Wohlschlegel et al., 2001; Lowe et al., 2002). The data presented here suggest that cyclin E might use Cy-motif-mediated interaction with Ciz1 to function as an immobilized receptor for cyclin-A-associated kinase. We have no evidence to suggest that this function would require CDK2 to be complexed with cyclin E. Therefore, we suggest that Ciz1-dependent localization of cyclin A to sites where pre-RCs have formed is a candidate CDK-independent role for cyclin E during the replication initiation process.

When bound to Cy-ii or Cy-iii, cyclin-A–CDK2 could mediate distinct phosphorylations within Ciz1 or in adjacent proteins. Cyclin E would presumably also phosphorylate Ciz1 or adjacent proteins when associated with Cy-ii. It remains to be seen whether directed phosphorylation of associated substrates is the basis for the functional cooperation between Ciz1 and cyclin-A–CDK2 during initiation. However, localization of cyclins within the cell is known to contribute to CDK specificity by controlling access to substrates, thereby promoting phosphorylation of some proteins and preventing untimely phosphorylation of others. It was shown previously that cyclins are targeted to the vicinity of replication origins through interaction with pre-RC proteins, for example cyclin E and CDC6 (Furstenthal et al., 2001) and Cln5 and ORC6 in budding yeast (Wilmes et al., 2004). In fact, localization strategies that can deliver kinase activity to the right place at the right time might be the primary source of CDK target specificity in vivo. This was clearly demonstrated by addition of a cyclin-E-derived nuclear-localization sequence to cyclin B1. Unlike cyclin-E–CDK2 or

