C-terminal domains deliver the DNA replication factor Ciz1 to the nuclear matrix

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Summary

Cip1-interacting zinc finger protein 1 (Ciz1) stimulates DNA replication in vitro and is required for mammalian cells to enter S phase. Here, we show that a significant proportion of Ciz1 is retained in nuclear foci following extraction with nuclease and high salt. This suggests that Ciz1 is normally immobilized by interaction with non-chromatin nuclear structures, consistent with the nuclear matrix. Furthermore, matrix-associated Ciz1 foci strikingly colocalize with sites of newly synthesized DNA in S phase nuclei, suggesting that Ciz1 is present in DNA replication factories. Analysis of green fluorescent protein-tagged fragments indicates that nuclear immobilization of Ciz1 is mediated by sequences in its C-terminal third, encoded within amino acids 708-830. Immobilization occurs in a cell-cycle-dependent manner, most probably during late G1 or early S phase, to coincide with its reported point of action. Although C-terminal domains are sufficient for immobilization, N-terminal domains are also required to specify focal organization. Combined with previous work, which showed that the DNA replication activity of Ciz1 is encoded by N-terminal sequences, we suggest that Ciz1 is composed of two functionally distinct domains: an N-terminal replication domain and a C-terminal nuclear matrix anchor. This could contribute to the formation or function of DNA replication factories in mammalian cells.

Key words: DNA replication, Cell cycle, Ciz1, Nuclear matrix

Introduction

In eukaryotic cells DNA replication takes place at discrete sites in the nucleus called replication foci or factories, which change in number and distribution as S phase progresses (Taddei et al., 2004). In cultured HeLa cells each factory is estimated to consist of around 10 replication origins and all the proteins that are required for their activity (Jackson and Pombo, 1998), as well as accessory factors involved in maintaining or changing heritable patterns of DNA replication and transcription. Non-chromatin nuclear structures organize replication factories by providing a framework upon which they are immobilized, although the composition and even the existence of the nuclear framework has been controversial. Depending on the method of preparation it has been given several names including nuclear matrix (Belgrader et al., 1991b), nuclear scaffold (Paulson and Laemmli, 1977) and nucleoskeleton (Jackson et al., 1990).

The first experiments to suggest a link between DNA replication and the nuclear framework looked at the DNA sequences that remain attached to the residual proteinaceous nuclear core after extraction. These studies linked A-T-rich sequences, known replication origins or newly synthesized DNA and replication intermediates with the nuclear matrix (Berezney and Coffey, 1975; Buckler-White et al., 1980; Dijkwel et al., 1979; Dijkwel et al., 1991; Razin et al., 1986; van der Velden et al., 1984). Strong evidence that this occurs at specific nuclear sites is provided by electron microscopy, which showed that in chromatin-depleted nuclei newly synthesized DNA is associated with electron-dense bodies that are immobilized on a core structure (Hozak et al., 1993). These data suggest that initiation occurs in close proximity to the nuclear matrix, and that the spatial and temporal organization of DNA replication may be related to this interaction. Furthermore, recent experiments showed that residual matrix-associated chromatin can support cell-cycle-regulated initiation (Radichev et al., 2005), arguing that the machinery that restricts initiation of DNA replication to once per cell cycle is itself associated with the nuclear matrix.

Analysis of the composition of nuclear matrix preparations revealed the matrin class of proteins which, like lamins A, B and C, are resistant to high salt extraction of isolated nuclei (Nakayasu and Berezney, 1991). Matrins are located in the interior of the nucleus and appear to be part of a core structure. Matrin 3 contains a matrin 3 type zinc finger (MH3) domain (Belgrader et al., 1991a), which is shared by at least seven human proteins. Although the function of matrin 3 has yet to be determined, many of the other MH3-containing proteins (SAP62, SAP61, U1 small nuclear ribonucleoprotein C and WW domain-binding protein 4) are involved in pre-mRNA splicing (Bedford et al., 1998; Das et al., 2000; Sillekens et al., 1988). The remaining two, NP220 and Cip1-interacting zinc finger protein 1 (Ciz1), share other regions of similarity and have both been shown to bind double-stranded DNA, NP220 to cytidine clusters (Inagaki et al., 1996) and Ciz1 to a weak consensus sequence (Warder and Keherly, 2003).

