The ORC1 homolog *orp1* in fission yeast plays a key role in regulating onset of S phase

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In a screen for new cell-cycle genes in *Schizosaccharomyces pombe* we have isolated *cdc30*, which is identical to *orp1*, a putative homolog of the *Saccharomyces cerevisiae* ORC1 gene. Analysis of the temperature-sensitive *orp1-4* and the *orp1Δ* mutants indicates that *orp1* is required at the onset of S phase for an early step of DNA replication. Orp1p is found in the nucleus and is present at a constant level throughout the cell cycle. Genetic interactions occur between *orp1* and *cdc18* and *cdc21* (an MCM homolog). Orp1p forms protein complexes with both *cdc18p* and *cdc21p* in vivo, suggesting that interactions between these proteins and ORC are important for controlling the initiation of DNA replication at the onset of S phase. The *orp1* gene is also required for the control that prevents entry into mitosis in the absence of DNA replication, suggesting a role for ORC in this checkpoint pathway.

[Key Words: ORC1 homolog; fission yeast; S-phase onset; checkpoint]

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Control of the G₁- to S-phase transition is an important regulatory point in the eukaryotic cell cycle, because successful replication of the chromosomes is a prerequisite for producing two normal daughter cells at division. Understanding how S phase is regulated requires characterization of the molecular mechanism that carries out DNA replication, and also a description of the regulatory processes that ensure that replication occurs once and only once every cell cycle. Here we characterize a fission yeast gene that may be part of the link between the mechanism of DNA replication and overall cell-cycle control.

Much progress has been made in describing the components of the eukaryotic DNA replication machinery using in vitro model systems, primarily SV40, and genetic systems, primarily budding yeast. One component of particular interest for understanding the initiation of replication is the complex of six proteins called the origin recognition complex (ORC) purified from budding yeast (Bell and Stillman 1992). The genes encoding these proteins have been cloned (Bell et al. 1993, 1995), and it has been demonstrated that the frequency of initiation events decreases in *orc* mutants (Fox et al. 1995; Liang et al. 1995). The ORC complex binds origins throughout the cell cycle (Diffley and Cocker 1992; Diffley et al. 1994), and therefore changes in origin binding of the six proteins making up the ORC complex seem unlikely to

control the initiation of replication. The genomic footprint on replication origins changes prior to replication as a result of the formation of a prereplicative complex (Diffley et al. 1994). Components of this prereplicative complex additional to the six proteins making up the ORC have yet to be defined. Homologs of two components of the ORC complex have been identified in a number of organisms: ORC1 homologs have been found in *Kluyveromyces lactis*, human cells, and *Schizosaccharomyces pombe* (Gavin et al. 1995; Muzi-Falconi and Kelly 1995), and ORC2 homologs in *Arabidopsis thaliana*, *Caenorhabditis elegans*, human cells (Gavin et al. 1995), *Xenopus laevis* (Carpenter et al. 1996), fruit fly (Gossen et al. 1995), and *S. pombe* (Leatherwood et al. 1996). The existence of these homologs suggests that the ORC complex is likely to be conserved during evolution.

In fission yeast *cdc18* is a crucial regulator of the initiation of DNA replication. The levels of *cdc18* mRNA and of p65^{cdc18} encoded by *cdc18* both increase prior to entry into S phase (Kelly et al. 1993; Nishitani and Nurse 1995; Muzi-Falconi et al. 1996). In the absence of *cdc18* the cells cannot replicate their DNA, blocking initially with a 1C DNA content and then proceeding to mitosis. This last observation indicates that p65^{cdc18} is also necessary to prevent mitosis until S phase is complete (Kelly et al. 1993). Importantly, overexpression of the *cdc18* gene is sufficient to drive cells into S phase in the absence of mitosis and without continuing protein synthesis (Nishitani and Nurse 1995), indicating that p65^{cdc18} is likely to play a rate determining role in the onset of S phase.

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The regulatory processes governing DNA replication ensure that S phase is confined to a specific phase of the cell cycle and is coordinated with other major cell-cycle events. Two main control mechanisms are emerging that have been proposed to limit DNA replication to once per cell cycle. One is the global cell-cycle control acting over entry into S phase that involves cyclin-dependent kinases (CDKs). Identification of a role of CDKs in ensuring that there is only one S phase per cell cycle was made in fission yeast. Deletion of the *cdc13* gene, which encodes the major mitotic B cyclin in fission yeast, leads to repeated rounds of replication without intervening mitoses, suggesting that the presence of the *cdc2*/cyclinB protein kinase prevents a G_2 cell from undergoing another S phase (Hayles et al. 1994). Overexpression of the *rum1* gene also results in repeated rounds of replication (Moreno and Nurse 1994) as a consequence of $p25^{rum1}$ acting as an inhibitor of the *cdc2*/cyclinB complex (Correa-Bordes and Nurse 1995; Martin-Castellanos et al. 1996). The second control mechanism has emerged from experiments with *Xenopus* extracts that have identified an activity required to "license" DNA, such that replication occurs only once in each cell cycle. Several lines of evidence suggest that proteins of the MCM family may have a role in this licensing control. MCM genes were first identified in budding yeast in genetic screens for mutants that lose minichromosomes at high frequency (Maine et al. 1984). The MCM proteins show a cell cycle-dependent localization in the yeast nucleus (Hennessy et al. 1990; Yan et al. 1993; Dalton and Whitbread 1995), and have been identified in a large number of organisms (for review, see Chong et al. 1996). In multicellular eukaryotes, MCM proteins are generally found to be nuclear throughout the cell cycle, although they show cell cycle-dependent chromatin binding (Kimura et al. 1994; Chong et al. 1995; Kubota et al. 1995; Madine et al. 1995; Todorov et al. 1995; Coué et al. 1996; Fujita et al. 1996; Krude et al. 1996). The exact role of MCMs and the mechanism by which they contribute to proper regulation of S phase is as yet unclear.

