Apc10 and Ste9/Srw1, two regulators of the APC–cyclosome, as well as the CDK inhibitor Rum1 are required for G₁ cell-cycle arrest in fission yeast

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Many eukaryotic cells arrest the cell cycle at G₁ phase upon nutrient deprivation. In fission yeast, during nitrogen starvation, cells divide twice and arrest at G₁. We have isolated a novel type of sterile mutant, which undergoes one additional S phase upon starvation and, as a result, arrests at G₂. Three loci (*apc10*, *ste9/srw1* and *rum1*) were identified. The *apc10* mutants, previously unidentified, show, in addition to sterility, temperature-sensitive growth with defects in chromosome segregation. *apc10*⁺ is essential for viability, encodes a conserved protein (a homologue of budding yeast Apc10/Doc1) and is required for ubiquitination and degradation of mitotic B-type cyclins. Apc10 does not co-sediment with the 20S APC–cyclosome, a ubiquitin ligase for B-type cyclins, and in the *apc10* mutant the 20S complex is intact, suggesting that it is a novel regulator for this complex. A subpopulation of Apc10 does co-immunoprecipitate with the anaphase-promoting complex (APC). A second gene, *ste9⁺/srw1⁺*, encodes a member of the fizzy-related family, also regulators of the APC. Finally, Rum1 is a cyclin-dependent kinase (CDK) inhibitor which exists only in G₁. The results suggest that dual downregulation of CDK, one via the APC and the other via the CDK inhibitor, is a universal mechanism that is used to arrest cell cycle progression at G₁.

Keywords: APC/CDK-inhibitor/cyclin/cyclosome/fission yeast/G₁-arrest

Introduction

The molecular bases of commitment to the proliferative cycle or controlled cell-cycle arrest are of fundamental importance in eukaryotic growth control. Depending on the external cues, dividing cells cease division and arrest at a specific cell-cycle stage. Negative growth factors, tumour suppressor genes and deleterious stress all lead to such stage-specific cell-cycle arrest. For example, during development in multicellular organisms, developing cells arrest at G₁ before S phase in response to nutritional deprivation or differentiation stimuli following a limited number of cell divisions. Mutations that are unable to do this result in a failure to differentiate normally (Thomas

et al., 1994; Kipreos *et al.*, 1996). Exposure to DNA-damaging agents or spindle drugs results in a transient G₁ or G₂ arrest or mitotic blockage, respectively, to ensure the repair of DNA lesions or the fidelity of chromosome segregation (collectively called checkpoint; Hartwell and Weinert, 1989; Murray, 1995). At the heart of cell-cycle regulation lie a number of master regulating cyclin-dependent kinases (CDKs) and their regulatory partner cyclins (Nurse, 1990). Cells have developed a number of molecular strategies to regulate CDK activities and the endpoint of checkpoint pathways often lies in regulation of CDKs (Morgan, 1995; Nurse, 1997; Hwang *et al.*, 1998; Kim *et al.*, 1998).

Recent molecular analysis has shed light upon the importance of the ubiquitin-dependent proteolysis in the regulation of cell-cycle progression, which is catalysed by a large mechanochemical complex called a proteasome. Ubiquitin, a 76-residue ubiquitous protein, acts as a recognition tag for proteasome-dependent proteolysis (Hochstrasser, 1996; Hershko, 1997). Ubiquitin is transferred to substrate proteins in a multiubiquitinated form via a series of enzymatic reactions comprising E1 (a ubiquitin-activating enzyme), E2 (a ubiquitin-conjugating enzyme) and sometimes E3 (a ubiquitin ligase). E3 is a crucial determinant of substrate selectivity and the timing of degradation. Classical E3s include Ubr1, which targets proteins for degradation by the N-end rule pathway (Varshavsky, 1996), and HECT-domain-containing proteins such as E6AP, which is responsible for the ubiquitination of p53 in the presence of human papilloma virus-encoded protein E6 (Huibregtse *et al.*, 1995). Recent analysis indicates that there are more E3s than was originally thought, both HECT-related members and other novel E3s.

The anaphase promoting complex (APC)–cyclosome is a large E3 complex which was discovered as a factor required for the ubiquitin-dependent degradation of B-type cyclins at anaphase (Hershko *et al.*, 1994; King *et al.*, 1995; Sudakin *et al.*, 1995). In sharp contrast to classical E3s, which are composed of a single component, the APC–cyclosome is a 20–22S multiprotein complex. Recent molecular analyses, both genetic and biochemical, have begun to identify components of this complex. Many of the components isolated to date are well conserved in evolution from yeasts to vertebrates (Irniger *et al.*, 1995; King *et al.*, 1995; Tugendreich *et al.*, 1995; Peters *et al.*, 1996; Yamashita *et al.*, 1996; Zachariae *et al.*, 1996, 1998; Yamada *et al.*, 1997; Yu *et al.*, 1998). These include members of a TPR-repeat family, Cdc27/Nuc2/Apc3, Cdc16/Cut9/Apc6, Apc7 and Cdc23/Apc8, and non-TPR members such as Cut4/Apc1, Rsi1/Apc2, Apc4 and Apc5. Apart from the E3 activities of the APC–cyclosome as a whole complex, however, the specific or distinct functions, if any, of each subunit in the ubiquitination reaction remain

to be addressed. Moreover, mechanisms of how the APC–cyclosome is activated and inactivated during cell-cycle progression are unknown.

Mitotic cyclins were the first substrates to be identified for the APC–cyclosome, the destruction mechanisms of which have been most extensively characterized (Glutzer *et al.*, 1991; King *et al.*, 1996). A nine-residue sequence in B-type cyclins is recognized for ubiquitination and degradation, and is called the ‘destruction box’. The destruction box exists not only in B-type cyclins but also in several other proteins including budding yeast Pds1 and Ase1 and fission yeast Cut2, which have also been shown to be degraded via the APC–cyclosome (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996; Juang *et al.*, 1997). Accordingly, the APC–cyclosome is required for the timely destruction of a group of proteins whose role is to induce anaphase, by different means, including inactivation of CDK/cyclins, sister chromatid separation and spindle morphogenesis.