Ciz1 is a nuclear protein with homologues in mouse, man
and other vertebrates. It was initially identified as a protein that interacts with the cell-cycle regulator p21Cip1 in a modified yeast two-hybrid system (Mitsui et al., 1999), and separately from a medulloblastoma derived cDNA library (Warder and Keherly, 2003). In a third approach, we analysed the products of cell-free DNA replication experiments to identify Ciz1, and used its cyclin A-cdk2-dependent behaviour to implicate it in the activation step of replication initiation (Coverley et al., 2005). Cell-free and cell-based experiments showed that recombinant Ciz1 stimulates DNA replication, whereas depletion of Ciz1 restrains DNA replication. Furthermore, analysis of the sub-nuclear localization of Ciz1 with specific antibodies showed that it occupies nuclear foci that overlap with the replication factory marker PCNA during S phase. However, unlike PCNA, Ciz1 occupies foci throughout the cell cycle, raising the possibility that it marks sites on the nuclear framework at which DNA replication factories will form in S phase.

We have investigated the nuclear binding characteristics of Ciz1, and dissected its domain organization. We show that the DNA replication activity that resides in the N-terminal half of Ciz1 becomes anchored to non-chromatin nuclear structures through C-terminal sequences, and that this occurs in an S-phase-dependent manner. As Ciz1 is both a nuclear matrix component and has intrinsic replication activity, we suggest that it is a molecular link between the DNA replication machinery and the sub-nuclear structures that organize their function.

Results

Ectopic Ciz1 and ECiz1 form sub-nuclear foci

Endogenous Ciz1 resides in nuclear foci when visualized by immunofluorescence. These show significant overlap with the replication factory marker proliferating cell nuclear antigen (PCNA) in S-phase nuclei (Coverley et al., 2005). We tagged Ciz1 and the embryonic splice variant ECiz1 with green fluorescent protein (Fig. 1A) to dissect their domain function and begin to investigate differences in behaviour between these isoforms. Like endogenous Ciz1, both GFP-tagged proteins form multiple sub-nuclear foci upon transfection into NIH3T3 cells (Fig. 1B), or HEK293 cells (not shown). Unlike GFP alone, which distributes evenly throughout the cell, fluorescent Ciz1/ECiz1 appears in the nucleus as a diffuse pattern within 12 hours of transfection but both variants become focal within 20 hours. For ECiz1 but not Ciz1, some diffuse nuclear fluorescence remains, whereas little or no cytoplasmic fluorescence is seen with either variant. By day 2 after transfection, the foci formed by both variants are larger and fewer. Based on visual analysis of transfected cultures, and limited time-lapse imaging, transfected cells appear not to proliferate. The observations suggest that once inside the nucleus, Ciz1 is concentrated at discrete locations, and that ECiz1 may be less efficiently concentrated than full-length Ciz1.

Unlike full-length Ciz1 and ECiz1, truncated forms of either variant, which lack 273 amino acids from the C terminus, do not form a focal pattern in the nucleus in NIH3T3 cells. Instead they form a small number of larger bodies (Fig. 2A,B), but at noticeably different rates. Most cells transfected with Ciz1-derived N572 form a single blob within 20 hours of transfection, however ECiz1-derived N572 is diffuse on day 1, only accumulating at discrete sites by day 2. Like GFP-Ciz1 and ECiz1, cells transfected with GFP-N terminal fragments appear not to proliferate (data not shown). The tendency of truncated Ciz1 and ECiz1 (N572) to form a large body suggests that domains in the C-terminal third of the protein act to limit and localize focal accumulation, resulting in much smaller speckles. This could be achieved if Ciz1 is normally attached to a fixed structure in the nucleus via C-terminal anchoring sequences. In their absence, the tendency of Ciz1 fragments to coalesce would not be restricted.

C-terminal domains anchor Ciz1 within the nucleus

The C-terminal 275 amino acids are identical in Ciz1 and ECiz1. When tested separately this fragment (GFP-C275) formed a non-focal textured pattern in the nucleus that is distinct from the pattern seen with full-length Ciz1 or ECiz1 (Fig. 2C, middle panel). Unlike the N-terminal constructs described above, C275 was initially restricted from entering the nucleus, resulting in many cells (approximately half at 20 hours) in which cytoplasmic fluorescence was greater than nuclear fluorescence. However, on the second day after transfection virtually all GFP-C275-expressing cells

![Fig. 1.](image-url)
contained fluorescent protein in the nuclear compartment. This accumulated above cytoplasmic levels, suggesting that once inside the nucleus, C275 is stabilized by binding to insoluble nuclear structures. Consistent with this, C275 does not become distributed throughout the cytoplasm during subsequent mitoses but partitions with condensed chromosomes (Fig. 2C).