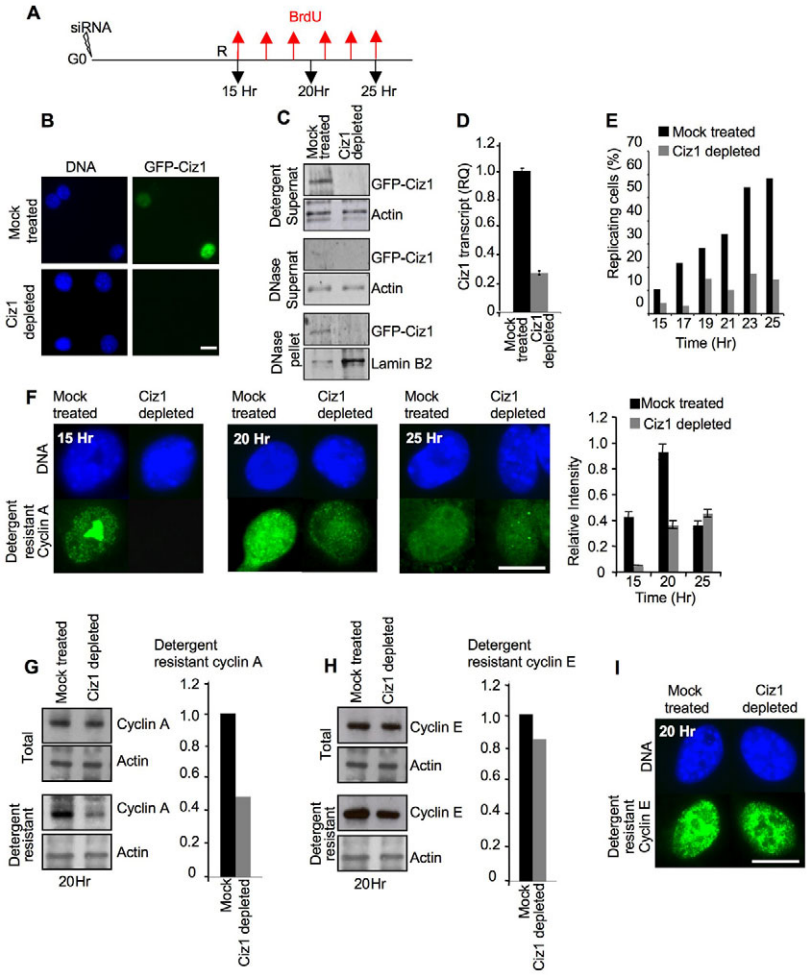

Fig. 5. Depletion of Ciz1 reduces nuclear immobilization of cyclin A. (**A**) Experimental overview. 3T3 cells were synchronised in G0, transfected with anti-Ciz1 siRNA, incubated with BrdU and harvested at the indicated times. (**B**) Fluorescence microscopy showing GFP-ECiz1-transfected cells (upper panels) and co-transfected GFP-ECiz1 and anti-Ciz1 siRNA cells (lower panels). GFP fluorescence is green, total DNA is counterstained in blue. Scale bar: 10 µm. (**C**) Western blots showing sequential fractions derived from 3T3 cells expressing GFP-ECiz1, with or without anti-Ciz1 siRNA. The detergent-soluble fraction, the nuclease-soluble fraction and the nuclease-resistant 'nuclear matrix' associated fraction are shown. (**D**) Quantitative real-time PCR showing a 72% reduction in endogenous Ciz1 transcript levels 20 hours after transfection, relative to mock-depleted cells. (**E**) Histogram showing DNA synthesis and incorporation of BrdU in Ciz1-depleted cells or mock-depleted cells between 15 and 25 hours after release from quiescence. (**F**) Immunofluorescence images of detergent-resistant cyclin A in Ciz1-depleted and mock-depleted cells at 15, 20 and 25 hours. Scale bar: 10 µm. The histogram shows mean fluorescence intensities with standard error, where n=20. (**G**) Western blot showing total and detergent-resistant fractions of cyclin A and actin, 20 hours after release from quiescence. Total levels of cyclin A are unaffected by Ciz1 depletion, but detergent-resistant cyclin A is reduced by 50%. The histogram shows densitometry quantification of detergent-resistant cyclin A after normalization to actin. (**H**) Western blot showing total and detergent-resistant fractions of cyclin E and actin at 20 hours. The histogram shows densitometry quantification of detergent-resistant cyclin E after normalization to actin. (**I**) Representative immunofluorescence images of detergent-resistant cyclin E at 20 hours. Scale bar: 10 µm.

cyclin-A–CDK1, cyclin-B1–CDK1 is normally unable to promote DNA synthesis in *Xenopus* egg extracts, but given timely entry into the nucleus, it is able to activate nuclear DNA replication (Moore et al., 2003). In mammalian somatic cells, DNA replication is subject to multiple layers of regulation that impose a hierarchy on initiation events. It is also capable of adapting to changes in growth conditions or gene expression. The requirement during the mammalian initiation process for (at least) two cyclins in a defined order will almost certainly involve a range of targeting strategies and, in addition, would necessitate a mechanism for linking their activity in time and space.

In addition to linking the activities of cyclins E and A, Ciz1 might also play a role in localizing these temporal regulators to the structures that organize DNA replication in nuclear space. We showed previously that newly synthesized Ciz1 becomes assembled into nuclease-resistant structures in late G1 and early S phase (Ainscough et al., 2007); therefore, Ciz1 might help to deliver associated factors, such as cyclin A, into nuclear-matrix-associated DNA replication factories. In fact, incorporation of cyclin A into replication factories at G1-S was described some time ago (Cardoso et al., 1993). However, immobilization of Ciz1 by association with the nuclear matrix is clearly not essential for it to function in DNA replication or for it to interact with cyclin E or cyclin A, because the ECiz1 fragments used here (N471 and N391) both lack the C-terminal sequences that support immobilization (Ainscough et al., 2007). Therefore, Ciz1 appears to have two distinct functions that can be uncoupled: (i) cyclin interaction and a function in DNA replication that is mediated by N-terminal sequences; and (ii) immobilization domains that might restrict replication activity to specific subnuclear sites.

Taken together, the data are consistent with a model (Fig. 6) that describes how Ciz1 could function at the interface between cyclins E and A during the initiation process. We suggest that Ciz1 is a

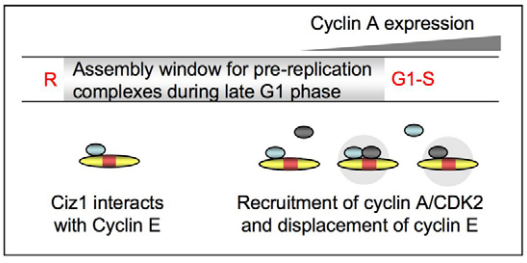

Fig. 6. Model of Ciz1-mediated cyclin exchange during initiation of DNA replication. The data suggest that Ciz1 would interact with cyclin E (blue oval) through Cy motif Cy-ii during mid G1 phase. As cyclin A levels rise in late G1 phase, cyclin E would be displaced from Cy-ii by cyclin A (grey oval). Interaction with Ciz1 could localize cyclin-A–CDK2 complexes to sites that contain cyclin E, promoting phosphorylation of nearby substrates (indicated by pale grey circle). Thus, Ciz1 helps to coordinate the sequential functions of cyclin E and cyclin A during the switch from pre-RC assembly to activation of DNA synthesis.

platform for cyclin exchange that interacts first with cyclin E during pre-RC assembly. Later in G1, when cyclin A levels rise, interaction with Ciz1 recruits cyclin A to the same sites as cyclin E, resulting in displacement of cyclin E and possibly phosphorylation of replication proteins by cyclin-A-associated kinase. In this way, Ciz1 could contribute to the switch from cyclin-E-dependent pre-RC assembly to cyclin-A-dependent DNA synthesis, ensuring that both events occur in the same place and in the correct order.

