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Control of metaphase–anaphase progression by proteolysis: cyclosome function regulated by the protein kinase A pathway, ubiquitination and localization

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Ubiquitin-mediated proteolysis is fundamental to cell cycle progression. In the fission yeast *Schizosaccharomyces pombe*, a mitotic cyclin (Cdc13), a key cell cycle regulator, is degraded for exiting mitosis, while Cut2 has to be destroyed for the onset of sister chromatid separation in anaphase. Ubiquitination of these proteins requires the special destruction box (DB) sequences locating in their N-termini and the large, 20S complex called the anaphase-promoting complex or cyclosome. Here we show that cyclosome function during metaphase–anaphase progression is regulated by the protein kinase A (PKA) inactivation pathway, ubiquitination of the cyclosome subunit, and cellular localization of the target substrates. Evidence is provided that the cyclosome plays pleiotropic roles in the cell cycle: mutations in the subunit genes show a common anaphase defect, but subunit-specific phenotypes such as in G1/S or G2/M transition, septation and cytokinesis, stress response and heavy metal sensitivity, are additionally produced, suggesting that different subunits take distinct parts of complex cyclosome functions. Inactivation of PKA is important for the activation of the cyclosome for promoting anaphase, perhaps through dephosphorylation of the subunits such as Cut9 (Apc6). Cut4 (Apc1), the largest subunit, plays an essential role in the assembly and functional regulation of the cyclosome in response to cell cycle arrest and stresses. Cut4 is highly modified, probably by ubiquitination, when it is not assembled into the 20S cyclosome. Sds23 is implicated in DB-mediated ubiquitination possibly through regulating de-ubiquitination, while Cut8 is necessary for efficient proteolysis of Cdc13 and Cut2 coupled with cytokinesis. Unexpectedly, the timing of proteolysis is dependent on cellular localization of the substrate. Cdc13 enriched along the spindle disappears first, followed by decay of the nuclear signal, whereas Cut2 in the nucleus disappears first, followed by decline in the spindle signal during metaphase–anaphase progression.

Keywords: anaphase; sister chromatid separation; phosphorylation; dephosphorylation; ubiquitination; de-ubiquitination

1. INTRODUCTION

Sister chromatid separation is a cell cycle step to separate correctly each set of duplicated sister chromatids into the daughter nuclei by the spindle force (Inoué 1997). This event takes place once during the cell division cycle of somatic cells in a brief period called anaphase. During meiotic divisions, sister chromatid separation occurs in meiosis II, but is suppressed in meiosis I since chromosomes have to be segregated in a reductive fashion (Miyazaki & Orr-Weaver 1994). Basically two mechanistic events are directly related to sister chromatid separation (Yanagida 1995): one is that whole chromatids including centromeres, telomeres and the arms of each chromosome must be disconnected at the onset of anaphase, followed by fast movements of the separated chromatids towards the opposite poles of the spindle, and the other is that sister chromatid separation must be restrained in metaphase, a stage immediately prior to anaphase, and triggered only after all the chromosomes are structurally, and the spindle functionally, ready for concerted separation. Molecular bases for setting up metaphase and leading to anaphase are of great interest.

In this article, we describe regulatory mechanisms for metaphase–anaphase progression in *Schizosaccharomyces pombe*, an excellent model organism to study mitosis. Emphasis is placed on ubiquitin-mediated protein degradation, now known to be an essential feature for anaphase (Holloway *et al.* 1993; Funabiki *et al.* 1996*a*; Cohen-Fix *et al.* 1996; Ciosk *et al.* 1998; Kumada *et al.* 1998). The work in this laboratory was initiated by screening temperature-sensitive (ts) or cold-sensitive (cs) mutations defective in the onset and/or the progression of sister chromatid separation (Toda *et al.* 1984; Hirano *et al.* 1986; Ohkura *et*

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Table 1. *Gene products related to anaphase-promoting proteolysis*

fission yeast	budding yeast	human	function
Apc10	Doc1/Apc10	Apc10	cyclosome subunit
Cdc13	Clbs	cyclin B	mitotic cyclins
Cig2	Clbs	Clbs	S-phase cyclin
Cut1	Esp1	KIAA0165	complexed with Cut2
Cut2	Pds1	UN[a]	degrades in anaphase
Cut4	Apc1	Tsg24/Apc1	cyclosome subunit
Cut9	Cdc16	Apc6/Cdc16Hs	cyclosome subunit
Cut20	Apc4	Apc4	cyclosome subunit
Cut23	Cdc23	Apc8/Cdc23Hs	cyclosome subunit
Dis2	Glc7	PP1	one of two PP1
Cut8	Dbf8	UN	regulator
Hcn1	Cdc26	UN	cyclosome subunit
Mts2	Yta5	S4	26S proteasome ATPase subunit
Mts3	Nin1	S1426S	proteasome non-ATPase subunit
Nuc2	Cdc27	Apc3/Cdc27Hs	cyclosome subunit
Scn1	YSCY164_3	KIAA0218	regulator
Scn2	UN	UN	regulator
Sds21	Glc7	PP1	one of two PP1
Sds23	23YBR214w YGL056c	UN	regulator
Slp1	Cdc20	p55[cdc]/hCDC20	bound to Mad2, WD protein
Ste9/Srw1	Hct1/Cdh1	hCdh1	WD protein