To begin to define the sequences that mediate this interaction we looked at the behaviour of an internal 122 amino acid fragment designated 708-830 (numbers define fragment boundaries in full-length Ciz1 protein), which contains the 53 amino acid MH3 homology domain as well as 21 amino acids upstream and 48 amino acids downstream. Like C275, GFP 708-830 is initially excluded from the nucleus, but by day 2 it also forms a non-focal nuclear pattern in the majority of cells, and partitions with condensed chromosomes at mitosis (Fig. 2D). Therefore, this fragment appears to contain all the information necessary to immobilize Ciz1.

Taken together, the opposing behaviours of Ciz1 C-terminal and N-terminal fragments lead us to suggest that Ciz1 is normally spatially restricted within the nucleus via ‘anchoring’ domains in its C-terminal end.

Endogenous Ciz1 resists high-salt and nuclease extraction

The interaction of proteins with the nuclear architecture has been studied using nuclease treatments that release proteins attached primarily to chromatin, and also by high-salt treatments that solubilize most of the proteins in the nucleus. Those that remain are classified as nuclear matrix proteins (reviewed by Mika and Rost, 2005). We have applied similar techniques, previously used to study the interaction properties of the DNA replication factors Cdc6 and ORC1 (Fujita et al., 2002), to investigate both endogenous Ciz1 and ectopic Ciz1 fragments.

Western blot analysis of NIH3T3 whole cell lysates, with anti-Ciz1 antibody 1793, reveals two major species, designated p100 and p125 (Coverley et al., 2005). Similar results were achieved with a second polyclonal antibody (anti-Ciz1 1791) raised against recombinant mouse ECiz1 (not shown), and in an independent investigation that probed human U2OS cell lysates with a monoclonal antibody to human Ciz1 (Mitsui et al., 1999). In our hands, p100 often resolves into two closely migrating species, whereas p125 is accompanied by a slower migrating form, which is usually less abundant (Fig. 3A). We have designated these p125a and p125b. We have also detected several other minor bands of similar apparent molecular mass, as well as putative breakdown products. The ratio of these bands varies significantly between preparations, suggesting that Ciz1 may be subject to extensive secondary modification.

Upon separation into detergent-soluble and detergent-insoluble fractions, p100 and p125 antigens partition primarily with the insoluble pellet fraction after centrifugation (Fig. 3A). By contrast, Mcm2 protein is distributed between the two phases as expected, indicating successful extraction of soluble proteins. Therefore, the majority of Ciz1 protein appears to be bound to detergent-resistant components of the cell. Using immunofluorescent techniques, endogenous Ciz1 protein was previously shown to reside in the nuclear compartment (Coverley et al., 2005). Therefore, Ciz1 in the detergent-insoluble pellet fraction is most likely attached to insoluble structures in the nucleus, rather than those derived from the rest of the cell.

Serial extraction with increasing concentrations of sodium chloride reveals a complex pattern of Ciz1 bands. These have differing extraction characteristics, indicating that detergent-
washed nuclei contain distinct sub-populations of Ciz1 (Fig. 3B,C). Half of the Ciz1 protein in the nucleus of G0 cells is extracted by 0.4-0.5 M NaCl falling to 36% in cycling cells and 31% in thymidine arrested cells, however in all cases a significant proportion, notably the p100 isoform remains resistant to 2 M NaCl (Fig. 3D). Therefore, some of the Ciz1 in the cell appears to associate strongly with salt-resistant sub-nuclear structures. It remains to be seen whether there is a functional difference between the salt-resistant and salt-soluble populations of Ciz1.

A number of previously characterized nuclear matrix-associated proteins undergo regulated interactions that are cell-cycle stage specific (reviewed by Mika and Rost, 2005). Given the evidence for a role in DNA replication this was possible for Ciz1. However, the data show that the high-salt-resistant population does not vary dramatically between nuclei prepared from cycling cells, thymidine arrested cells or quiescent cells (Fig. 3D). In all three populations, half or more of the Ciz1 in the nucleus resisted extraction, and in all cases this was primarily the p100 isoform. If Ciz1 normally associates and dissociates from salt-resistant structures in a cell-cycle-dependent manner, more significant differences in the proportion of resistant protein would be evident in this experiment. It should be noted however, that this analysis does not reveal how and when newly synthesized Ciz1 protein becomes associated with the nuclear matrix (see below).