Materials and Methods

Cell culture and synchrony

3T3 and HeLa cells were cultured as described (Coverley et al., 2002). Mouse 3T3 cells were synchronized in G_0 by contact inhibition and serum depletion followed by release into fresh media as described (Coverley et al., 2002). For replication extracts, HeLa cells were synchronized at the start of S phase by double-thymidine (2.5 mM) arrest and released for 1 hour into fresh media (Krude et al., 1997). G1 cells were obtained by releasing thymidine-arrested cells into medium supplemented with 0.04 μg/ml nocodazole for 10 hours. Cells were released from mitotic arrest into fresh medium and harvested at intervals. DNA content and cell synchrony were verified by flow cytometry using a Dako CyAn ADP cytometer and analysed with Summit version 4.2 software. Cells were fixed in 80% ice-cold ethanol, then stained with 0.1% v/v Triton X-100, 0.1 mg/ml propidium iodide in PBS.

Antibodies

Mouse monoclonal antibodies anti-cyclin A (CY-1A, Sigma Aldrich), anti-cyclin E (HE12, Santa Cruz Biotechnology), anti-actin (AC40, Sigma Aldrich) and anti-lamin B2 (Zymed-Invitrogen) and rabbit polyclonal anti-CDK2 (ab7954, Abcam) were used as directed for western blotting. Ciz1 was detected with anti-Ciz1 polyclonal antibody 1793 (Coverley et al., 2005). Goat anti-mouse IgG-POD and goat anti-rabbit IgG-POD (both Sigma Aldrich) were used with the ECL detection system (GE Lifescience). Densitometry was performed using ImageJ software (NIH). For immunofluorescence, monoclonal anti-cyclin A (1:500, CY-1A; Sigma Aldrich) and monoclonal anti-cyclin-E (1:2500, E4; Santa Cruz Biotechnology) were used and detected with a goat polyclonal anti-mouse-AlexaFluor488 conjugate (Invitrogen). Fluorescence intensities were extracted using Openlab software (Improvision).

Cloning and site-directed mutagenesis

The ECiz1 N471 and N391 deletion fragments were PCR amplified from pGEX-ECiz1 (Coverley et al., 2005) using Advantage cDNA polymerase (BD Clontech). Incorporation of unique flanking *Xma*I and *Xho*I restriction sites facilitated in-frame ligation into pGEX-6-P3 and pEGFP-C3 vectors. ECiz1 double-alanine substitution mutations of the Cy motifs at R87 (Cy-i), K321 (Cy-ii) and K447 (Cy-iii) were generated so that R/KXL was changed to AXA. Numbers refer to the amino acid position in full-length Ciz1. Mutagenesis was performed using the Stratagene QuikChange method, as recommended, using pGEX-ECiz1 N471 as template. All constructs were sequence verified (Eurofins MWG).

Protein production

All ECiz1 fragments and derived mutants were expressed as GST fusions in pGEX-6-P3 vectors in *Escherichia coli* BL21 (DE3) and purified as described (Coverley et al., 2002; Coverley et al., 2005). For binding studies, proteins were retained on glutathione beads. Fragment designations refer to the position of the amino acid at the fragment boundary, relative to full-length mouse Ciz1 (NP_082688). Cy motif mutant designations refer to the location of the Cy motif in full-length Ciz1. GST–cyclin-A, GST–cyclin-E and their CDK2 complexes were expressed in *E. coli* and purified as described (Coverley et al., 2002).

Cell-free replication

'Replication-competent' late G1-phase nuclei and mid G1-phase extracts were prepared from 3T3 cells released from quiescence for 17 and 15 hours, respectively, as described (Coverley et al., 2002). S-phase extracts were prepared from HeLa cells 1 hour after release from double-thymidine arrest (Krude et al., 1997). Cyclin concentrations in S-phase extracts were quantified by comparison to purified recombinant cyclins A and E in western blots, and band intensities were measured using ImageJ software (NIH).