In this paper we describe the isolation of *cdc30* from fission yeast and show that it is identical to *orp1*, a gene proposed to encode a homolog of ORC1. Analysis of the phenotypes of temperature-sensitive and deletion mutants of *orp1* indicates that the gene is required at an early step of DNA replication and is also necessary to restrain mitosis until DNA replication is complete. We show genetic and physical interactions between *orp1* and *cdc18*, and between *orp1* and *cdc21*, an MCM4 homolog in fission yeast, and propose that these interactions are important for linking the initiation of DNA replication to overall cell-cycle control.

Results

Isolation of mutants acting at G_1/S

To identify new genes acting at the G_1/S -phase (G_1/S) control in fission yeast we screened a bank of 2000 temperature-sensitive lethal mutants for those that arrested

at the G_1/S transition. We isolated 172 mutants displaying an elongated phenotype at the restrictive temperature, and 65 of these were found by DAPI staining to arrest with a single interphase nucleus. These were subjected to FACS analysis to identify those which arrested with a 1C DNA content or close to a 1C DNA content, and 12 such mutants were isolated. Complementation analysis between these 12 mutants and mutants of previously described genes required at the G_1/S transition identified novel alleles of *cdc6*, *cdc23*, and *cdc20* and five new genes. The mutants in the five new genes showed various phenotypes: Two arrested with a 1C DNA content, two showed S-phase defects, and one arrested with a mixed population of cells having 1C and 2C DNA contents. One of the mutants that arrested with a 1C DNA content, *cdc30-4*, was chosen for further study because it showed uniform first cell-cycle arrest. Cell division stopped after a cell number doubling, and cells became elongated with a uniform 1C DNA content (Fig. 1).

cdc30-4 arrests early in the cell cycle

To map the cell-cycle arrest point of the *cdc30-4* mutant more accurately we carried out a reciprocal shift experiment with a hydroxyurea block. DNA replication in *cdc30⁺* and *cdc30-4* cells was inhibited using hydroxyurea at 25°C, the permissive temperature for *cdc30-4*. The cells were then shifted to the restrictive temperature 36°C, and the hydroxyurea removed. The two cultures underwent cell division with the same kinetics (Fig. 2A), indicating that *cdc30-4* functions either upstream or at the hydroxyurea block. The same result was obtained when the cells were kept at 36°C during the last hour of the hydroxyurea block to ensure that the temperature-sensitive protein was fully denatured before re-

Figure 1. *cdc30-4* arrests with a 1C DNA content. (A) *cdc30-4* cells after 5 hr at the restrictive temperature. (B) Cell number increase of *cdc30-4* cells upon shift to 36°C. (C) FACS analysis of *cdc30-4* cells at permissive temperature and 2, 4, and 5 hr after shift to the restrictive temperature.

Figure 2. *cdc30-4* arrests at an early stage of S phase. (A) Cell number increase in a hydroxyurea reciprocal shift experiment. (▲) *cdc30-4* cells; (●) *cdc30+* cells. Cells were arrested with 12 mM hydroxyurea at the permissive temperature of 25°C, then shifted to the restrictive temperature of 36°C and the hydroxyurea removed. (B) *cdc30-4* cells can conjugate from the arrest point. *cdc30-4*, *cdc10-129*, *cdc25-22*, and *leu1-32* cells were mixed with *mat2-102 ade6⁻* cells to determine the conjugation frequency as described in Materials and Methods. Open bars: incubated at 25°C, hatched bars: shifted to 36°C for 4 hr and then to 32.5°C to allow conjugation. (C) Level of *cdc18* message in *cdc30-4*. RNA was isolated from cells shifted to 36°C for 0, 3, 4, and 5 hr. *ura4* transcript level was measured as loading control. (D) Tyrosine phosphorylation of *cdc2* in *cdc30-4*. Protein extracts were prepared from cells shifted to 36°C for 0, 3, 3.5, 4, 4.5, and 5 hr for Western blotting with α -*cdc2* (upper lanes) and α -tyrosine phosphorylated *cdc2* (lower lanes). Panel on left shows *cdc30-4* extracts, panel on right shows *cdc25-22* (lane 1) and *cdc10-129* (lanes 2,3) incubated at 36°C for 4, 0, and 4 hr, respectively, to demonstrate the specificity of the antibody for the tyrosine phosphorylated form. (E) PFGE of chromosomes prepared from *orp1-4* (*cdc30-4*) cells 0, 3, 4, and 5 hr after shift to 36°C. (F) Plasmid stability in *orp1-4*. *leu1-32* and *leu1-32 orp1-4* cells were transformed with a plasmid carrying one copy of *ARS1* and a *LEU2* marker (see text). The histogram shows the number of *leu+* cells as a percentage of total cells at the time of shift back to 25°C.

removal of the hydroxyurea (data not shown). This excludes the possibility that the observed replication and cell division were due to residual activity of the temperature-sensitive protein following shift-up. The reciprocal experiment was also performed when *cdc30-4* was incubated at 36°C for 4 hr before shift to 25°C in the presence and absence of hydroxyurea. However, even the cells shifted to 25°C in the absence of hydroxyurea did not undergo cell division within a 5-hr incubation (data not shown), indicating that the *cdc30-4* cells cannot rapidly recover from the 36°C arrest. This lack of recovery pre-

vents a distinction being made as to whether *cdc30+* functions upstream or at the hydroxyurea block. From these experiments we conclude that *cdc30+* functions early in the cell cycle, either before or at the start of S phase.