Consistent with a probable interaction with the nuclear matrix, endogenous Ciz1 also resists nuclease digestion. DNase 1 fails to release Ciz1 into the detergent-soluble fraction, despite efficiently releasing PCNA (Fig. 4A), and dramatically reducing the DNA content of nuclei (Fig. 4B). Thus, the reported interaction between Ciz1 and DNA (Warder and Keherly, 2003), or a possible interaction with chromatin, is unlikely to account for the observed immobilization of Ciz1 in the nucleus. Instead the data indicate that detergent-resistant Ciz1 is immobilized by attachment to non-chromatin nuclear structures. This does not rule out associations with DNA or chromatin in addition to non-chromatin structures, which would not be revealed by these experiments.

After extraction with detergent, nuclease and high-salt, the resistant Ciz1 fraction continues to occupy sub-nuclear speckles (Fig. 4C). Resistant foci are evident in all cells in cycling populations, indicating that they are not restricted to a particular phase in the cell cycle. Therefore, nuclear matrix-associated Ciz1 foci may mark the sites at which replication factories form in S phase nuclei.

When cells were treated with the protein-protein cross-linker DTSP prior to extraction with detergent, nuclease and high salt, the Ciz1 speckles were noticeably different in quality and quantity (Fig. 4D), being smaller, more abundant and generally more like those detected in unextracted nuclei (Coverley et al., 2005). One possible explanation is that a sub-population of foci, possibly the smaller most abundant ones are more readily stripped away during nuclease and high-salt extraction so that only larger foci remain.

Strikingly, the extraction-resistant nuclear matrix-associated Ciz1 foci strongly colocalise with sites of newly synthesized DNA in early S phase nuclei (Fig. 4E), reinforcing our previous
observations of colocalization with PCNA (Coverley et al., 2005). This adds weight to the idea that Ciz1 foci represent the nuclear bodies that will become the sites of early S phase replication factories.

C-terminal domains mediate interaction with the nuclear matrix

The extraction studies described above indicate that a proportion of endogenous Ciz1 protein in each cell interacts with the nuclear matrix. In order to determine which part of Ciz1 mediates this interaction we used similar extraction techniques to investigate the binding of selected ectopically expressed ECiz1 fragments. Ectopic GFP-ECiz1 (Fig. 5A) and some cells expressing C-terminal fragment GFP-C275 (Fig. 5B) resist detergent and nuclease/high-salt extraction, indicating attachment to a core nuclear structure. By contrast, GFP-ECiz1 N471 (which is the smallest fragment we have generated that is active in DNA replication; unpublished data), washes out freely and requires only detergent treatment for full solubilization (Fig. 5C). This indicates that sequences in the C-terminal third of the protein are responsible for immobilization of Ciz1 on the nuclear matrix.

In vivo cross-linking with DTSP supports this conclusion. After cross-linking, GFP-ECiz1 N471 resisted detergent extraction, but not salt extraction (Fig. 6), suggesting that it becomes attached to a detergent-resistant platform that is itself fully solubilized by 0.5 M NaCl. As expected, GFP-C275 continued to resist both treatments (Fig. 6). These comparisons argue that immobilization on the nuclear matrix is not specified by sequences contained within N471.

Immobilization of C-terminal fragment C275 is cell-cycle dependent

We have shown that endogenous Ciz1 protein associates with the nuclear matrix, and that the proportion of Ciz1 protein engaged in this interaction does not vary greatly in the cell cycle. However, this does not address when and how new Ciz1 protein becomes attached.
Inhibition of new Ciz1 production by RNA interference prevents cells from entering S phase, however, this has little impact on total nuclear Ciz1 levels (Coverley et al., 2005). These observations suggest that Ciz1 persists in the nucleus once incorporated into nuclear structures, but that the presence of ‘old’ Ciz1 from previous cycles is not sufficient to support initiation of DNA replication. Therefore, how and when new Ciz1 is immobilized on the nuclear framework are important questions. We have used C-terminal fragments to begin to study this interaction in the absence of other possible interactions mediated by the replication activity-encoding domains in the rest of the protein.