[a] UN, not found.

al. 1988; Takahashi *et al.* 1994). A large number of genes were isolated by transformation of mutants using a genomic DNA library, and their gene products were characterized. Some of the genes thus identified (reviewed in Su & Yanagida 1997; Yanagida 1998) were found to be the subunits of the 20S anaphase-promoting complex (APC), or cyclosome, (hereafter designated the cyclosome) required for mitotic cyclin destruction (Sudakin *et al.* 1995; Irniger *et al.* 1995; King *et al.* 1995). Most components of the cyclosome are evolutionarily conserved. Phosphorylation takes place specifically on the mitotic cyclosome. In fission yeast, the cyclosome in mitotically arrested cells sediments slightly faster than that in interphase. We describe results of our attempts to identify genes which may regulate cyclosome functions and also factors that affect the destruction of Cdc13 and Cut2, the target substrates of the cyclosome (Funabiki *et al.* 1996*a*, 1997; Yamano *et al.* 1996). The names of fission yeast genes discussed in this article, and their homologues in budding yeast and human, are shown in table 1.

2. CYCLOSOME SUBUNIT MUTATIONS BLOCK ANAPHASE

Twelve and eight subunits, respectively, are present in the purified preparations of budding yeast and human cyclosomes (Yu *et al.* 1996, 1998; Zacchariae *et al.* 1996, 1998). In fission yeast, purification of cyclosomes has not been done, but six gene products are identified as the subunits by investigating anaphase-defective mutant strains (figure 1). They are Nuc2 (APC3), Cut4 (APC1), Cut9 (APC6), Cut20 (APC4), Cut23 (APC8) and Hcn1 (APC12) (Yamashita *et al.* 1996, 1999; Yamada *et al.* 1997). Fission yeast Apc10 is a regulator rather than the subunit of the cyclosome reported for budding yeast (Kominami *et al.* 1998). Null mutants of these seven genes are all non-viable, and ts mutants exist except for Hcn1. These proteins, except Apc10, co-sediment with the 20S cyclosome complex in sucrose-gradient centrifugation. They are co-precipitated with anti-Cut9 or anti-Nuc2 antibodies. A small fraction of Apc10 is co-precipitated with the cyclosome, however (Kominami *et al.* 1998).

The cyclosome subunit mutant, *nuc2-663*, was originally isolated after cytological characterization of many ts mutants which displayed, at the restrictive temperature, the cell cycle arrest with highly condensed chromosomes arranged around the short metaphase spindle (Hirano *et al.* 1988*a*). This is the first clear demonstration that yeast cells could be blocked at metaphase. However, the arrest is not strictly tight so that the septum is later formed in the absence of nuclear division. A similar metaphase-block phenotype is found in the *cut9-665* mutant (Samejima & Yanagida 1994*a*). Nuc2 and Cut9 proteins are thought to be functionally related as they physically and genetically interact with each other and contain the trichotetrapeptide repeat (TPR) sequences. Further studies of these and several other cyclosome mutants indicate that, although anaphase is invariably defective, their additional phenotypes are substantially different from one another (§ 3).

3. PROPERTIES OF THE CYCLOSOME SUBUNITS AND THEIR MUTANTS

The cyclosome subunits may be classified into at least four distinct groups.

(*a*) *Nuc2, Cut9 and Cut23*

These three subunits contain the TPR repeats (Hirano *et al.* 1990; Samejima & Yanagida 1994*a*, Yamashita *et al.* 1999), which are functionally relevant. The ts mutation sites of *nuc2* and *cut9* reside in the repeats, causing the