Cell-free initiation experiments were performed and analysed as described previously (Coverley et al., 2002; Coverley et al., 2005). Where indicated, recombinant proteins (ECiz1 or derived fragments N471 and N391) were added to extracts at the indicated concentrations (20 pM to 2 nM), followed by addition of nuclei. For cell-free reactions with G1 extracts, purified recombinant cyclin-A–CDK2 was included at between 0.001 ng/μl and 1 ng/μl in the presence or absence of 1 nM or 10 nM ECiz1, as indicated. Reactions were incubated at 37°C for 60 minutes and biotin-labelled nascent DNA was visualized by high-magnification (600×) fluorescent microscopy after detection with streptavidin-conjugated fluorescein isothiocyanate (Amersham). When 3T3 cells are released from quiescence by the protocol used here, no more than 70% of the total population enters S phase (Coverley et al., 2002). However, the highest observed replication frequency in vitro is nearer 50%. For the G1 population of 3T3 nuclei used here, 15% were in S phase (%S) and the maximum number that replicated in any assay in vitro was 47%. Therefore, 32% of this population is competent to initiate replication in vitro (%C). Thus, for each data point, % initiation = (% replication – %S)/%C × 100, where '% replication' is variable and depends on the conditions of the reaction.

Alternatively, products were analysed by western blot to determine the subnuclear distribution of proteins. Detergent-washed nuclei were generated by adding an equal volume of 0.5% Triton X-100 in PBS, followed by centrifugation at 13,000 ***g*** at 4°C to generate supernatant and pellet fractions.

Binding reactions

Immunoprecipitation reactions were performed in 200 μl binding buffer [50 mM HEPES pH 7.8, 10 mM $MgCl_2$, 20 mM $CaCl_2$ 10 mM magnesium acetate, 0.04% NP40, 2× complete protease inhibitor cocktail (Roche), 10 mM ATP and 200 mM KCl] and 100 μl S-phase HeLa extracts. Recombinant protein (0.4 μg) and 2 μl rabbit polyclonal 1793 antibody (Coverley et al., 2005) were added as indicated, and incubated for 2 hours at 4°C. Antibodies were immobilized on Immunopure protein A beads (Pierce Bioscience) for 1 hour, washed five times (10 × bead volume) in a binding buffer diluted 2:3 with distilled water, and analysed by western blot.

Interaction studies using GST-ECiz1 derivatives were performed using either endogenous proteins from 9 hour HeLa cell extracts or addition of 2 μg recombinant protein to reactions containing bait protein immobilized on glutathione beads, as indicated, followed by incubation in binding buffer at 4°C for 1 hour with agitation. Beads were washed five times and bound protein was analysed by western blot. For competition assays, purified cyclin E or cyclin-E–CDK2 was pre-incubated with GST-tagged ECiz1 derivatives bound to glutathione in binding buffer for 15 minutes at 4°C, before addition of cyclin A or cyclin-A–CDK2. After 1 hour, beads were washed and the recovered protein analysed by western blot.

Surface plasmon resonance

GST–cyclin-A and GST–cyclin-E (in 50 mM HEPES pH 7.8, 150 mM NaCl, 1 mM DTT and 5 mM glutathione) were immobilized onto a BIAcore CM5 chip by standard amine coupling chemistry and quenched with ethanolamine. Binding reactions were performed at varying concentrations of N471 or derived mutants in wash buffer (see above) at 25°C, 30 μl/min flow rate using a BIACORE T-100. Apparent dissociation constants (k_D) were determined by plotting response units for each binding reaction at equilibrium against concentration of ligand. The data were fit with two-site (cyclin A) or one-site (cyclin E) ligand-binding equations using SigmaPlot (SPSS).

Depletion of Ciz1 by siRNA

Asynchronous 3T3 cells were transfected with pEGFP-ECiz1 (Coverley et al., 2005) in the presence or absence of anti-Ciz1 siRNAs 4 and 8 to validate siRNA efficiency. The nuclear matrix fraction was prepared as described (Ainscough et al., 2007). Quiescent 3T3 cells were released by trypsinization and transfected using anti-Ciz1 siRNAs 4 and 8 (Coverley et al., 2005) or mock transfected (without siRNA) using Amaxa Kit R, program U-30, according to the manufacturer's instructions. Transfected cells were cultured for 15, 20 or 25 hours before harvest to allow passage through G1 phase. Cells were resuspended in PBS, divided to generate a total fraction and a detergent-resistant fraction by incubating in CSK buffer (10 mM PIPES pH 6.8, 300 mM sucrose, 100 mM NaCl, 1 mM $MgCl_2$, 0.1 mM ATP, 1 mM DTT, 0.1% v/v Triton X-100) for 5 minutes before centrifugation (10,000 ***g***), and finally analysed by western blot.

Quantitative real-time PCR

Total RNA was extracted using NucleoSpin RNA II (Macherey-Nagel) and 1 μg was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) with random hexamers (Promega) as recommended. Ciz1 transcript levels were quantified relative to GAPDH using Taqman probes (Applied Biosystems) for Ciz1 exon 7 (Mm00503762_g1) and GAPDH (Mm99999915_g1), according to instructions, using an ABI 7000 RT-PCR machine (Applied Biosystems).

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