Using resistance to detergent as an indicator of immobilization, the data show that after 24 hours GFP-C275 is immobilized in only 27% of cells that express it (Fig. 7A), despite nearly 80% of them having bright fluorescence in the nuclear compartment prior to extraction (not shown). Similarly, GFP-708-830 becomes immobilized in 29% of transfected cells within the same time window, confirming that the sequence information necessary to immobilize Ciz1 is contained within this fragment. The number of transfected cells is determined for each construct in parallel samples that are not extracted with detergent. Failure of the majority of nuclei to immobilize the C-terminal fragments could be because the interactions that are responsible occur as part of a regulated, possibly cell-cycle stage-specific process. Consistent with this, when transfected populations were incubated for a further 24 hours the proportion with detergent resistant GFP-C275 in the nucleus increased to almost 80% of those that were transfected. Furthermore, this increase is not simply a function of time, but is dependent on cell-cycle progression, as exposure to thymidine during the second 24 hours significantly reduced the increase in the proportion of cells with immobilized Ciz1 so that it reached only 42% of transfected cells (Fig. 7B). These data strongly suggest that immobilization of Ciz1 through C-terminal interactions occurs as cells progress through a discrete point in the cell cycle. To more closely define when this transition occurs, cells were first restrained in early S phase using thymidine, then transfected with GFP-C275 before release. Samples were analysed at intervals after release (Fig. 7C), and unreleased control populations were taken at the start and the end of the time course. In unreleased cells, neither transfection frequency (not shown) nor the proportion of cells with immobilized Ciz1 changed greatly between the start of the time course (10 hours) and the end (22 hours), despite prolonged exposure to an S phase environment and gradual accumulation of C275 in the nucleus. However, released cells accumulated GFP-C275 in the insoluble fraction as they progressed through the cycle (Fig. 7D). Significant increases in the proportion of cells with immobilized Ciz1 took place in populations that were enriched in early S phase cells, reaching a peak in the 22-hour population (which contains some cells that have reached G2). Taken together the data are most consistent with incorporation of Ciz1 into insoluble nuclear structures as cells traverse S phase.

Discussion
In cell-free experiments, Ciz1 promotes initiation of DNA replication through domains encoded within the most N-terminal 435 amino acids of the ECiz1 isoform; those that remain after deletion of 273 amino acids from the C-terminal end (Coverley et al., 2005). Here, we have shown that interaction with insoluble nuclear structures is mediated through these C-terminal sequences. Thus, Ciz1 is immobilized in the nucleus via a C-terminal ‘anchor’ (Fig. 8A). Because Ciz1 can promote initiation even in the absence of its nuclear anchor we can infer that it plays a role in replication that is separate from its ability to become localized on the nuclear matrix. Thus, the major function of the C-terminal anchor may be to restrict the replication activity of Ciz1 to specific sites in the nucleus.

Over three hundred proteins are believed to be part of, or interact with, the nuclear matrix (Mika and Rost, 2005). For several of these, including Runx/AML (Vradii et al., 2005;
Zaidi et al., 2001; Zeng et al., 1997), Nxp2 (Kimura et al., 2002), Hairless (Djabali and Christiano, 2004) and Sp2 (Moorefield et al., 2006) the interaction is driven by what has been termed a ‘nuclear matrix targeting signal’ or NMTS. Whereas some NMTSs have been narrowed down to less than 50 amino acids, little sequence homology has been identified between them, and we can find no striking similarity with sequences contained within Ciz1 708-830. This is perhaps not surprising, as these proteins most likely interact with different components of the matrix, as implied by their varied functions.

The sequences responsible for immobilization of Ciz1 appear to be contained within a 122 amino acid fragment that contains the conserved zinc-finger motif (C, X2, C, X12-13, H, X5, H) first identified in the inner nuclear matrix protein matrin 3 (Matsushima et al., 1997). Therefore, this domain could represent a new class of NMTS. The MH3 domain of Ciz1 shares 36% homology with matrin 3 over 53 amino acids, and intriguingly overlaps with a 30 amino acid sequence with some similarity to a nucleic acid binding module, the SAP or SAF motif (Aravind and Koonin, 2000). First identified in SAF-A, where it specifically recognizes scaffold attachment region (SAR) DNA (Kipp et al., 2000), SAP/F modules are found in a wide range of proteins with functions in nucleic acid metabolism, and are thought to deliver associated functional domains to SAR sequences. For Ciz1, the validity and function of the SAP-like sequence has not been tested, and the similarity to the matrin-type zinc finger is more compelling.