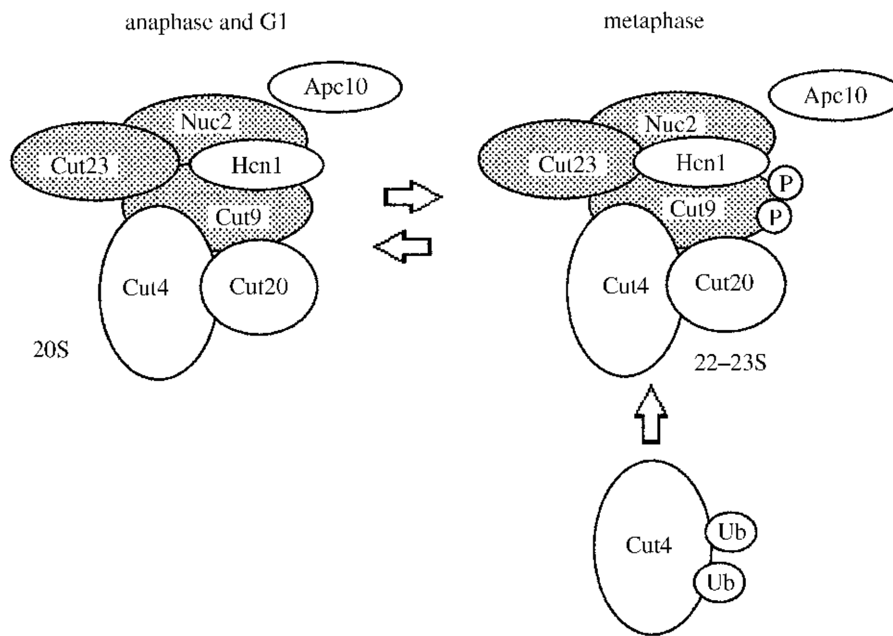

Figure 1. Fission yeast APC or cyclosome consists of at lease six gene products. Three of them, Nuc2, Cut9 and Cut23 (hatched) contain the TPR repeats essential for cyclosome assembly. Cut9 bound to the cyclosome is hyperphosphorylated at metaphase, and its phosphorylation is greatly diminished in the absence of PKA (Yamada *et al.* 1997). On the other hand, Cut4 free from the cyclosome is ubiquitinated and may form an initial core complex for cyclosome assembly (Yamashita *et al.* 1999). Apc10 is loosely bound to the cyclosome (Kominami *et al.* 1998). The sedimentation rate of the cyclosome at metaphase is greater than that at interphase.

failure to form the 20S complex (Yamashita *et al.* 1996; Yamada *et al.* 1997). In immunoprecipitation using anti-cut9 and anti-nuc2 antibodies, Nuc2 and Cut9 are co-precipitated. They may share a common essential role, as their mutants are synthetically lethal.

Cut9 is hyperphosphorylated in mitotic prophase–prometaphase and dephosphorylated in anaphase (Yamada *et al.* 1997). Phosphorylation of Cut9, occurring only in the 20S complex, is dependent on the presence of protein kinase A (PKA) (encoded by the *pka1*$^+$ gene), and the ts phenotype of *cut9* is suppressed by the deletion of *pka1*$^+$. Moreover, inactivation of PKA partly restores the cyclosome assembly in *cut9-665* mutant cells. These results strongly suggest that PKA negatively regulates the cyclosome, and that phosphorylation of Cut9 is functionally important. In contrast, protein phosphatase (PP1) mutation (*dis2-11*) showed the reverse effect on the phenotype of *cut9.* The double mutant *cut9-665 dis2-11* is synthetically lethal.

nuc2-663 is sterile and arrests in G2 at the permissive temperature upon nitrogen starvation (Kumada *et al.* 1995), suggesting that a functional cyclosome is required for the G1 arrest. As this sterility is suppressed by the deletion of an S-phase cyclin, Cig2 (K. Kumada, unpublished data), destruction of Cig2 might be inefficient in *nuc2-663* so that cells fail to arrest at G1 after nitrogen starvation. Nuc2 is an inhibitor of septum formation. Overproduction of Nuc2 completely blocks septation, while nuclear division is not inhibited, producing giant cells containing numerous nuclei (Kumada *et al.* 1995). In contrast, overproduction of Cut9 shows no phenotype (Yamada *et al.* 1997). However, the N-truncated Cut9 blocks cells at G2 when overexpressed. Cells are highly elongated and contain the 2c DNA. These results show distinct roles of Cut9 and Nuc2 for cell cycle progression.

cut23 mutant cells at the restrictive temperature showed a mitotic delay with condensed chromosomes and the metaphase spindle, followed by the cut phenotype (Yamashita *et al.* 1999). Gene cloning and characterization of the gene product show that Cut23 containing the TPR repeats is similar to budding yeast Cdc23.

(b) *Cut4 and Cut20*

Cut4, the largest cyclosome subunit, has no repeat sequence. Cut4 free from the cyclosome displays heavily modified bands in sodium dodecyl sulphate gel electrophoresis (figure 2, top panel), and this is due to ubiquitination (Yamashita *et al.* 1999). The modified band can also be observed when the *cut4*$^+$ gene is tagged with haemagglutinin antigen (HA) at the C-terminus and detected by anti-HA antibody. This modified Cut4 polypeptide is associated with large, fast-sedimenting materials in interphase cells. The modification pattern is strikingly altered during the cell cycle. In mitotically arrested *mts3-1* mutant cells (figure 2, bottom panel), a greater part of the modified Cut4 bands sediment at rates smaller than 20S. Note that Cut4 bound to cyclosome complex does not show extensive modification. Modified Cut4 is thus de-ubiquitinated when assembled into the cyclosome. This modification may be functionally relevant as overproduction of Mad2, a spindle check protein, or the loss of Fizzy-like Slp1 leads to the increase in the level of modified forms (Yamashita *et al.* 1999).