Cells become committed to the mitotic cycle at the G₁/S boundary at the control called START. In fission yeast three genes have been found to operate at or before START, *cdc2*, *cdc10*, and *res1* (Nurse and Bissett 1981; Tanaka et al. 1992; Caligiuri and Beach 1993). To determine whether the *cdc30-4* mutant is committed to the cell cycle at its arrest point, we asked whether such arrested cells were able to conjugate. The conjugation efficiency of *cdc30-4* was compared with that of *cdc10-129*, which is able to conjugate from its arrest point in G₁ (Nurse and Bissett 1981). Arrested *cdc30-4* cells form about half as many diploids as *cdc10-129*, and far more than those formed with the G₂-arrested *cdc25-22* cells (Fig. 2B). Therefore *cdc30-4* appears to arrest before or at the point of commitment to the mitotic cell cycle.

cdc10 is required for the transcriptional activation of genes necessary for the initiation of S phase (Lowndes et al. 1992), including transcription of the *cdc18* gene (Kelly et al. 1993). *cdc30-4* arrests with *cdc18* mRNA present at a level comparable to that seen in exponentially growing cells (Fig. 2C), suggesting that the *cdc30-4* mutant arrests later than *cdc10-129*. Consistent with this conclusion is the observation that the tyrosine phosphorylated form of p34^{cdc2} accumulates in *cdc30-4* cells 4.5–5 hr after the shift to 36°C (Fig. 2D, lower left) when they have all arrested in G₁ (Fig. 1C). This result is in contrast to a *cdc10-129* mutant 4 hr after the shift to 36°C when no tyrosine phosphorylated form is detectable (Fig. 2D, lower right; Hayles and Nurse 1995).

Overall, these experiments establish that *cdc30* functions early in the cell cycle, close to the point of cell-cycle commitment at START and to the onset of S phase.

cdc30 encodes a homolog of ORC1

The *cdc30* gene was cloned by complementation of the temperature-sensitive lethal phenotype using a fission yeast genomic library. The gene encodes a putative protein of 707 amino acids that is 31% identical to the product of the *S. pombe cdc18* gene and 30% identical to the product of the *S. cerevisiae ORC1* gene. Recently the gene has been isolated independently by Gavin et al. (1995) and Muzi-Falconi and Kelly (1995) as *orp1*. Furthermore, a temperature-sensitive mutant has been isolated by K. Suto, H. Murakami, and H. Okayama (pers. comm.), which also defines the same gene. Given that the name *orp1* has already been used for this gene, from now on we shall use the name *orp1* instead of *cdc30*.

The *orp1-4* mutant is defective in DNA replication

The early arrest point of the *orp1-4* (originally *cdc30-4*) mutant suggests a role at the G₁/S transition. Given the sequence similarity to the *S. cerevisiae ORC1*, we inves-

tigated whether DNA replication is defective in this mutant. DNA was prepared for pulsed field gel electrophoresis from *orp1-4* cells grown at permissive and restrictive temperatures. When the cells were grown at the permissive temperature, the three chromosomes entered the gel and could be separated as three distinct bands. In contrast, when the cells were grown at the restrictive temperature, the chromosomes could not enter the gel (Fig. 2E). This indicates that the topology of the DNA is altered, suggesting that some early stages of the initiation process have occurred at the nonpermissive temperature. Since these cells have an approximately 1C DNA content, this indicates that the *orp1-4* mutant has a defect at an early stage of DNA replication.

Several *S. cerevisiae* mutants defective in DNA replication have been reported to lose episomal plasmids at high frequency (Hogan and Koshland 1992; Foss et al. 1993; Micklem et al. 1993; Loo et al. 1995). We examined whether this is also true for the *orp1-4* mutant. *orp1⁺* and *orp1-4* cells were transformed with a plasmid carrying one copy of ARS1 and a selectable marker. The transformants were shifted to the restrictive temperature and selection for the plasmid relaxed. After 4 hr at 36°C the cells were shifted to the permissive temperature and plated onto appropriate media to determine the rate of plasmid loss. While the temperature shift had no effect on plasmid loss in *orp1⁺* cells, less than a quarter of the *orp1⁻* cells retained the plasmid after the 4-hr incubation at 36°C (Fig. 2F). This result indicates that the *orp1* gene function is required for efficient replication of plasmids.

Synthetic checkpoint defects in *orp1-4 cdc18-K46* and *orp1-4 cdc21-M68* double mutants

Between 3 to 5 hr after shifting the *orp1-4* mutant to the restrictive temperature, the cell number increase reached a plateau, and after this time, from 5 to 8 hr, cell number increased again (Fig. 3Aa). This increase was due to mitosis and cell division as judged by spindle formation and the appearance of postanaphase arrays (Fig. 3Ab,c). Despite undergoing mitosis, the cells had still failed to undergo DNA replication, and divided with a 1C DNA content, producing daughter cells with an uneven distribution of DNA (Fig. 3Ac). This so-called "cut" phenotype was eventually exhibited by up to 50% of the population (Fig. 3Aa). This suggests that in addition to being required for DNA replication, the *orp1* gene also may have some role in the checkpoint pathway that prevents mitosis until S phase is complete.