Fission yeast cells arrest cell-cycle progression at G₁ phase upon nutritional starvation. This arrest is a prerequisite for sexual differentiation (Egel and Egel-Mitani, 1974; Kumada *et al.*, 1995). We have previously proposed the existence of mechanisms that actively restrain DNA replication under starvation. This proposal was based on the analysis of sterile mutant *nuc2-663* (Kumada *et al.*, 1995). In *nuc2-663* mutant cells, instead of arresting at G₁, one additional S phase occurs under starvation conditions, and results in a G₂ arrest with defects in sexual differentiation. Interestingly, Nuc2 is also required for the mitotic cycle, since *nuc2-663* shows, in addition to sterility, temperature-sensitive growth with metaphase arrest at the restrictive temperature (Hirano *et al.*, 1988). It is of particular interest that a subsequent study showed that Nuc2, a homologue of budding yeast Cdc27 and vertebrate Apc3, is an ubiquitous subunit of the APC–cyclosome (King *et al.*, 1995; Yamada *et al.*, 1997). This has prompted us to perform systematically a novel genetic screen, in the hope that we could identify more genes that are involved in the degradation of B-type cyclins in order to maintain the G₁ arrest required for sexual differentiation.

In the current study, we have undertaken a large-scale screen of mutants which show phenotypes similar to *nuc2*, and are sterile due to an inability to arrest in G₁ phase. We have identified *apc10*⁺, which encodes a novel regulator of the APC–cyclosome. In addition, genetic analysis of isolated mutants adds support for the simple scheme, in which G₁ cell-cycle arrest requires dual approaches to the downregulation of CDK activity, one acting via the degradation of B-type cyclins and the other via a direct physical inhibition by the CDK inhibitor.

Results

Isolation of novel types of sterile mutants which fail to arrest at G₁, with defects in the inhibition of S phase

In order to identify systematically genes that are required for proper cell-cycle arrest at G₁ phase following nitrogen starvation, we undertook a large-scale screen for sterile mutants. We focused on mechanisms that restrain DNA replication under starvation conditions. To isolate mutants which are defective in this block to DNA replication, we

chose the following strategy. Sterile mutants were first isolated by iodine staining and then the DNA content of each mutant under starvation conditions was measured with flow cytometry (FACS). To distinguish mutants which are defective in restraining DNA replication from those defective in entering mitosis, which also arrest at G₂ (Shiozaki and Russell, 1996), the morphology was examined. We sought strains that arrested at a size as small as that of the wild type (<8 µm), which usually divides twice and arrests at G₁ before S phase; mutants that are defective in the G₂/M transition typically arrest at a larger cell size (Kumada *et al.*, 1995).

A total of 1008 independent sterile mutants were identified following iodine staining (see Materials and methods). FACS analysis showed that, as expected, most of these mutants (95%) were normal in terms of cell-cycle arrest at G₁; however, 56 mutants arrested with the 2C DNA content typical of G₂ rather than G₁ (1C) cells. Cell morphology showed that 28 of these mutants appeared to arrest at a small cell size (<8 µm) and so were selected for further analysis. Figure 1 shows a typical example of DNA content (left panels), cell size distribution (middle) and cell morphology (right) in wild type (A) and three sterile mutants that typify the class, we isolated (B, *apc10*; C, *rum1*; and D, *ste9/srw1*, see below). For comparison, an example of a G₂-arrest mutant with a larger cell size is also shown (E).

Classification of mutants defective in G₁-arrest and S-phase inhibition

In addition to the sterile phenotype, 14 out of 28 strains showed temperature-sensitive (ts) growth. In particular, nine of these mutants showed the ‘cut’ phenotype at the restrictive temperature (Figure 2), as septation and cytokinesis occurred in the absence of chromosome segregation. This resulted in two incompletely separated daughter cells associated in a side-by-side manner (Hirano *et al.*, 1986). These phenotypes are extremely similar to the phenotype of *nuc2-663* cells grown at the lower range of its restrictive temperature (33°C; Kumada *et al.*, 1995). However, plasmids containing *nuc2*⁺ did not suppress either the temperature sensitivity or the sterility of these nine mutants, indicating that they are unlikely to be allelic to *nuc2*. Pair-wise complementation tests using protoplast fusion showed that nine mutants were all allelic and represent a novel locus, designated *apc10* (Table I).

Our preliminary analysis of known sterile mutants by FACS analysis showed that, in addition to the *nuc2* mutant, *ste9* and *rum1* are defective in arresting at G₁ under nutritional starvation. Complementation tests of the remaining 19 mutants with the *ste9* and *rum1* mutants showed that eight were allelic to *ste9* and three were allelic to *rum1*, and that none of them was allelic to *nuc2* (Table I). The other eight mutants all belonged to distinct complementation groups, which were designated *sat1-8* (starvation-induced arrest).

Next we asked whether multicopy plasmids containing either *rum1*⁺, *nuc2*⁺ or *ste9*⁺/*srw1*⁺ suppressed the sterile mutants that were isolated in this study. The sterile, but not ts phenotypes of five out of nine *apc10* alleles were suppressed by elevating the dosage of the *rum1*⁺ gene (Table II). This result suggests that since Rum1 is the CDK-inhibitor of Cdc2/Cdc13 and Cdc2/Cig2 (Correa-

A wild type **B *apc10-27*** **C *rum1-7*** **D *ste9-12*** **E 6-42**

Fig. 1. DNA content and cell size of novel sterile mutants under nitrogen starvation. (A) Wild type (TP108-3A, Table III), (B) *apc10-27*, (C) *rum1-7*, (D) *ste9-12* and (E) 6-42 cells were grown in a low nitrogen medium supplemented with leucine and uracil (25 µg/ml) for 48 h at 27°C. DNA content (left), forward scattering (middle) and cell morphology (right panels) are shown. Bar, 10 µm.

Bordes and Nurse, 1995; Jallepalli and Kelly, 1996; Martin-Castellanos *et al.*, 1996), inhibition of CDK activity could partially bypass the normal requirement of Apc10 for the mating pathway under starvation conditions.

Cloning of the *apc10*⁺ gene

The *apc10*⁺ gene was cloned by complementation of the temperature sensitivity using a fission yeast genomic library. Five different plasmids which contained overlapping inserts were obtained. These plasmids suppressed both the ts and the sterile phenotype. Restriction mapping and subsequent subcloning indicated that the internal *Xho*I site was essential for suppressing activity (Figure 3A). The nucleotide sequence of this region revealed one 189-amino-acid open reading frame (ORF), which was interrupted by two putative introns. The identity of this

A **B**

Fig. 2. Cut phenotypes of the ts *apc10* mutant. The *apc10-27* mutant (KK1427, Table III) was grown in rich medium at 26°C and shifted to 35.5°C. Cells were collected hourly, fixed with 3.7% formaldehyde, stained with DAPI [(A) shows 4 h samples, with cut cells indicated by arrows] and (B) the percentage of cut cells was also measured. Bar, 10 µm.