Cyclosome formation is greatly impaired in *cut4-533* mutant (Yamashita *et al.* 1996; figure 2, middle panel). Cut4, Nuc2 and Cut9 are all sedimented at slow rates around 4–8S, indicating that the assembly of Nuc2 and Cut9 into the cyclosome requires functional Cut4. In contrast, Cut4 is assembled into a subcomplex around 15S in *nuc2* and *cut9* mutant cells (Y. Yamashita, unpublished

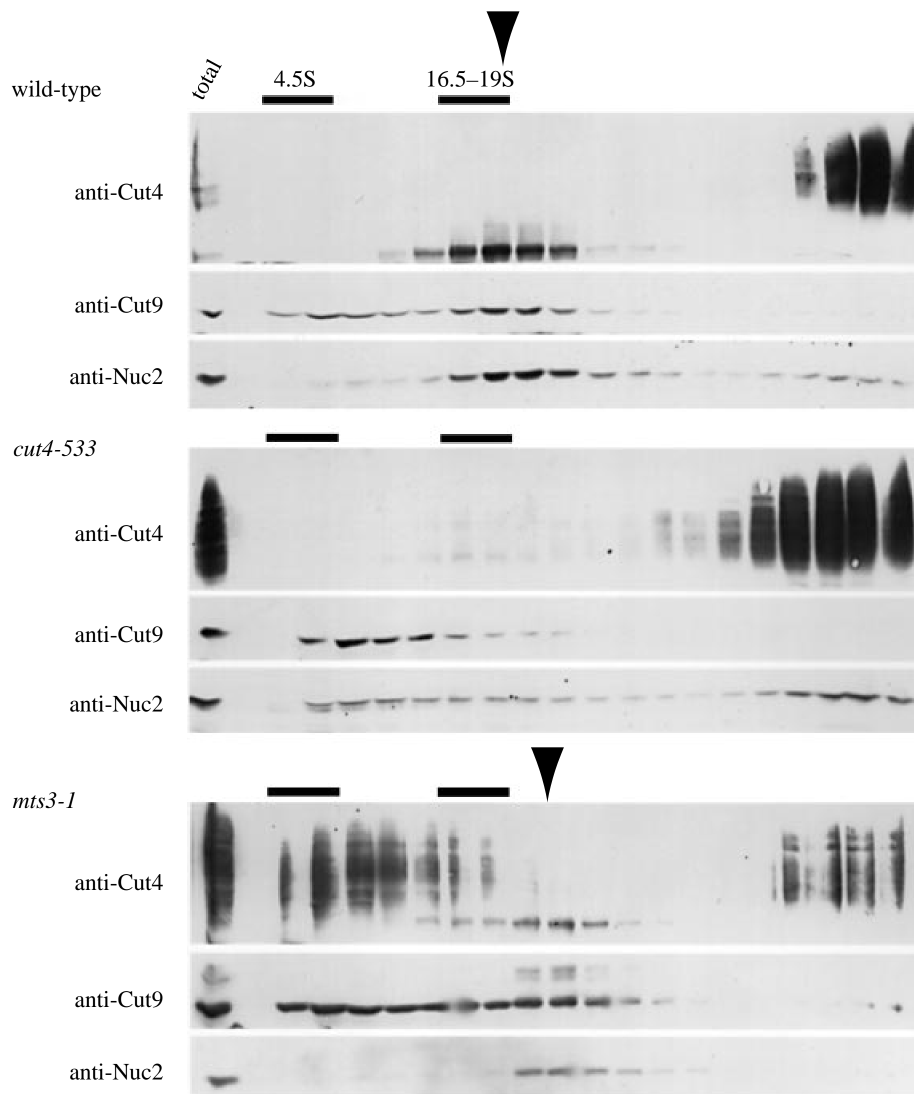

Figure 2. Sucrose-gradient centrifugations of Cut4, Cut9 and Nuc2, the cyclosome subunits, in extracts of the wild-type and *cut4-533* and *mts3-1* mutants. Antibodies against Cut4, Cut9 or Nuc2 were used. The arrowheads indicate the positions of the cyclosome (20S for the wild-type interphase and 22S for the mitotically arrested cells). *mts3-1* is mitotically arrested due to the defect in the 26S proteasome (Gordon *et al.* 1993). Hypermodified Cut4 is seen as the intense upper bands in fast-migrating fractions (top panel). These upper bands became slowly sedimenting in the *mts3* mutant cell (bottom panel). These upper bands were derived from Cut4 as the C-terminally HA-tagged Cut4 showed the same upper bands by anti-HA antibodies. The fast-migrating Cut4 contained ubiquitin (Yamashita *et al.* 1999). In *cut4-533* mutant cells, Cut4 at 20S is negligible, and most Cut4 was hypermodified in the fast-sedimenting fractions, while Cut9 and Nuc2 sedimented slowly.

data), suggesting that Cut4 may be required for an early assembly step of the cyclosome.