This G₁ arrest and cut phenotype of the *orp1^{ts}* mutant is reminiscent of that observed with *cdc18Δ*. Therefore we asked whether there was a genetic interaction between *orp1* and *cdc18*. We constructed a double mutant carrying both the temperature-sensitive *cdc18-K46* and *orp1-4* alleles. The temperature-sensitive allele *cdc18-K46* is leaky, and unlike the full deletion of *cdc18* it arrests with an approximately 2C DNA content and fails to enter mitosis (Nasmyth and Nurse 1981). While the *orp1-4* mutant arrests at G₁/S and only then proceeds

Figure 3. Synthetic checkpoint defect in the double mutants *orp1-4 cdc18-K46* and *orp1-4 cdc21-M68*. (A) *orp1* is required for the checkpoint preventing entry into mitosis until S phase is complete. (a) Cell number increase (shaded boxes) and percent of cut cells (Δ) in *orp1-4* following shift to the restrictive temperature. (b) DAPI-stained cells 7 hr after shift up. Cell 1 arrested in interphase, cells 2–4 cut. (c) DAPI (left) and tubulin (right) staining of cells 7 hr after shift to the restrictive temperature. Cell 1 shows a postanaphase array, cell 2 contains a mitotic spindle, and chromatin is distributed unevenly. Bar, 10 μ m. (B) Genetic interaction between *orp1* and *cdc18*. Cell number increase (solid lines) and % cut cells (dashed lines) after shift to the restrictive temperature. (Shaded boxes) *orp1-4*; ($\blacklozenge$) *cdc18-K46 orp1-4*; (Δ) percent cuts in *orp1-4*; ($\ast$) percent cuts in *cdc18-K46 orp1-4*; ($\times$) percent cuts in *orp1-4* overexpressing *cdc18⁺*. (C) Genetic interaction between *orp1* and *cdc21*. Cell number increase (solid lines) and percent cut cells (dashed lines) after shift to the restrictive temperature. (Shaded boxes) *orp1-4*; ($\blacklozenge$) *cdc21-M68 orp1-4*; (Δ) percent cuts in *orp1-4*; ($\ast$) percent cuts in *cdc21-M68 orp1-4*; ($\oplus$) percent cuts in *cdc21-M68*.

slowly to mitosis and cell division, the double mutant proceeds rapidly to mitosis and cell division (Fig. 3B). Overexpression of *cdc18* in the *orp1-4* mutant rescues the checkpoint defect (Fig. 3B). These observations sug-

gest that *orp1* and *cdc18* might interact in the process that prevents mitosis in the absence of DNA replication.

In addition to the synthetic checkpoint defect of *cdc18* and *orp1*, a genetic interaction was also observed between *orp1* and *cdc21*, which encodes the fission yeast homolog of MCM4 (Coxon et al. 1992). The *cdc21-M68* and *orp1-4* mutants are synthetically lethal; at 29°C the double mutant is unable to form colonies, unlike the single mutants. Furthermore, the checkpoint phenotype of the *cdc21-M68 orp1-4* double mutant at 36°C is enhanced, although only a smaller percentage of 1C cells undergo mitosis (Fig. 3C) compared with the *cdc18-K46 orp1-4* double mutant.

These results establish that *orp1* plays a role in preventing mitosis when S phase is incomplete, and are suggestive that *orp1* may interact with *cdc18* and the MCM4 homolog *cdc21* in bringing about this checkpoint control.

Characterization of *orp1Δ* and *orp1* overexpressing strains

We deleted the region coding for amino acids 195–607 of *orp1* and replaced it with the *ura4⁺* gene; the deletion removes the region most highly conserved between *cdc18* and *ORC1*. One of the wild-type copies of the gene was replaced with the deletion construct in a diploid strain homozygous for *ura4-D18*. When the diploid was sporulated and tetrads analyzed, only two spores were able to form colonies. Both of these were *ura4⁻*, showing that the *orp1* gene is essential. To analyze the phenotype of the deletion mutant, the diploid was sporulated and the spores inoculated into medium that allows germination of only the spores carrying the deletion. The *orp1Δ* spores germinated and underwent the first cell cycle with a similar kinetics to *orp1⁺* spores (Fig. 4A), but at later timepoints they underwent cell-cycle arrest. However, unlike the temperature-sensitive *orp1-4* mutant, the deletion mutant cells arrested with a 2C DNA content. Furthermore, only a few percent of the cells showed aberrant nuclear morphology indicative of a premature entry into mitosis, even 18 hr after inoculation (Fig. 4A). These results are similar to the findings of Muzi-Falconi and Kelly (1995).