Table I. Complementation groups and characteristics of G₁-arrest-defective mutants

Loci	Alleles	Suppression by multicopy		ts ^a
		<i>rum1</i> ⁺	<i>ste9</i> ⁺	
<i>apc10</i>	3, 5, 27, 71, 202	yes ^b	no	–
	104, 127, 193, 207	no	no	–
<i>ste9</i>	12, 39, 44, 53, 120, 150, 165, 223	no	yes	+
<i>rum1</i>	7, 173, 204	yes	no	+
<i>sat1</i>	11	yes ^b	no	–
<i>sat2</i>	32	no	yes	+
<i>sat3</i>	48	no	no	+
<i>sat4</i>	86	no	no	–
<i>sat5</i>	100	no	no	+
<i>sat6</i>	103	no	no	+
<i>sat7</i>	110	no	no	–
<i>sat8</i>	146	no	no	+

^aTemperature sensitive; + indicates growth at 36°C.

^bOnly sterile phenotypes were suppressed.

ORF as the *apc10*⁺ gene was confirmed by subcloning the ORF into a plasmid under the direct control of the thiamine-repressible *nmt1* promoter (pREP1-*apc10*⁺, Figure 3A; see Materials and methods). pREP1-*apc10*⁺ was slightly toxic when transformed into wild-type cells and modest cell elongation was observed (on average cells

Table II. Suppression of sterile phenotypes by high gene dosage of *rum1*⁺

Mutants ^a	% of conjugation ^b	
	Vector	<i>rum1</i> ⁺
<i>Δrum1</i>	<1	100 ^c
<i>rum1-7</i>	<1	63
<i>apc10-3</i>	2.5	30
<i>apc10-5</i>	3.9	39
<i>apc10-27</i>	5.5	24
<i>apc10-71</i>	5.8	45
<i>apc10-104</i>	<1	<1

^aThe following strains were used: *Δrum1* (PN1012, Table III), *rum1-7* (KK137), *apc10-3* (KK143), *apc10-5* (KK135), *apc10-27* (KK1427), *apc10-71* (KK1371) and *apc10-104* (KK13104).

^bMutant cells containing pREP3-*rum1*⁺ were streaked on minimal plates supplemented with uracil and thiamine and incubated for 3 days at 28°C. The percentage of cells which had conjugated or sporulated was scored. At least 500 cells were observed in each case.

^cA conjugation frequency of ~10% (65 out of 698 cells) was obtained. In the table, this value is shown as 100%, and the efficiency of conjugation of each mutant is shown relative to this value.

Fig. 3. Cloning, overexpression and disruption of the *apc10*⁺ gene. (A) The restriction map and subcloning strategy for the *apc10*⁺ gene are shown. Two introns are depicted with open boxes. A single *Xho*I site was essential for complementation as, when it was disrupted (shown with closed triangle), the resulting subclone lost the complementing activity. (B) Cells which had been transformed with a vector (pREP1, left, or pREP1-*apc10*⁺, right) were streaked on minimal medium (derepressed) and incubated at 30°C for 2 days. (C) Tetrad analysis of a heterozygous *apc10*⁺/Δ*apc10* diploid (KK300, Table III) is shown. (D) Germinating cells from a nonviable *apc10*⁻ deleted spore are shown. After germination, cells divided several times and arrested with either 'cut' (arrow heads) or elongated cell (arrow) phenotypes. Bar, 10 μm.

were 30% longer than the wild type; Figure 3B). FACS analysis indicated that no increase in ploidy was evident, suggesting G₂ delay in the cell cycle (data not shown).

The *apc10*⁺ gene was disrupted with the PCR-based

gene targeting method (Bähler *et al.*, 1998). Two viable and two inviable spores were obtained from 20 dissected tetrads and all the viable colonies were Ura⁻, indicating that the *apc10*⁺ gene is essential for cell viability (Figure 3C). Inviabile spores germinated, divided several times and then ceased division. Two kinds of terminal morphology was observed; elongated cells which occasionally had multiple septa (Figure 3D, arrow) or with a cut phenotype (arrow head), which was similar to the defective phenotypes of the *ts apc10* mutants.

The *apc10*⁺ gene encodes an evolutionarily conserved protein

A database search revealed that Apc10 is a highly conserved protein: homologues exist in vertebrates, invertebrates and budding yeast, with 49% identity between the fission yeast and human proteins in the central 80 amino acid residues and 38% between fission and budding yeasts in the central 150 amino acid residues (the reported nucleotide sequence of the human homologue of Apc10 is incomplete and its C-terminus is unsequenced; Figure 4A and B). During the preparation of this manuscript, we learned that the budding yeast homologue of Apc10, called Doc1, had been identified and proposed to be a component of the APC–cyclosome (Hwang and Murray, 1997).

In addition to homologues from different organisms, there is a human gene (KIAA0076) which encodes a protein distantly related to Apc10 (Figure 4B). Although the function of this human protein is not known, it is interesting to note that it also contains a region of 200 amino acid residues near the C-terminus which is related to sequences in cullins, a protein family involved in ubiquitin-dependent protein degradation (Figure 4A, 30% identity to cullin-1; Kipreos *et al.*, 1996; Mathias *et al.*, 1996; Willems *et al.*, 1996).

In the *apc10* mutant the level of mitotic B-type cyclin Cdc13 is not downregulated at G₁ phase

Given the phenotypic similarity between *apc10* and the *nuc2* mutant that is defective in one of the APC–cyclosome subunits (Kumada *et al.*, 1995; Yamada *et al.*, 1997), the possibility that Apc10 is also involved in regulating the stability of B-type cyclin Cdc13 was addressed. The APC–cyclosome is active from the onset of anaphase throughout the G₁ phase and continuously degrades Cdc13 (Yamano *et al.*, 1996; Correa-Bordes *et al.*, 1997). The level of Cdc13 in G₁-arrested cells was measured in *ts* mutants defective in the Cdc10 transcription factor which arrest in G₁ at Start (*cdc10-129* and *apc10-27cdc10-129*). As shown in Figure 5A, Cdc13 levels decreased in the *cdc10* mutant at the restrictive temperature (lanes 1–3), whereas in the double mutant no significant decrease was observed (lanes 4–6). This was confirmed by densitometric measurement of the level of Cdc13 at each time point, using tubulin as an internal loading control (Figure 5B). The stability of Cdc13 in the *apc10cdc10* mutant is not ascribable to a failure of the cells to arrest at G₁ in the double mutant, as FACS analysis confirmed the accumulation of G₁-arrested cells at 2 and 4 h at the restrictive temperature (Figure 5C). It should be noted that in the *apc10cdc10* mutant, the kinetics of G₁ arrest were somewhat delayed compared with a single *cdc10* mutant. These data indicate

Fig. 4. Amino acid sequence comparison of fission yeast Apc10 with putative homologues from other organisms. (A) An overall homology shared between Apc10-related proteins is shown. Species are indicated by the following abbreviations: Sp, fission yeast; Hs, human (DDBJ/EMBL/GenBank accession No. AA234328); Mm, mouse (AA472445); Dm, fly (AA141768); Ce, worm (Z73972); and Sc, budding yeast (Z72762). A human protein (KIAA0076, D38548) which shows a homology to both Apc10 (closed box) and the C-terminal region of cullins and Apc2 (shadowed box) is also shown. (B) The amino acid sequence of the central 150 amino acid residues of Apc10 is compared with that of Apc10 homologues in other species and to the human ORF KIAA0076. Closed boxes with white letters show identical amino acids, while shadowed boxes with black letters represent conservative amino acids.

that Apc10 is required for the degradation of Cdc13 at G₁ phase.