The metaphase arrest of *cut4-533* is dependent on the culture medium, as *cut4* cells grow in the synthetic medium but are arrested in the rich medium or the synthetic medium with added heavy metals or stress-inducing agents (Yamashita *et al.* 1996). The addition of cAMP also produces the arrest phenotype. Under these condition, the cyclosome assembly was found to be impaired. In contrast, the *cut4* arrest phenotype is suppressed by increasing the gene dosage of cAMP-dependent phosphodiesterase (PDE) or the PKA inhibitor or by deleting the gene coding for PKA. Inactivation of the PKA pathway thus rescues the *cut4* phenotype. As suppression occurs at the level of cyclosome assembly, PKA may directly or indirectly act on the cyclosome subunits to confer the ability to form the complex. Kotani *et al.* (1998) showed that PKA suppresses the cyclosome activity by direct phosphorylation in an *in vitro* reconstituted system. The PKA activity falls at metaphase.

Cut20, also having no repeat motif, co-sediments with the 20S cyclosome, and is co-precipitated with Cut4, Cut9 and Nuc2 (Yamashita *et al.* 1999). Its amino-acid sequence is similar to budding yeast APC4. The mutant phenotype of *cut20-100* is similar to that of *cut4*, as it is suppressed by elevating the gene dosage of PDE or decreasing PKA. Cut4 and Cut20 may form an initial assembly complex.

(c) *Apc10*

A sterile mutant *apc10-27* fails to arrest in the G1 phase upon nitrogen starvation and it also shows the ts growth defect with the cut phenotype in the rich medium (Kominami *et al.* 1998). The phenotype is similar to *nuc2-663*. Apc10 is a relatively small protein, similar to budding yeast Apc10/Doc1. Apc10 in fission yeast is only loosely bound to the cyclosome, and may be a regulator of the cyclosome rather than a stable subunit.

(d) *Hcn1*

The *hcn1*$^{+}$ gene was isolated as a multicopy suppressor for *cut9* mutation (Yamada *et al.* 1997). The cyclosome defect in the *cut9* mutant was almost completely restored, suggesting that Hcn1 might have a chaperon-like activity for mutant Cut9 protein. However, Hcn1 is a subunit of the cyclosome, as it co-sediments with 20S cyclosome and co-immunoprecipitates with Cut9 and Nuc2 (K. Kumada, unpublished data). Hcn1 polypeptide is small, consisting of 80 amino acids, and its sequence resembles budding yeast Cdc26. Cdc26 is a functional homologue of Hcn1, as its overproduction can suppress the ts

phenotype of *cut9* (Yamada *et al.* 1997). The null phenotype of Hcn1 is non-viable.

4. REGULATORY GENES OF CYCLOSOME FUNCTION

We have identified a number of genes which genetically interact with cyclosome mutations, either by extragenic suppression, synthetic lethality or high-dosage suppression. These gene products may regulate directly or indirectly the cyclosome activity.

(a) *PKA-related genes*

Three PKA-related genes, *pka1*$^{+}$ (encoding the catalytic subunit of PKA), *cgs1*$^{+}$ (inhibitory regulatory subunit), and *cgs2*$^{+}$/*pde1*$^{+}$ (cAMP-dependent PDE) interact with *cut4, cut9* and *cut20* mutations (Yamashita *et al.* 1996, 1999). The deletion mutant of adenylate cyclase (*Δcyr1*) shows a positive effect on growth of cyclosome mutations, as in the case of *Δpka1*. The addition of cAMP to the culture medium was found to be inhibitory as described for the *cut4* mutation. Inactivating the PKA activity partly suppresses *cut4*, whereas enhancing PKA is inhibitory.

The *pka1*$^{+}$ gene is non-essential for cell viability although its gene disruption causes slow growth and derepresses conjugation and sporulation when cells with the opposite mating type are present; the phenotypes are similar to *Δcyr1* (Maeda *et al.* 1994). No other PKA-like gene has been found in the genome of *S. pombe* in contrast to the three PKA-like genes in budding yeast. In *Δpka1* cell extracts, the majority of cAMP-dependent protein kinase activity is lost (H. Yamada and Y. Nakaseko, unpublished data). The loss of Pka1 affects mitotic progression: the arrest phenotype of *nda3-KM311* is suppressed by *Δpka1*; *nda3-KM311 Δpka1* forms colonies at 20 °C (H. Yamada, unpublished data). The loss of Pka1 appears to facilitate microtubules to change into the anaphase state structure.