Nevertheless, whichever of these structures exist in the native protein, they both imply that Ciz1 may also interact with nucleic acids. In fact, because scaffold- or matrix-associated DNA normally resists nuclease digestion we cannot rule out the possibility that Ciz1 interacts specifically with a protected fraction of chromatin, or indeed matrix-associated RNA. However, our salt-extraction experiments indicate that such interactions are unlikely to be solely responsible for nuclear immobilization of Ciz1.

In the case of Ciz1, fragments containing the nuclear matrix attachment domain do not confer the sub-nuclear spatial organization that is seen with the complete protein; N-terminal domains are also required to induce foci formation. This is similar to the behaviour of Sp2, for which two independent regions are required to specify correct matrix-associated patterning (Moorefield et al., 2006). Thus, it seems likely that the concept of a simple NMTS will have to give way to a more complex model in which higher order structural determinants influence patterning.

GFP-Ciz1 displays a strong tendency to become concentrated into intra-nuclear foci that are very similar in size and number to endogenous Ciz1 foci. Furthermore the rate at which ectopic Ciz1 forms foci is diminished in the embryonic splice variant, indicating that one or more of the three sequences spliced out of ECiz1 must influence their formation. Differences in the rate of foci formation may be a consequence of protein aggregation and be related to the glutamine content of Ciz1 (Kim et al., 2002). The N-terminal half of Ciz1 is glutamine rich; exon 2 encodes 53% glutamine residues, whereas exon 8 encodes 25% glutamines and both are spliced out of ECiz1. Thus, alternative splicing appears to modulate glutamine content, and could therefore influence possible aggregation of Ciz1 molecules within the nucleus.

Fig. 6. GFP-ECiz1 N471 resists detergent extraction after cross-linking with DTSP, but remains susceptible to salt extraction whereas GFP-C275 is resistant to both treatments. Twenty-four hours after transfection with the indicated C- or N-terminal Ciz1 fragments, cells were cross-linked with DTSP then extracted with Triton X-100 (left panels) or Triton X-100 with 0.5 M NaCl (right panels). Total nuclei were visualised after staining with Hoechst 33258 (blue) and those expressing GFP are indicated with arrows. Bar, 10 μm.

What is the function of Ciz1? To begin to address what its role might be we looked at the behaviour of Ciz1 in synchronised cells. We found no evidence that total Ciz1 levels vary significantly in the cell cycle, however, previous work showed that depletion of newly synthesized Ciz1 protein prevents cells from entering S phase (Coverley et al., 2005). To reconcile these observations we suggest that newly synthesized Ciz1 protein contributes relatively little to the total Ciz1 complement in the cell, but that this new protein is required for cells to replicate. We showed here that immobilization of newly synthesized C-terminal fragment
occurs in a cell-cycle-dependent manner, coinciding with its inferred point of action. Thus newly synthesized Ciz1 could act to deliver associated factors to the nuclear matrix, possibly to existing Ciz1-containing sites. We hypothesize that this might be involved in delivering origin-bound pre-replication complexes to replication factories (Fig. 8B).

Although there has been extensive analysis of the eukaryotic pre-replication complex and the proteins that regulate its assembly on chromatin, mostly from yeast and Xenopus model systems (Blow and Dutta, 2005; Diffley, 2004), published work does not adequately address how chromosomal replication origins become organized into replication factories. The pre-replication complex proteins Orc1 (part of the six subunit origin recognition complex) and Cdc6 play fundamental roles in the selection of replication origins and the subsequent assembly of pre-replication complexes in late G1 phase. Both Orc1 and Cdc6 associate with the nuclear matrix in mammalian cells (Fujita et al., 1999), but their interaction appears to be partial (Cdc6) or transient (Orc1), rather than stable and salt-resistant like Ciz1. Replication origins themselves also become transiently associated with the nuclear matrix in late G1 phase, and appear to dissociate after activation (Djeliova et al., 2001). Although it is not yet clear how this occurs and what role this association plays in initiation, Orc1 is likely to be involved, as its appearance in the cell cycle correlates with recruitment of the rest of the Orc complex to nuclease-resistant structures (Tatsumi et al., 2003).