Apc10 is required for the ubiquitination of Cdc13

The results presented above suggest that Apc10 is involved in some step in the pathway resulting in the proteolysis of Cdc13. If Apc10 is a subunit or a regulator of the APC-cyclosome, Cdc13 should not be ubiquitinated in the *apc10* mutant. To address this question, a double mutant containing mutations in *apc10* and a proteasome mutant were constructed. *mts2-1* is a ts mutant which is defective in subunit 4 of the 26S proteasome (Gordon *et al.*, 1993) and polyubiquitinated intermediate forms of Cdc13 accumulate in this mutant at the restrictive temperature (Yamashita *et al.*, 1996). Cdc13 was expressed under the control of the thiamine-repressible *nmf1* promoter in *mts2-1* and *apc10-27mts2-1* mutants, and the temperature of the culture was shifted to the restrictive temperature.

Immunoblot analysis of protein extracts prepared from samples at different time points at the permissive (25°C) and at the restrictive temperature (35.5°C) is shown in

Figure 5D. Consistent with a previous report (Yamashita *et al.*, 1996), the higher molecular forms of Cdc13, which were detected with anti-ubiquitin antibody, appeared after temperature shift-up of the *mts2-1* mutant culture (Figure 5D, compare lanes 1, 2, 5 and 6). In sharp contrast, however, no higher forms of ubiquitinated Cdc13 were detectable in the *apc10mts2* double mutant (Figure 5D, lanes 3, 4, 7 and 8). These results demonstrate that Apc10 is required for ubiquitination and degradation of Cdc13.

Apc10 genetically and physically interacts with a universal subunit of the APC-cyclosome in vivo

As a first step towards addressing the question as to whether Apc10 is a subunit or a regulator of the APC-cyclosome, the possibility of a physical interaction between Nuc2 and Apc10 was examined. The N-terminus of Apc10 was tagged with a c-myc peptide (pREP41-myc-apc10⁺). This plasmid rescued the *apc10* deletion, indicating that tagging did not interfere with the Apc10 function. Immunoprecipitation was performed with the deletion strain containing pREP41-myc-apc10⁺ using anti-myc antibody. A non-related anti-HA antibody (or mock) was used as a

Fig. 5. Apc10 is required for the degradation and ubiquitination of Cdc13. (A) *cdc10* single (lanes 1–3, *cdc10*, Table III) or *apc10cdc10* double mutants (lanes 4–6, TP426–9C) were grown in rich medium at 26°C and shifted to 35.5°C. Cell extracts were prepared at 0 (lanes 1 and 4), 2 (lanes 2 and 5) and 4 h (lanes 3 and 6) at 35.5°C, run on SDS–PAGE, immunoblotted with anti-Cdc13 (upper) and anti-tubulin antibodies (lower panel). (B) Densitometric calibration of the data in (A) is shown. At each time point, the relative level of the Cdc13 was measured by using the level of tubulin as an internal control. The level at time 0 was assigned 100%. (C) Samples used in (A) were fixed and processed for flow cytometry. (D) *mts2* single (*mts2*, Table III) or *apc10mts2* double mutants (TP429–2A) containing pREP1-*cdc13*⁺ were grown in minimal medium at 25°C in the presence of 2 μ M thiamine. Cells were harvested by filtration, and expression of the *cdc13*⁺ gene was induced by replacing the medium with minimal medium which lacked thiamine (at a density of 2×10^5 cells/ml). After further growth for 16 h at 25°C, the culture was divided into two, and one half was incubated at 25°C (lanes 1, 3, 5 and 7), whilst the other half was shifted to 35.5°C (lanes 2, 4, 6 and 8). Both cultures were then incubated for a further 4 h. Cell extracts were prepared from these cultures and immunoblotted with either anti-Cdc13 (lanes 1–4) or anti-ubiquitin antibody (lanes 5–8).

control. As shown in Figure 6A, Apc10 formed a complex with Nuc2 (lanes 3). Co-precipitation was not observed when anti-HA (or mock) was used as a primary antibody (lanes 2 and 4). Unfortunately, myc–Apc10 migrated as a 24 kDa band, which overlapped with a light chain of IgG in these extracts (25 kDa). However, the precipitation of myc–Apc10 was evident because the intensity of the band around 24 kDa was much stronger with anti-myc antibody than that with anti-HA (Figure 6A, compare lanes 3 and 4). This result supports the notion that Apc10 is a component of the APC–cyclosome, although it does not mean that it is a stoichiometric subunit of this complex (see below).

Next, the possibility of a genetic interaction between *nuc2* and *apc10* was addressed. It was found that *ts nuc2-663* and *apc10-27* were synthetically lethal: no double mutants were viable at 26°C, the permissive temperature for both single mutants. It should be noted that a similar synthetic lethality was also reported between *nuc2-663* and *cut9-665*, which is defective in another subunit of the APC–cyclosome (Samejima and Yanagida, 1994; Yamada *et al.*, 1997).

Apc10 is not a core component of the APC–cyclosome

To examine whether Apc10 exists in the 20S APC–cyclosome complex, a sucrose gradient centrifugation was

performed with cell extracts prepared from exponentially growing cells which contain the myc-tagged *apc10*⁺ gene (in the chromosomal *apc10* gene deletion background, KK301, Table III). As reported previously (Yamashita *et al.*, 1996), the majority of the Nuc2 protein sedimented around 20S (Figure 6B, top panel). Also, Cut9 co-sedimented with Nuc2, although compared with Nuc2, Cut9 also sedimented in a smaller fraction, as shown previously (Figure 6B, middle panel; Yamashita *et al.*, 1996). In sharp contrast, however, myc–Apc10 sedimented at a velocity much slower than the 20S complex, as a diffuse pattern. The majority of myc–Apc10 sedimented between the top fraction and that of about 4.5S (Figure 6B, bottom panel). This result indicates that Apc10 is not a stoichiometric complex of the APC–cyclosome, but rather that it is a positive regulator of this complex.