(b) *Dis2*

PP1 phosphatase is required for exiting mitosis (Ohkura *et al.* 1989; Ishii *et al.* 1996) and its activity is negatively regulated by Cdc2 (Yamano *et al.* 1994), but its actual role in initiating anaphase is little understood. The following results suggest that PP1 might be involved in cyclosome function. *S. pombe* has two PP1 phosphatase genes, *dis2*$^{+}$ and *sds21*$^{+}$, and the double gene disruption leads to the metaphase arrest. The single semi-dominant *dis2-11* mutation is synthetically lethal with *cut9* mutation (Yamada *et al.* 1997) and also with *cut4* (Y. Yamashita, unpublished data). In addition, the *sds23*$^{+}$ gene (described in § 4(c)) links PP1 to the cyclosome. However, the inhibitors of PP1 do not affect cyclin B destruction, but those for PP2A do (Vorlaufer & Peters 1998). The role of PP1 in cyclosome function, if it exists, may be indirect or dispensable, as other principal regulators, such as PKA, are downregulated. PP1 is alternatively required for establishing the link between the kinetochore structure and spindle microtubules, as the phenotype of *dis2-11* is indistinguishable from that of kinetochore microtubule-defective *dis1* mutants (Nabeshima *et al.* 1998). Inactivating PP1 during mitosis is possibly a requirement for restraining the kinetochore–microtubule interaction in anaphase. PP1 might have two execution points during mitosis, one to control the kinetochore function by dephosphorylating the spindle components and the other to promote anaphase proteolysis by dephosphorylating proteins related to anaphase-promoting ubiquitination.

(c) *Sds23*

Sds23 is neither a component of the 20S cyclosome nor the stable subunit of PP1 holoenzyme, but it strongly interacts with *nuc2-663, cut9-665* and *dis2-11,* as these mutants are suppressed by multicopy plasmids carrying the *sds23*$^{+}$ gene (Ishii *et al.* 1996). Sds23 is a conserved polypeptide, and its null mutant grows poorly at 26–30 °C, but not at 20 °C and 36 °C. Mutant cells reveal an anaphase defect and aberrant cell morphology at 20 °C, and this cs phenotype is suppressed by plasmid overproducing Nuc2, Cut9 or Sds21, indicating that the high-dose suppression is mutual among these genes. Thus Sds23 has a positive effect on cyclosome-mediated proteolysis.

Does Sds23 affect the assembly of the cyclosome? This possibility is unlikely, as the assembly of the 20S cyclosome is apparently normal in the *Δsds23* null mutant (K. Ishii, unpublished data). Moreover, the defects in cyclosome assembly in *cut9* or *nuc2* mutant cells are not restored by overexpressing Sds23. Therefore, suppression of *cut9* and *nuc2* mutants by the elevated dosage of Sds23 should occur through a pathway other than the cyclosome assembly.

A clue to understanding the role of Sds23 may be the finding that overproduction of Cut2 or Cdc13 can also suppress the phenotypes of *Δsds23*. Experimental results are shown in figure 3. The N-terminal 73 amino acid Cut2 fragment (Cut2N73) containing the destruction box (DB) sequences also has the ability to suppress *Δsds23*, but the same fragment containing the substitution mutations in DB (Cut2N73ddm; Funabiki *et al.* 1996*a*, 1997) has not. This mutant fragment is neither destructible nor ubiquitinated. These results strongly suggest that Sds23 may directly or indirectly interact with the DB sequences.

Further work is definitely needed to understand the role of Sds23 and obtain any firm conclusion on the above suppression. A simple hypothesis that can be tested is that Sds23 is an inhibitor of de-ubiquitinase, which putatively removes ubiquitin from Cut2 and Cdc13, and competes with cyclosome activity (figure 4). If this was the case, ubiquitination of Cut2 and Cdc13 would be inhibited in *Δsds23* cells due to an unbalanced increase of the de-ubiquitinating activity in these cells. Overproducing Cut2N73 might compete out de-ubiquitinase. The mutant form Cut2N73ddm would not interact with de-ubiquitinase so that it has no suppressing activity. The de-ubiquitination activity towards DB-containing substrates might change during the progression from metaphase to anaphase. High dosage of Sds23 could suppress *nuc2* and *cut9* mutants, which contain a weak cyclosome activity, by reducing the de-ubiquitinating activity.