Our observations suggest that Ciz1 is involved in a late stage of the replication initiation process. In vitro, Ciz1 antigen is most prevalent in nuclei exposed to cyclin A-cdk2 concentrations that activate DNA synthesis and which is non-permissive for Cdc6-dependent pre-replication complex assembly (Coverley et al., 2002; Coverley et al., 2005), suggesting that Ciz1 is involved in an event that coincides with activation of replication complexes. Furthermore, recombinant ECiz1 increases the number of nuclei that incorporate labelled nucleotides in vitro, indicating that it is active in a step that converts nuclei that are competent to begin DNA synthesis into nuclei that are actively synthesizing DNA. Our RNA interference studies tell a similar story because depleted cells fail to enter S phase, but accumulate after Mcm3 has assembled onto chromatin. Together these experiments indicate that Ciz1 acts after or independent from pre-replication complex assembly and are consistent with our suggestion that Ciz1 may be involved in recruiting pre-replication complex-containing replication origins to the nuclear matrix. The precise mechanism by which Ciz1 stimulates DNA replication remains to be determined, but taken together our results describe a role for Ciz1 at the interface between initiation of DNA replication and the sub-nuclear structures that organise this process.
Table 1. Amino acid composition of ECiz1, Ciz1 and derived fragments

| Fragment    | Amino acids present | Total fragment size (aa) |
|-------------|---------------------|--------------------------|
| Ciz1        | Met1-845            | 845                      |
| Ciz1N572    | Met1-572            | 572                      |
| ECiz1       | Met84-196 ^ 202-342 ^ 391-845 | 708                      |
| ECiz1N572   | Met84-196 ^ 202-342 ^ 391-572 | 435                      |
| ECiz1N471   | Met84-196 ^ 202-342 ^ 391-471 | 334                      |
| C275        | 570-845             | 275                      |
| 708-830     | 708-830             | 122                      |

Numbers refer to amino acids (aa) at fragment boundaries, relative to full-length Ciz1 (Accession number NP_082668). In all ECiz1-derived fragments residues 1-83 (exon 2), 197-201 (exon 6) and 343-390 (exon 8) are absent and indicated by chevrons. The total number of residues in each fragment is also indicated.

5 minutes after adjustment of the NaCl concentration to 0.5 M or 2 M. Soluble and insoluble fractions were separated by centrifugation and the pellets were washed once with the same buffer without DNase 1. Digested nuclei were monitored by fluorescence microscopy (600x), after addition of Hoechst 33342. For immunofluorescence analysis, cells were grown in 1 cm wells on glass coverslips. These were flooded with 100 µl of CSK with or without DNase 1 or high salt, incubated as above, then rinsed twice before fixation with 4% paraformaldehyde.

Growing cells were washed three times with phosphate-buffered saline (PBS) at room temperature, then incubated with cross-link buffer (PBS, 1 mM MgCl₂, 0.01% Triton X-100) with or without dithio-bis-succinimidyl-propionate (DTSP, Sigma) which was dissolved in dimethylsulfoxide (DMSO) and added at a final concentration of 200 µg/ml (Sigma). 15 µl of buffer was used on each 15 cm dish and incubated on a rotary shaker for 10 minutes. Reactions were stopped with 15 µl quench buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA), then rinsed in CSK before extraction with NaCl or DNase 1. The method was modified from (Fujita et al., 2002). Volumes were scaled down 15 fold for cells on coverslips.

Imaging

Images of fluorescence were taken using a Zeiss Axioscope microscope fitted with a 63x/1.40 oil immersion objective. Data was collected using an AxiosCam camera and OpenLab software. GFP fluorescence was captured with invariant exposure time (500 milliseconds) to represent the level of GFP in nuclei before and after extraction. Similarly, where the effect of nuclease extraction was recorded, images of nuclei treated with and without DNase 1 were generated under identical conditions to represent the quantity of DNA remaining in each sample. Images were manipulated using Adobe Photoshop Elements in order to reduce background to black. Where nuclear fluorescence intensity is integral to the message (Fig. 3C, Fig. 4B,C,D, Fig. 5 and Fig. 6) manipulations were applied identically to all samples including controls within an experiment. High resolution thin section (0.5 µm) images for Figs 1 and 2 were taken by confocal microscopy using a Zeiss LSM Axiolab 200M microscope fitted with a 63x water objective and Zeiss LSM510 confocal microscopy software.