We were concerned that the phenotype of the temperature-sensitive mutant was different from that of the *orp1* deletion. The spores carrying the deletion underwent at least one complete normal cell cycle after germination, indicating that the spores might have contained residual *orp1* protein carried over from the parental diploid into the spore, masking expression of the deletion phenotype. We therefore constructed a diploid strain heterozygous for the deletion and the temperature-sensitive *orp1-4* allele. Spores derived from this diploid were germinated at the restrictive temperature to inactivate any residual temperature-sensitive *orp1* protein. The spores carrying the *orp1Δ* arrested in G₁ and then proceeded to mitosis primarily with a 1C DNA content, with over 90% of cells displaying the cut phenotype 10 hr after inoculation (Fig. 4B). This result confirmed

Figure 4. Deletion and overexpression of the *orp1* gene. (A) FACS analysis of *orp1⁺* (left) and *orp1Δ* (right) spores germinating at 30°C. DAPI-stained cells are shown 18 hr after inoculation. The arrowheads point at rare cells with aberrant nuclear morphology. Bar, 10 μ m. (B) FACS analysis of *orp1⁺* (left) and *orp1Δ* spores carrying the temperature-sensitive *orp1-4* protein (right), germinating at 36°C. Smearing of the FACS profile is often seen in germinating spores undergoing cutting. DAPI-stained cells are shown 10 hr after inoculation. Arrowheads point at cells showing the cut phenotype. Bar, 10 μ m. (C) Overexpression of the *orp1⁺* gene from the *nmt1* promoter. (Left) FACS of cells grown in the presence of thiamine (promoter off). (Middle) FACS analysis of cells 22 hr after induction. (Right) DAPI-stained cells 22 hr after induction. Bar, 10 μ m.

our conclusions drawn from the phenotype of the temperature-sensitive mutant.

We next tested the effect of overexpressing *orp1* to high levels in cells. The *orp1* gene was expressed using the full-strength *nmt1* promoter. Cells become elongated with a 2C DNA content and the nuclei showed an unusual, enlarged, diffuse morphology (Fig. 4C). The cells cannot form colonies and thus high levels of the *orp1* gene product are lethal.

Regulation of *orp1* transcription, protein level, and intracellular localization

To investigate whether *orp1* is regulated at the transcriptional level during the cell cycle, the *orp1* message level was measured in cells arrested at various cell-cycle stages, using cell-cycle mutants and hydroxyurea. The stage of the cell-cycle block is shown in brackets in the following list. The level of the *orp1* transcripts was the same in cells arrested with the *cdc10-V50* mutant (G_1), *cdc25-22* mutant (G_2/M), hydroxyurea (S), *nda3-KM311* mutant (metaphase), and *dis3-54* mutant (anaphase) (Fig. 5A), indicating that there was unlikely to be any periodic changes in transcript level during the cell cycle. To follow the protein level during the cell cycle the *orp1* gene was tagged on its carboxyl terminus with a triple HA tag. The *orp1* gene carrying the HA tag under control of the *orp1* promoter was introduced into the chromosome by gene replacement, and this strain is referred to as *orp1-HA* (Fig. 5B). The apparent molecular weight of the tagged protein is 90 kD. The protein level was monitored during the cell cycle in a *cdc25^{ts}* block and release experiment to synchronize the cells. The level of *orp1* protein did not change significantly during the cell cycle as cells proceeded through mitosis and S phase, either in soluble (Fig. 5C, top) or insoluble (Fig. 5C, bottom) fractions (see Materials and Methods) of protein extracts.

To investigate the intracellular localization of *orp1p*, exponentially growing *orp1-HA* cells were fixed and examined by immunofluorescence. Consistent with *orp1p* being a component of the ORC complex, the protein was localized primarily to the nucleus with some weak staining in the cytoplasm (Fig. 5D). However, we did not detect the *orp1* protein in cells undergoing mitosis (Fig. 5D, arrowed cells). This may be a result of an altered exposure of the epitope, possibly because of chromatin condensation, or a result of relocalization of the protein from the nucleus into the cytoplasm during mitosis. As a control, wild-type cells lacking *orp1-HA* were examined in a similar way by immunofluorescence, and although the cytoplasmic staining was detected and so is presumably nonspecific, no distinct nuclear staining was observed (Fig. 5D, inserts).

orp1p interacts physically with *cdc18p* and *cdc21p*

To investigate whether the genetic interactions between the *cdc18*, *cdc21*, and *orp1* genes result from direct protein-protein interactions, protein extracts prepared from protoplasts were immunoprecipitated with α -HA antibody and Western blotted with α -cdc18p (gift of H. Nishitani, Imperial Cancer Research Fund, London, UK) and α -cdc21p antibodies (gift of D. Maorano and S. Kearsey, Oxford University, UK). The *orp1-HA* cells were used, and wild-type and *cig2-HA* cells used as controls. Extracts from all three strains were Western blotted using the α -cdc18p (Fig. 6A, lanes 5–7) and α -cdc21p (Fig. 6B, lanes 5–7) antibodies. Both antibodies detected proteins of the correct molecular weight, confirming previous characterization carried out by Nishitani and Nurse

Figure 5. Cell-cycle regulation of the *orp1* gene. (A) Transcript level of *orp1* in cell-cycle blocks (see text). The upper panel shows Northern blot analysis of the *orp1* transcript, the lower panel shows the ethidium bromide-stained gel as loading control. (B) Detection of the HA-tagged *orp1*. Protein extract was prepared from exponentially growing *orp1-HA* and untagged wild-type cells, run on SDS-PAGE gel, and Western blotted with α -HA antibody. (C) Level of the *orp1* protein during the cell cycle. *cdc25-22 orp1-HA* cells were arrested in G_2 at the restrictive temperature of 36°C for 4 hr, then released at 25°C. Samples were taken every 10 min after the release. The graph shows the septation index during the release and S phase occurs at ~60–80 min. Protein was loaded from the soluble (upper two panels) and insoluble (lower two panels) fractions of the cell extracts for Western blotting. α -tubulin antibody was used to check even loading. (D) Immunofluorescence of exponentially growing *orp1-HA* cells. Upper panels show α -HA immunofluorescence; lower panels show DAPI staining. The arrowheads point to anaphase cells. Inserts show untagged control cells. Bar, 10 μ m.