(d) *Slp1, Ste9/Srw1*

Fizzy and Cdc20 containing a WD repeat are the activators of the cyclosome by direct interaction (Fang *et al.* 1998*a*,*b*). In fission yeast, Slp1 and Ste9/Srw1 are similar

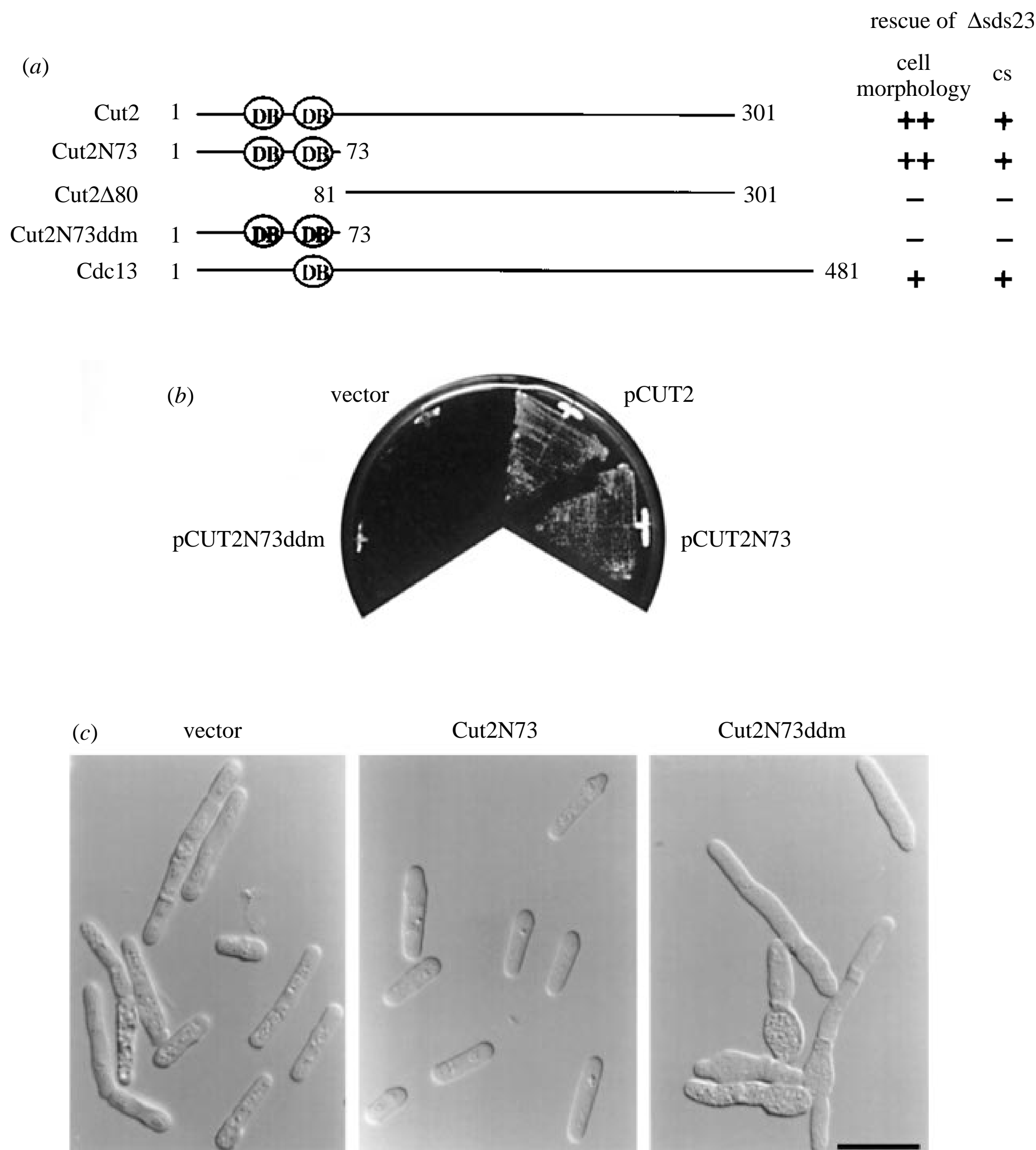

Figure 3. Suppression of the phenotypes of *Δsds23* null mutant by overproducing Cut2 and Cdc13. (*a*) The cs and aberrant cell morphology phenotypes of *Δsds23* were suppressed by multicopy plasmids carrying the *cut2*$^+$ or *cdc13*$^+$ gene as indicated. The short fragment Cut2N73 containing the two DBs also suppressed the phenotype, but the Cut2N73ddm mutant, which contains alanine substitutions in the DB sequences and cannot be polyubiquitinated (Funabiki *et al.* 1996*a*, 1997), did not suppress the phenotype of *Δsds23*. (*b*) Colony formation of *Δsds23*-carrying plasmid as indicated. pCut2 and pCut2N73 could suppress the cs phenotype of *Δsds23*, whereas pCut2N73ddm did not. (*c*) Cells in liquid cultures of *Δsds23* mutant carrying vector (left panel), pCut2N73 (middle) or pCut2N73ddm (right) at 20 °C are shown. Cell division of *Δsds23*-carrying vector or pCut2N73ddm was severely inhibited, whereas *Δsds23*-carrying pCut2N73 grew. Scale bar, 10 μm.