Immunofluorescence

Whole or extracted cells on coverslips were fixed with 4% paraformaldehyde, rinsed twice with PBS, then blocked with 10% BSA, 0.02% SDS, 0.1% Triton X-100 in PBS. Primary antibody anti-Ciz1 1793 was used at 1:1000 as described previously (Coverley et al., 2005).

Western blot analysis

Sample volumes were adjusted to represent cell-equivalents (approximately 10⁶ per lane), separated by 8% SDS-PAGE, transferred to nitrocellulose, blocked with PBS, 10% dried milk, 0.1% Tween 20, and probed with anti-Ciz1 1793 at 1/1000 (Coverley et al., 2005), anti-MCM2 BM28 (Transduction Laboratories, Lexington, KY) at 1/100, or anti-PDNA PC10 (Santa Cruz Biotechnology) at 1/500. Blots were developed using enhanced chemiluminescence (ECL) solution (Amersham).

In vitro DNA synthesis

In vitro synthesis was performed essentially as described (Nakayasu and Berezney, 1989). Asynchronously growing cells were grown on coverslips, washed 2 x with PBS, then permeabilized in G buffer (20 mM Tris pH 7.4, 25% glycerol, 5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM PMSF) plus 0.5% Triton X-100, by incubation for 5 minutes at room temperature. Coverslips were rinsed in G buffer then overlaid with 25 µl of G-buffer supplemented with nucleotides, including biotinylated dUTP and an energy regenerating system as for in vitro initiation experiments (Krude et al., 1997) for 30 minutes at room temperature. To digest non-matrix-associated

Materials and Methods

Mammalian expression constructs

GFP-tagged mouse Ciz1 and ECiz1 were described previously (Coverley et al., 2005). GFP-Ciz1 N572 and the equivalent fragment from ECiz1 (termed GFP-ECiz1 N572), were made by ligating the 1 kb C-terminal XhoI fragment from GFP-Ciz1 and GFP-ECiz1, respectively, into the XhoI site of pEGFP-C3. Previously described versions of this fragment were inserted into a bacterial expression vector and designated pGEX ECiz1 Nterm 442 (Coverley et al., 2005). For ease of comparison between Ciz1 splice variants we have now adopted a new nomenclature that reflects the position of fragment boundaries relative to full-length Ciz1 (Table 1). For GFP-ECiz1 N471 a 1.1 kb N-terminal SalI fragment from pTriplEx-clone L (Coverley et al., 2005), digested with SalI and XhoI, and transferred into the SalI site of pEGFP-C3. GFP-C275 was made by ligating the 1 kb C-terminal XhoI fragment from pTriplEx-clone L (Coverley et al., 2005) into the XhoI site of pEGFP-C2 (Clontech). For GFP-708-830 the 0.35 kb EcoRI-BamHI fragment (spanning the MHS homology domain) was ligated into the respective sites in pEGFP-C2. All clones were checked for orientation, and reading frame was verified by sequencing.

Cell culture

NIH3T3 cells (ECACC CB2975) were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% foetal calf serum and 1% penicillin/streptomycin on coverslips. NIH3T3 cells grown on coverslips were transfected using TransIT-3T3 (Mirus, Madison, WI). To determine immobilization of GFP-Ciz1 fragments, coverslips from the same transfection were processed in parallel to reveal either the transfection frequency (no detergent), or the proportion of cells with immobilized Ciz1. (B) Speculative model showing possible role for Ciz1 as a mediator between replication factories (grey circles) and chromatin-associated replication complexes (grey barrels).
DNA, coverslips were rinsed in TBS and overlaid with 20 µl of TBS RNAse-free DNAse (Roche) at a final concentration of 1000 IU/ml. After 10 minutes at room temperature coverslips were washed with ammonium sulphate buffer (20 mM Tris pH 7.4, 0.2 M ammonium sulphate, 0.2 mM MgCl₂), followed by TBS/0.2% Tween 20, then fixed with 4% paraformaldehyde. Biotinylated dUTP was detected with streptavidin-FITC (Amersham) as described previously and DNA content in digested and undigested cells was visualized with Hoechst 33258.

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