To address further a relationship between Apc10 and the APC–cyclosome, a sucrose gradient centrifugation was performed in cell extracts prepared from the *ts apc10* mutants (TPR27-1, Table III) incubated at the restrictive temperature. As shown in Figure 6C, the sedimentation profile of the Nuc2 protein did not change significantly compared with wild-type cells (upper panel, compare with Figure 6B). Nor did a pattern of Cut9 differ significantly from that of wild-type cells (lower panel). Therefore, we conclude that inactivation of *apc10*⁺ does not cause any

Fig. 6. Apc10 interacts with a subunit of the APC–cyclosome but is not a core component. **(A)** A $\Delta apc10$ strain containing a plasmid carrying myc-tagged $apc10^+$ (pREP41–myc– $apc10^+$, KK301, Table III) was grown in minimal medium in the absence of thiamine (derepressed conditions) at 30°C. A cell extract (1 mg) was prepared and immunoprecipitation was performed using either anti-myc (lane 3), anti-HA antibody (lane 4) or mock treatment of no antibody (lane 2). Total cell extracts (30 μ g) were also run (lane 1). Immunoblotting was performed with anti-Nuc2 (upper panel) or anti-myc (lower) antibody. Due to a similar size between myc–Apc10 (24 kDa) and the IgG light chain (25 kDa, IgG-L), these two proteins overlapped on the gel (lanes 3 and 4). However, note that in contrast to a clear difference in the intensity of bands around 24 kDa which were precipitated with anti-myc and with anti-HA (lower panel in lanes 3 and 4), the amount of the IgG heavy chain (upper panel) was indistinguishable with these two antibodies (IgG-H, 55 kDa, cross-reacted with anti-rabbit secondary antibody, upper panel). **(B)** Cell extracts prepared from the same strain used in **(A)** were separated through a 15–40% sucrose gradient centrifugation and fractions were analysed by immunoblotting with anti-Nuc2 (top), or anti-myc antibody (bottom panel). The same filter used in immunoblotting with anti-Nuc2 was stripped off and reprobed with anti-Cut9 antibody (middle). Positions of sedimentation markers (19.2S, thyroglobulin; 4.5S, BSA) and fraction numbers (frac. no.) are also shown. Total cell extracts (15 μ g) used in centrifugation were run in the leftmost panel (crude). The second lane was blank (no proteins were run). **(C)** Cell extracts prepared from an $apc10-27$ strain (TPR27-1, Table III) incubated at 35.5°C for 4 h were separated through a sucrose gradient as in **(B)**, and immunoblotting was performed.

significant alterations in a physical state of the 20S APC–cyclosome.

The sterile phenotype of $apc10$ mutants is suppressed by deletion of S-phase B-type cyclin-encoding $cig2^+$

The sterile phenotypes of the $rum1$ and $ste9/srw1$ mutants are suppressed by deletion of the B-type cyclin-encoding

Table III. Strain list

Strains	Genotypes	Derivations
HM123	h^+leu1	our stock
TP108–3A	$h^+leu1ura4$	our stock
TP114–2A	$h^{90}leu1ura4$	our stock
KK135	$h^{90}leu1ura4apc10-5$	this study
KK137	$h^{90}leu1ura4rum1-7$	this study
KK143	$h^{90}leu1ura4apc10-3$	this study
KK612	$h^{90}leu1ura4ste9-12$	this study
KK1371	$h^{90}leu1ura4apc101-71$	this study
KK1427	$h^{90}leu1ura4apc10-27$	this study
KK13104	$h^{90}leu1ura4apc10-104$	this study
C761–5D	$h^{90}his1ste9-B36$	from Dr C.Shimoda
TP316–16C	$h^{90}leu1ura4nuc2-663$	our stock
PN1012	$h^{90}leu1ura4ade6rum1::ura4^+$	from Dr P.Nurse
TPR27-1	$h^+leu1his2apc10-27$	this study
cdc10	$h^+leu1cdc10-129$	from Dr P.Nurse
TP426–9C	$h^+leu1apc10-27cdc10-129$	this study
mts2	$h^+leu1mts2-1$	from Dr C.Gordon
TP429–2A	$h^+leu1his2apc10-27mts2-1$	this study
KK300	$h^+/h^+leu1/leu1ura4/ura4his7/his7ade6-M210/ade6-M216apc10::ura4^+/+$	this study
KK301	$h^+leu1ura4his7ade6apc10::ura4^+$ carrying pREP41–myc– $apc10^+$	this study
PN1076	$h^+leu1ura4ade6cig2::ura4^+$	from Dr P.Nurse
TP441–1B	$h^{90}leu1ura4apc10-27cig2::ura4^+$	this study

gene $cig2^+$ (Martin-Castellanos *et al.*, 1996; Yamaguchi *et al.*, 1997; Kitamura *et al.*, 1998). Cig2 is important for regulating S-phase progression. To examine whether $\Delta cig2$ suppresses the sterility of the $apc10$ mutants in a similar manner, the double mutant between $apc10$ and $\Delta cig2$ was constructed. As expected, $apc10\Delta cig2$ double mutants were no longer sterile, they conjugated and sporulated almost as efficiently as wild type, as manifested by the strong iodine staining (Figure 7A) and cell morphology (Figure 7B). Importantly, however, the double mutant strain was still temperature sensitive and the cut phenotype was still observed at the restrictive temperature (data not shown). These results indicated that the sterility of $apc10$ was, at least in part, ascribable to the existence of the B-type cyclin Cig2, but that the ts cut phenotype must arise as a result of an inability to carry out degradation of proteins other than Cig2, although it is possible that Apc10 may have a role in mitosis other than that in the proteolysis pathway.

Discussion

Divergence and generality of G_1 phase regulation

Unlike budding yeast and other eukaryotes, and like early frog embryos, fission yeast has no appreciable G_1 phase in actively dividing conditions (Nurse, 1990). In parallel with this phenomenon, the level of Rum1 is extremely low in the vegetative cycle and only accumulates in G_1 -arresting conditions (Correa-Bordes and Nurse, 1995). This contrasts with a pattern of Sic1, the CDK inhibitor of budding yeast, which has a longer G_1 phase (Schwob *et al.*, 1994). It is of interest how this apparent divergence of cell-cycle regulation, in particular the duration of G_1 , arises in different organisms. It is possible that the difference of G_1 -phase duration is ascribable to a distinct regulation of the APC–cyclosome and CDK inhibitors.

Fig. 7. *cig2* deletion rescues the sterility defect of the *apc10* mutant. (A) Homothalic strains of *apc10* (KK1427, Table III) and *apc10cig2* (TP441-1B) were streaked on malt extract plate, incubated at 27°C for 3 days and exposed to iodine vapour. Sporulating cells stained darkly (*apc10cig2*), whereas sterile *apc10* strain did not stain (*apc10*). (B) The cell morphology of *apc10* (left) and *apc10cig2* (right panel) is shown. Bar, 10 µm.