(1995) and Maorano et al. (1996). The extracts were also Western blotted with α -HA antibodies that detected proteins of the correct molecular weight in extracts from the *orp1-HA* and *cig2-HA* stains but not wild-type (Fig. 6C, lanes 5–7).

The *orp1-HA* protein was immunoprecipitated from extracts of the *orp1-HA* strain, and this immunoprecipitation could be competed by excess HA peptide (Fig. 6, lanes 1,2). Both *cdc18p* (Fig. 6A, lane 2) and *cdc21p* (Fig. 6B, lane 2) could be detected in the *orp1-HA* immunoprecipitation but neither was present in the HA peptide

Figure 6. The *orp1* protein interacts with the *cdc18* and *cdc21* proteins. Protein extracts were prepared from exponentially growing *orp1-HA* (lanes 1,2,5) untagged wild-type (lanes 3,6) and *cig2-HA* (lanes 4,7) cells. α -HA immunoprecipitates from 10-mg soluble extracts were run in lanes 1–4. 50 μ g soluble extracts were run in lanes 5–7. The *orp1* protein was immunoprecipitated both in the presence (lane 1) and in the absence (lane 2) of HA peptide. The samples were run on SDS–PAGE gel, Western blotted, and probed with α -*cdc18* (A); α -*cdc21* (B); and α -HA (C) antibodies.

competition control (Fig. 6A,B, lane 1). Immunoprecipitation from an extract of either wild-type or *cig2-HA* cells failed to bring down either *cdc18p* or *cdc21p* (Fig. 6, lanes 3,4). These controls indicate that the complexes found between *orp1-HA* and *cdc18p* and *cdc21p* are specific. We also attempted to do the reciprocal immunoprecipitation experiments using α -*cdc18p* antibodies, but this antibody failed to immunoprecipitate effectively. We conclude that at least some fraction of the *cdc18*, *cdc21*, and *orp1* proteins interact physically in vivo.

Discussion

In this paper we characterize a gene that we propose has a role at the onset of S phase in fission yeast. The gene, initially called *cdc30*, was found to be identical to the recently described *orp1* gene that encodes a protein homologous to *S. cerevisiae* ORC1 (Gavin et al. 1995; Muzi-Falconi and Kelly 1995). Given that *orp1* already has been described, and the name is more consistent with the nomenclature in budding yeast where the ORC complex is best characterized, we use the name *orp1* instead of *cdc30*.

We have presented several lines of evidence that *orp1* acts at the G_1 /S-phase transition. Analysis of temperature-sensitive and deletion mutants of *orp1* show that they arrest with a 1C DNA content, prior to bulk DNA synthesis. This result is consistent with the fact that the

arrest point is at or before the arrest point of the DNA synthesis inhibitor, hydroxyurea. The chromosomes prepared from the temperature-sensitive *orp1-4* mutant incubated at the restrictive temperature fail to enter the gel during pulsed field gel electrophoresis, indicating that at the arrest point some topological changes of the DNA have occurred that prevent entry into the gel. The *orp1-4* mutant also fails to maintain autonomously replicating episomal plasmids, indicating a defect in DNA replication. These various observations establish that *orp1* acts at the G_1 /S-phase transition at a stage close to the initiation of DNA replication. Given the homology of *orp1* to ORC1, we conclude that an equivalent of the ORC complex is present in fission yeast and that this has an important role in the initiation of DNA replication. Similar conclusions have been suggested by others (Gavin et al. 1995; Muzi-Falconi and Kelly 1995; Leatherwood et al. 1996).

We also have presented evidence that *orp1* interacts genetically with both *cdc18* and *cdc21*, the latter showing homology to the *MCM4* gene from budding yeast. The associations between *orp1*, *cdc18*, and *cdc21* indicated by these genetic interactions have been confirmed by immunoprecipitation experiments. Immunoprecipitates of HA-tagged *orp1p* from cell extracts contain the proteins encoded by both *cdc18* and *cdc21*. In these experiments all three genes are controlled by their endogenous promoters and thus the three proteins are expressed at normal levels. Therefore the observed interactions are not a result of an artefact of overexpression. We conclude that *orp1p* forms complexes in vivo with both $p65^{cdc18}$ and the MCM homolog *cdc21p*, suggesting that these latter two proteins become associated with the ORC complex. Expressing $p65^{cdc18}$ to high levels is sufficient to drive cells into DNA synthesis, and the level of $p65^{cdc18}$ is periodic during the cell cycle, peaking at the G_1 - to S-phase transition (Nishitani and Nurse 1995; Muzi-Falconi et al. 1996). In contrast, we have shown here that the levels of *orp1p* are constant during the cell cycle; *orp1p* is also found in the nucleus, although it may undergo some change during mitosis that makes it less easy to detect by immunofluorescence. Given the physical association between *orp1p* and $p65^{cdc18}$ reported here and the interaction between *orp2p* and $p65^{cdc18}$ reported by Leatherwood et al. (1996), we propose that the increase in level of $p65^{cdc18}$ at G_1 /S results in $p65^{cdc18}$ becoming associated with *orp1p* and *orp2p*, transiently activating a putative ORC and bringing about the initiation of DNA replication. Sometime toward the end of S phase, $p65^{cdc18}$ levels drop, and thus further activation of ORC complexes is prevented. The *MCM4* homolog *cdc21* encodes a protein that also appears to be complexed with *orp1p*. Given its role in other systems, *cdc21p* may be one of the components that distinguish G_1 but not G_2 cells to undergo DNA replication. The biochemical mechanisms involved in these processes are unclear, but *orp1p* contains a nucleotide binding site and so its action may involve ATP or GTP hydrolysis.