Unlike other organisms, in fission yeast the APC–cyclosome may be turned on and off very rapidly in order to ensure a temporal ordering of the cell cycle.

A requirement of the dual downregulation of CDK is also operative in G₁ arrest in budding yeast. The *cdc16* and *cdc23* mutants, which are defective in the APC–cyclosome, not only are incapable of exiting from mitosis but also commit unscheduled additional DNA replication in G₁-arresting conditions (Irniger and Nasmyth, 1997). The similarity between the two systems extends further to regulation of the CDK inhibitors. In budding yeast, the two CDK inhibitors, Far1 and Sic1, regulate G₁ progression and both of the proteins are degraded through the action of another E3 complex, the SCF^{Cdc4}/PULC pathway (Peter and Herskowitz, 1994; Schwob *et al.*, 1994; Henchoz *et al.*, 1997; Herskowitz, 1997). Again, this is highly reminiscent of the situation in fission yeast where Rum1 is degraded by the SCF^{Pop1} pathway (Kominami and Toda, 1997).

In animal cells, depletion of serum or mitogens results in arrest in G₁ phase at a specific point (the restriction point; Pardee, 1989). p27^{Kip1} and related CDK inhibitors have been shown to be crucial for the connection between mitogenic signals and the cell cycle (Coats *et al.*, 1996; de Nooij *et al.*, 1996; Lane *et al.*, 1996). p27^{Kip1} is also important for cell-cycle arrest during differentiation (Casaccia-Bonnel *et al.*, 1997; Durand *et al.*, 1998). A role for the APC–cyclosome in cell-cycle arrest, on the other hand, has not been directly addressed in mammalian cells. However, as in yeast, the APC–cyclosome in vertebrates is activated at the end of mitosis and this activity persists throughout G₁ (Amon *et al.*, 1994; Brandeis and Hunt, 1996; Yamano *et al.*, 1996). This suggests that the APC–cyclosome in higher eukaryotes also plays a crucial role in cell-cycle arrest at G₁.

Role of Apc10 in the degradation of B-type cyclins: a positive regulator of the APC–cyclosome

Apc10 is a highly conserved protein, from yeast to humans. The putative homologue in budding yeast, Doc1/Apc10, is also involved in the proteolysis of B-type cyclin Clb2 (Hwang and Murray, 1997). Strains deleted for *DOC1* are, although not lethal, severely retarded; they are temperature sensitive and even at a lower temperature are delayed in mitotic exit (Hwang and Murray, 1997). As shown in this study, *apc10*⁺ is absolutely required for viability of fission yeast, for chromosome segregation and for the degradation of Cdc13 at G₁. Thus in both yeasts, Apc10 and Doc1 are crucial for mitotic exit and for the degradation of mitotic B-type cyclins.

In budding yeast, a significant amount of Doc1/Apc10, if not all, has been shown to co-sediment with the APC–cyclosome as the ~20S complex (Hwang and Murray, 1997). In addition Doc1/Apc10 was independently identified as one of the proteins associated with core subunits of the APC–cyclosome (Zachariae *et al.*, 1998). In contrast, as shown in this study, in fission yeast the majority of Apc10 does not exist within the 20S APC–cyclosome. Furthermore, the physical state of the APC–cyclosome appears normal in the absence of the Apc10 function, as both Nuc2 and Cut9 exist in the 20S complex in ts *apc10* mutant cells incubated at the restrictive temperature. This clearly contrasts with the situation of other ts mutations in stoichiometric subunits of this complex (*cut4*, *cut9* and *nuc2*) in this organism, in which the 20S complex is physically disrupted (Yamashita *et al.*, 1996; Yamada *et al.*, 1997). Results in fission yeast, therefore, strongly suggest that Apc10 does not comprise a core subunit of the APC–cyclosome. This is consistent with previous biochemical data in frog, in which a core APC–cyclosome complex consists of eight stoichiometric subunits (Apc1-8, Peters *et al.*, 1996; Yu *et al.*, 1998). It would be of interest to see whether or not the complex formation of the APC–cyclosome is impaired in the budding yeast *doc1/apc10* mutants. Although fission yeast Apc10 does not exist in the 20S complex, it co-immunoprecipitates with Nuc2 as budding yeast Doc1/Apc10 does (Hwang and Murray, 1997; Zachariae *et al.*, 1998). It is highly possible that Apc10 associates transiently with the APC–cyclosome complex in a cell-cycle- or substrate-specific manner. Apparent contradiction of the behaviour of Apc10 in different organisms might arise from the difference in the duration of the cell-cycle stages in each organism. We propose that Apc10 is a positive regulator of the APC–cyclosome which is involved either in activation of this complex or in substrate recognition.

A predicted human protein (KIAA0076) has two domains that share a significant homology with Apc10 and cullins. cullin-1 is a member of a cullin family and acts in the ubiquitin-dependent proteolysis pathway as a structural platform for SCF/PULC (Herskowitz, 1997; Lisztwan *et al.*, 1998; Patton *et al.*, 1998). Of particular interest is the fact that Apc2/Rsi1, another universal subunit of the APC–cyclosome, contains a region that shows an homology to the cullin family (Figure 4A; Kramer *et al.*, 1998; Yu *et al.*, 1998; Zachariae *et al.*, 1998). It is, therefore, possible that Apc10 also has a structural role together with Apc2 in the formation and maintenance of the APC–cyclosome complex.

WD-repeat protein, fizzy/fzr/Ste9

ste9⁺/srw1⁺, which encodes a WD repeat protein is most homologous to metazoan fizzy-related protein fzr and budding yeast Hct1/Cdh1, both of which are involved in the proteolysis of B-type cyclins (Schwab *et al.*, 1997; Sigrist and Lehner, 1997; Visintin *et al.*, 1997; Yamaguchi *et al.*, 1997; Kitamura *et al.*, 1998). *Ste9/Srw1*, when ectopically overexpressed, stimulates an activity of the APC–cyclosome (Yamaguchi *et al.*, 1997; Kitamura *et al.*, 1998). Unlike *Apc10*, however, which is also required in the mitotic cycle, *Ste9/Srw1* is essential only for G₁ arrest under nutritional deprivation and subsequent sexual differentiation. In contrast, *Slp1*, which is more closely related to fizzy, is required for mitotic exit (Matsumoto, 1997). In the fly, fizzy is required for regulation during mitosis, whilst fzr mainly regulates differentiation from G₁ phase (Dawson *et al.*, 1993; Sigrist *et al.*, 1995; Sigrist and Lehner, 1997). It appears that each organism has at least two subfamilies of these related molecules that have a distinct role, one (*Slp1* in fission yeast, *Cdc20* in budding yeast and fizzy in fly) regulates the metaphase–anaphase transition during somatic cell cycles, whilst the other (*Ste9*, *Hct1* and *fzr*) functions in G₁ phase or upon differentiation.

Downregulation of CDK by the CDK-inhibitor

Rum1 plays a central role in the regulation of the G₁ phase (Moreno and Nurse, 1994). Although the APC–cyclosome and *Rum1* downregulate CDK in a distinct manner, these two pathways appear to overlap functionally. As shown in this study, the sterility of some of the *apc10* mutants is suppressed by the higher dosage of the *rum1⁺* gene. Also, it has been suggested that *Rum1* plays a specific role in the degradation of *Cdc13* (Correa-Bordes *et al.*, 1997; Stern and Nurse, 1998). Fission yeast cells, like other eukaryotes, arrest the cell cycle at G₁ not only in response to nutritional deprivation but also in response to extracellular differentiation factors, namely mating pheromones (Davey and Nielsen, 1994; Imai and Yamamoto, 1994). It is highly possible that G₁ arrest by pheromone is also achieved by the dual downregulation of CDK as neither the *nuc2-663* mutant nor *rum1*-deleted cells respond to pheromone exposure, and they fail to arrest at G₁ (Kumada *et al.*, 1995; Stern and Nurse, 1997, 1998). We propose that the dual downregulation of CDK might be a universal mechanism that specifically arrests the cell cycle at G₁ phase in response to intracellular and extracellular signals.

Materials and methods**Strains, media, genetic techniques and nomenclature**

The *Schizosaccharomyces pombe* strains used in this study are listed in Table III. Standard procedures for *S. pombe* genetics were followed as previously described (Moreno *et al.*, 1991). *Schizosaccharomyces pombe* cells were transformed via the lithium method (Ito *et al.*, 1983). Low nitrogen minimal medium (0.25% NH₄Cl instead of 0.5%) was used for nitrogen starvation experiments. Mating experiments were performed on malt extract plates (Moreno *et al.*, 1991). The plates contained 1.6% agar. Gene disruptions are abbreviated as the gene preceded by Δ, for example Δ*apc10*. Proteins are designated by an uppercase first letter, e.g. *Apc10*. Temperature sensitivity is abbreviated as ts.

Isolation of sterile mutants that are defective in G₁ arrest

A homothallic *h⁹⁰* strain (TP114–2A, Table III) was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Uemura and Yanagida, 1984).

Among 1.2×10⁵ mutagenized colonies, 1008 sterile mutants were first identified by iodine vapour treatment at 28°C. Then each sterile mutant was grown in low nitrogen medium for 3 days at 26°C and processed for FACS as described previously (Kumada *et al.*, 1995). Cell morphology was also observed in order to classify mutant types. Mutants which exhibited both small cell size and a significant peak of G₂ cells rather than the normal G₁ phase (more than 50%) were selected for further analysis.

Allelism tests

A cross between each mutant and the wild type or a pair-wise cross between the different mutants was performed using the protoplast fusion method (Alfa *et al.*, 1993). All the sterile mutants isolated were recessive, as the diploid between the mutant and wild type was able to sporulate. To positively select diploid between sterile mutants, one pair of mutants was transformed with *LEU2*-containing plasmid (pREP1) and the other was transformed with *ura4⁺*-containing plasmid (pREP2). Prototrophic diploid was selected on minimal plates containing 1.2 M sorbitol after the protoplast fusion. At least 10 independent diploid colonies were examined for conjugation and sporulation to determine allelism between mutants. For allelism to *rum1*, the *rum1*-deleted mutant (PN1012, Table III) was fused with three mutants individually (Table I). For allelism to *ste9*, the *ste9* mutant (C761–5D, Table III) was fused with eight mutants individually. In both cases, no sporulation was observed in the resulting diploid (>10⁴ cells observed). Also, multicopy plasmids containing *rum1⁺* and *ste9⁺* complemented sterility of these three and eight mutants, respectively.

Nucleic acids preparation and manipulation

Standard molecular biology techniques were followed (Sambrook *et al.*, 1989). Enzymes were used as recommended by the suppliers (New England Biolabs Co.).

Suppression by multicopy plasmids

pREP3-*rum1⁺* (obtained from the laboratory of Paul Nurse) was used for suppression experiments. As pREP3-*rum1⁺* is toxic (Moreno and Nurse, 1994), suppression of sterility was examined on minimal plates containing thiamine in order to repress gross overexpression of *rum1⁺* (Table II). Multicopy plasmids containing *ste9⁺* were obtained from Dr Hiroshi Murakami.

Cloning of the *apc10⁺* gene

The *apc10-27* strain (KK1427, Table III) was transformed with a fission yeast genomic library (obtained from Dr A. Carr) and transformants were first selected on minimal plates at 27°C, then replica-plated on YPD rich plates and incubated at 36°C. Twelve out of 50 000 transformants grew at 36°C and all of them had also simultaneously become fertile. Plasmid DNAs were recovered in *Escherichia coli* and restriction mapping showed that five different plasmids (PKK3, PKK7, PKK21, PKK22 and PKK23) had been isolated. Southern hybridization using an insert (a *Pst*I–*Sma*I fragment) from PKK7 as a probe indicated that all five plasmids contained overlapping inserts. The DNA fragment that we isolated is located adjacent to the *leu1⁺* gene on chromosome II. Genetic analysis indicated that *apc10* is tightly linked with *his2* (PD:TT:NPD = 13:2:0), which is also linked to *leu1*, indicating that the isolated gene was indeed *apc10⁺*.

Nucleotide sequence determination

The nucleotide sequence of a 2.3 kb *Eco*RI–*Hind*III fragment was determined using the dideoxy method (Sanger *et al.*, 1977). The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession No. AB012919.