The proposal that associations between $p65^{cdc18}$,

cdc21p, and ORC are important steps in the initiation of DNA replication can be incorporated into models of S-phase control presented by ourselves and others (Liang et al. 1995; Nishitani and Nurse 1995; Cocker et al. 1996; Wuarin and Nurse 1996). The association between *p65^{cdc18}* and *orp1p* may be necessary to generate a pre-replicative complex similar to that proposed for budding yeast. The formation of this complex during *G*₁ of budding yeast requires the *CDC6* gene function (Cocker et al. 1996), which is a close homolog of *cdc18*, and other genes may also be involved such as those encoding the MCM proteins. This process would license the DNA for replication. The generation of this complex would be the first stage in a two-step process for the initiation of DNA replication, with the second step being the onset of DNA replication. The fall in *p34^{cdc2}* protein kinase activity as cells exit mitosis and enter *G*₁ would allow the association to occur and for the prereplicative complex to form on DNA origins. The complex could be similar to that found in *Xenopus* nuclei when *p34^{cdc2}* is inactivated (Adachi and Laemmli 1994). After this first step is completed the complex requires phosphorylation by *p34^{cdc2}* to bring about DNA replication, this process being the second step in the initiation of DNA replication. The rise in late *G*₁ of *p34^{cdc2}* protein kinase activity or of other S-phase promoting CDKs would bring about this second step and automatically prevent the formation of any further prereplicative complexes. As a consequence DNA would only be replicated once in any S phase. Such a model (Wuarin and Nurse 1996) could explain licensing and also links a number of molecules implicated in this control, including *p34^{cdc2}*, *p65^{cdc18}* (*CDC6*), *orp1p* (*ORC1*), *orp2p* (*ORC2*), and *cdc21p* (*MCM4*).

In addition to being required for initiation of DNA replication, the *orp1* gene is also required for the checkpoint pathway that prevents entry into mitosis until S phase is complete. The phenotypes of both the *orp1* temperature-sensitive and deletion mutants are similar to that of *cdc18Δ*, and several other mutations such as *cdc21Δ*, *cdt1Δ*, *cut5/rad4*, and *pol1Δ*, which not only block DNA replication but also allow the cells to enter mitosis with unreplicated DNA (Saka and Yanagida 1993; Hofmann and Beach 1994; D'Urso et al. 1995; Maiorano et al. 1996). We have also shown that the *cdc18^{ts} orp1^{ts}* and *cdc21^{ts} orp1^{ts}* double mutants show a synthetic checkpoint defect, and that the corresponding proteins interact physically within the cell. These observations suggest that a complex involving these proteins is necessary to generate a signal that S phase is in progress and as a consequence onset of mitosis should be blocked. The effects of the *orp1^{ts}*, *cdc18^{ts}*, and *cdc21^{ts}* single mutants are less severe than the double mutants or the corresponding null mutants. This may be because the single temperature-sensitive mutants have a less severe effect on the formation of the complex, which allows some defective DNA replication to take place and still generates a checkpoint signal that partially blocks mitosis. More severe defects produced by the double temperature-sensitive or null mutants might disturb formation of the complex more profoundly, blocking DNA

replication and preventing the checkpoint signal from being generated.

We have also shown that the *orp1-4* mutant is not yet committed to the mitotic cycle at its arrest point. Unlike the situation for previously identified mutants arresting pre-START, in *orp1-4* cells DNA replication has been initiated at the arrest point as judged from the inability of the chromosomes to enter the gel during pulsed-field gel electrophoresis (PFGE). This observation indicates that initiation of DNA replication does not require complete commitment to the mitotic cycle because cells can still be diverted away from the mitotic cycle to undergo conjugation. The window during which cells are able to undergo conjugation appears to extend into S phase. This finding suggests that the traditional view of START as an event in late *G*₁ that commits the cell to the mitotic cycle might require some re-evaluation. START is perhaps best considered as a window early in the cell cycle, during which cells can be diverted to undergo the alternative developmental pathway of conjugation. As cells proceed further toward S phase they complete more and more events required for progression through the cell cycle. Eventually sufficient events are completed for the cell to be put in a state that is incompatible for conjugation. Our experiments indicate that this window may be not closed until sometime just after the initiation of DNA replication. It is also possible that cells blocked using *orp1-4* are initially in a committed state for the mitotic cycle but that this state is reversible and cells can eventually slip back to the pre-START state from which they can conjugate. However, operationally it is very difficult to distinguish between these different interpretations.