Synthetic lethality between *apc10* and *nuc2* mutants

An *apc10-27* strain (TPR27–1, Table III) containing multicopy plasmids carrying *apc10⁺* and a *nuc2-663* strain (TP316–16C, Table III) containing multicopy plasmids carrying *nuc2⁺* were crossed and tetrad analysis was performed. Spores were germinated at 26°C. From nine tetrads dissected, we obtained six tetrads which showed a pattern of three viable (one Ts⁺ and two Ts[–]) and one inviable spore (assigned as Tetra Type); two tetrads showed two viable (Ts⁺) and two inviable spores (assigned as Non-Parental Ditypes), and one tetrad had four viable spores (all Ts[–], assigned as Parental Ditypes). The existence of *apc10-27* was confirmed, since *apc10* is tightly linked to *his2* and mating type loci, whilst the *nuc2-663* mutation was confirmed by transforming each segregant with multicopy plasmids carrying *nuc2⁺*. In each case, none

Table IV. Oligonucleotides used in this study

Name	Oligonucleotides ^a
KO1	5'GGGGGGGGGCATATGGCGCAAATTCGACAAG3'
KO2	5'GGGGGATCCCTATCGCGGGCTGCTCTCTG3'
KO3	5'GGGGGGGGGCATATGGATGATAATATCGATACAT3'
KO4	5'CATTGTAAATATAGATAAGGAAGCCTTGGGAGGACGTTATGTCAACATCTCACAAGTCCGCATTGTTTACATTTAGCTTTACAAAT- CCCCTGGCTATA3'
KO5	5'CCAGAGACATGACTTTTGCATTACCGTAATTGGTTTCTACTTATGAATTGTAAACTTGTGTAGGGAATTTATCCACCAGATTCTAA- TGCCTTCTGACA3'

^aThe restriction enzyme recognition sequences used for subcloning are underlined (*Nde*I in KO1 and KO3, *Bam*HI in KO2).

of the viable ts segregants was a double mutant, and inviable spores were judged to be double mutants based on the assumption of Mendelian segregation.

Construction of the *apc10*⁺ gene under control of the thiamine-repressible *nmf1* promoter

Two oligonucleotides were used to amplify the *apc10*⁺ gene by PCR, KO1 and KO2 (Table IV). A 640 bp amplified fragment was subcloned into pREP1, pREP2, pREP41 and pREP81 (Basi *et al.*, 1993), designated pREP1-*apc10*⁺, pREP2-*apc10*⁺, pREP41-*apc10*⁺ and pREP81-*apc10*⁺, respectively. All these plasmids were functional as they were capable of suppressing the *apc10-27* mutant. Plasmids (pREP1-*apc10*⁺ and pREP41-*apc10*⁺) suppressed the temperature sensitivity of the *apc10* mutant even in the presence of thiamine (repressed condition), whilst pREP81-*apc10*⁺, which is a compromised version of *nmf1* (Basi *et al.*, 1993) only suppressed in the absence of thiamine (derepressed). A fragment which encoded a form of the N-terminal truncated *apc10* gene (deletion of the first 47 residues) was cloned in pREP1 and pREP41 using PCR fragments amplified with the following primers: KO2 and KO3 (Table IV), designated pREP1-*apc10* and pREP41-*apc10*, respectively. The ORF which lacks the first 47 amino acid residues was not capable of rescuing temperature sensitivity or sterility of the *apc10* mutant when expression was regulated by any promoter (Figure 3A).

Gene disruption

Two 100-base-long oligonucleotides (KO4 and KO5, Table IV) each of which contained 80 bases of the *apc10*⁺ gene plus 20 bases of the *ura4*⁺ gene of 5' and 3' flanking regions, respectively, were used to amplify the *ura4*⁺ gene using Vent DNA polymerase (New England Biolabs Co.), essentially as described (Bähler *et al.*, 1998). Then 10 µg of amplified fragments were used for transformation to disrupt the *apc10*⁺ gene in a diploid strain. Correct disruption was verified by PCR. Tetrad analysis of the resulting heterozygous diploid (KK300, Table III) indicated that the *apc10*⁺ gene is essential for cell viability: 20 tetrads were dissected and in all cases we obtained two viable spores and two inviable spores, the two viable spores forming colonies auxotrophic for uracil (Ura⁻).

Construction and expression of the tagged *apc10*⁺ genes

pREP41-myc-*apc10*⁺ was constructed by subcloning a 640 bp *Nde*I–*Bam*HI fragment from pREP1-*apc10*⁺ into pREP41-myc (provided by Dr A.Carr). Tagging of the myc peptides in the N-terminus did not interfere with the normal function of the *Apc10* protein, as pREP41-myc-*apc10*⁺ suppressed the *apc10* mutation, both for temperature sensitivity and deletion.

Immunochemical assays

Antibodies used in this study were as follows: anti-Cdc2 antibody, anti-Cdc13 antibody (provided by Dr H.Yamano), anti-Cut9 and anti-Nuc2 antibodies (provided by Dr M.Yanagida), anti-ubiquitin antibody (MAB1510, Chemicon Inc.), anti-tubulin antibody (T-5168, Sigma), and anti-myc and anti-HA antibodies (9E10 and 16B12, respectively, BAbCO). Cell extracts were prepared with glass beads, and SDS–PAGE and immunoblotting were performed with either Tris–Glycine or CAPS buffer as described previously (Kominami and Toda, 1997). Prestained protein marker (New England Biolabs Co.) was used as a size marker. Horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Bio-Rad) and a chemiluminescence system (ECL, Amersham) were used to detect bound antibody. For immunoprecipitation, 1 mg of soluble cell extracts, 2.5 µg of anti-myc or anti-HA antibody and

30 µl slurry of Protein G–Sepharose beads (Pharmacia Biotech. Co.) were used.

To detect the ubiquitination of Cdc13, the method used previously in the case of Rum1 and Cdc18 (Kominami and Toda, 1997) was followed. *mts2-1* and *apc10-27mts2-1* mutant cells (TP429-2A, Table III) containing pREP1-cdc13⁺ (obtained from Dr H.Yamano) were grown in minimal medium in the presence of thiamine (2 µM) at 25°C and thiamine was removed from the medium when the culture was in the very early log phase (2×10⁵ cells/ml). After 16 h incubation at 25°C, the cultures were shifted to 35.5°C. Protein extracts were prepared and immunoblotting was performed with anti-Cdc13 and anti-ubiquitin antibodies.

Sucrose gradient centrifugation

A linear sucrose gradient (4.5 ml, 15–40%) was made in HB buffer (25 mM MOPS, pH 7.2, 60 mM β-glycerophosphate, 15 mM *p*-nitrophenyl phosphate, 15 mM MgCl₂, 15 mM EGTA, 1 mM DTT, 1% Triton X-100 and a cocktail of inhibitors; Moreno *et al.*, 1991). Then 2 mg (100 µl) of yeast cell extract (supernatant after centrifugation at 13 000 r.p.m. for 15 min) was loaded on the top of the gradient. The gradient was run at 40 000 r.p.m. for 12 h at 4°C using a Beckman SW50.1 rotor. Samples (260 µl each, in total 18 fractions) were collected manually from the top. To determine the sedimentation coefficient, a parallel gradient was run with standards consisting of thyroglobulin (19.2S), α-amylase (11.2S) and BSA (4.5S).

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