We conclude that *orp1p* is required for the onset of S phase and plays a role at an early step in the initiation of DNA replication. *Orp1p* is localized to the nucleus, consistent with a role in DNA replication, but its level remains unchanged through the cell cycle. However, *orp1p* is found physically associated with both *p65^{cdc18}* and *cdc21p*, and so a periodic change during the cell cycle in these protein associations could be important for controlling the onset of S phase. *Orp1p* is also required to signal to the cell that S phase is in progress, implicating this early step in the initiation of DNA replication in this checkpoint control. Given that *orp1p* is likely to be a component of ORC, these results indicate an important role for interactions between *cdc18p*, *cdc21p*, and ORC in controlling the initiation of DNA replication at the onset of S phase and in the associated checkpoint controls.

Materials and methods

S. pombe strains and methods

All strains were derived from the wild types L972 *h*[−] and L975 *h*⁺. Media and growth conditions were as described by Moreno et al. (1991). The permissive and restrictive temperatures used for the heat sensitive mutants *cdc18-K46*, *cdc21-M68* (Nasmyth and Nurse 1981), *cdc25-22* (Fantes 1979), *cdc10-129* (Nurse et al. 1976), and *cdc10-V50* (Marks et al. 1992) were 25°C and 36°C,

respectively. The permissive and restrictive temperatures used for the cold-sensitive mutants *nda3-KM311* (Hiraoka et al. 1984) and *dis3-54* (Ohkura et al. 1988) were 32°C and 20°C, respectively. Standard genetic techniques, 4', 6-diamidino-2-phenylindole (DAPI) staining, and cell number counting were carried out as described previously (Moreno et al. 1991). Cells were prepared for FACS analysis as described previously (Sazer and Sherwood 1990). Mating assays were carried out as described previously (Nurse and Bissett 1981) with the following modifications: Cells were grown to mid-log phase in minimal medium containing 2 mg/ml NH₄Cl. They were then washed and shifted to 36°C in minimal medium without a N-source. Samples were taken for FACS analysis at the time of shift to check that the cells were not accumulating in G₁ due to N starvation. After 4 hr the mating partners were mixed and shifted to 32.5°C to allow conjugation for 7 hr, then plated onto appropriate media to determine the number of diploid cells. Immunofluorescence was carried out as described (Moreno et al. 1991). Cells were fixed with methanol. TAT1 antibodies (gift from Keith Gull, Manchester University, UK) and CY3 conjugated goat antimouse secondary antibodies (Sigma) were used for tubulin staining. 12CA5 antibodies (Boehringer) and CY3 conjugated goat antimouse secondary antibodies were used to examine the localization of orp1-HA.

Cloning and sequencing

The genomic clone of *orp1*⁺ was obtained by transforming the *orp1-4 ura4-D18 h*⁻ strain with a genomic library in pUR19 provided by A.M. Carr (Barbet et al. 1992). Transformants were plated on minimal medium, incubated at 25°C overnight, and then shifted to 36°C. Plasmids were reisolated from 12 colonies which contained three overlapping clones. Sequencing was carried out using the Sequenase II and ΔTaq cycle sequencing kits United States Biochemical. Alignments were carried out using the GAP program of the GCG package of sequence software.

PFGE

Agarose plugs were prepared as described by Kelly et al. (1993). PFGE was carried out in 0.6% chromosomal grade agarose gel (BioRad), using a BioRad CHEF-DRII. The gel was run for 72 hr at 50 V with a switch time of 30 min, in 0.5× TAE.

Deletion and spore germination

A fragment carrying the *orp1:ura4*⁺ construct with 1 kb of flanking sequence was transformed into a diploid strain *ade6-M210/ade6-M216 ura4-D18/ura4-D18 h*⁺/*h*⁻. Stable transformants were isolated and gene replacement was confirmed by Southern analysis. Diploid cultures of the strain carrying the deletion and the wild-type control *ade6-M210/ade6-M216 ura4*⁺/*ura4-D18 h*⁺/*h*⁻ were sporulated in minimal medium lacking a nitrogen source. Spores were germinated in minimal medium at 30°C (*orp1*⁺/*orp1Δ*) or 36°C (*orp1*^{ts}/*orp1Δ*).

HA tagging of *orp1*

A restriction site for NotI was introduced before the STOP codon of the *orp1* ORF and was used to insert a fragment encoding three copies of the HA tag. A genomic fragment containing *orp1-HA* was transformed into the *orp1-4* mutant and transformants were incubated at 36°C. Integration was confirmed by Southern analysis.

Preparation of protein extracts, immunoprecipitations, and Western blots

The soluble and insoluble fractions were prepared as described previously (Nishitani and Nurse 1995). For immunoprecipitations the cells were protoplasted with Novozyme in 0.65 M KCl, washed in 1 M sorbitol and lysed in HB buffer. HB buffer contains 60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 25 mM MOPS (pH 7.2), 1% Triton, 15 mM MgCl₂, 15 mM EGTA, 1 mM DTT, 0.1 mM sodium-vanadate, 1 mM PMSF, 20 mg/ml leupeptin, and 20 mg/ml aprotinin. The lysate was incubated on ice for 30 min, then centrifuged at 15000 rpm using an Eppendorf microfuge for 20 min at 4°C. Ten milligrams of the supernatant was used for immunoprecipitations. orp1-HA was immunoprecipitated with 12CA5 Mab cross-linked to Dynabeads coated with α-mouse secondary antibody (Dyna). Protein extracts and immunoprecipitates were electrophoresed using an 8% SDS-polyacrylamide gel. Dilutions of the antibodies were 1:2000 for α-cdc18, 1:500 for α-cdc21, and 1:1000 for α-HA, and proteins were detected using ECL (Amersham).

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