to them. Radiation-sensitive *slp1-362* is suppressed by *wee1-50* or *cdc2-3w* and implicated in the checkpoint repair recovery (Matsumoto 1997). Slp1 is found to be bound to Mad2, a spindle checkpoint protein, which delays the onset of anaphase until all chromosomes are attached to the spindle (Kim *et al.* 1998). Disruption of the binding between Slp1 and Mad2 leads to the loss of the spindle checkpoint. Cyclosome mutants *cut4* and *cut9* are hypersensitive to overproduction of Mad2 in the presence of Slp1 but not sensitive in the background of the *slp1* mutant, strongly suggesting that Slp1 mediates the restraining signal of Mad2 to the cyclosome complex.

In contrast to Slp1, Ste9/Srw1 is non-essential for viability and the deletion mutant is sterile. Ste9/Srw1 is similar to budding yeast Hct1/Cdh1 and required for the G1 arrest under nitrogen starvation (Yamaguchi *et al.* 1997; Kitamura *et al.* 1998; Kominami *et al.* 1998).

(e) *Cut8*

Cut8 is required for efficient proteolysis of Cdc13 and Cut2 during anaphase but is not the component of the cyclosome judging from the results of immunoprecipitation and sucrose-gradient centrifugation (H. Tatebe and M. Yanagida, unpublished data). *cut8-563* mutant is defective in anaphase (Samejima & Yanagida 1994*b*). The phenotype, however, differs from that of cyclosome mutations, as sister chromatids are partly pulled apart and streaked by spindle extension. Sister chromatid separation and spindle extension do not occur in the cyclosome subunit mutations. The spindle extension might prematurely be

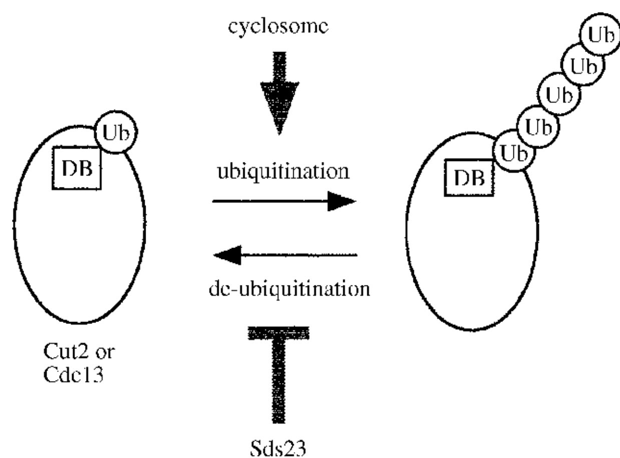

Figure 4. A hypothetical role of Sds23 for ubiquitination. Sds23 may negatively regulate de-ubiquitinating reactions of Cut2 or Cdc13, which are polyubiquitinated by the cyclosome. Polyubiquitination reduced in cyclosome subunit mutations may be considerably restored if de-ubiquitination is simultaneously inhibited. This would explain why Sds23 was isolated as a multicopy suppressor of cyclosome mutations, but did not restore the assembly of the cyclosome.

triggered in the *cut8* mutant before the degradation of Cdc13 and Cut2. This notion is supported by a huge delay in mitotic cyclin (Cdc13) destruction in *cut8* mutant cells, visualized by the use of green fluorescent protein (GFP)-tagged Cdc13 at 36 °C (Tatebe *et al.* 1999).

In the previous immunofluorescent study (Alfa *et al.* 1990), Cdc13 was enriched in the nucleolus and at the mitotic spindle pole bodies (SPBs). GFP-Cdc13 is seen in the whole nucleus of cells in G2 and mitosis (figure 5, top panel). The intense signal was also observed along the spindle (20–25 min) and at the SPB. Cdc13 is accumulated at the single SPB already in the late G2 (10 min). Spindle formation quickly takes place in 3 min (20–23 min) at 36 °C as reported in Nabeshima *et al.* (1998). The spindle signal first disappears. The nuclear signal then rapidly disappears in 2 min (26–28 min) accompanied by nuclear extension. This mode of destruction is strikingly different from that of Cut2 (Funabiki *et al.* 1996*a*,*b*; Kumada *et al.* 1998); the Cut2 nuclear signal first disappears whereas the spindle signals vanish later. These results suggest that different localizations of the substrates are responsible for the different timing of proteolysis.

In *cut8* mutant cells, proteolysis of GFP-Cdc13 is greatly delayed, whereas the duration of time for spindle formation is approximately the same as in wild-type (figure 5, bottom panel). The time required for degradation is roughly five times longer than that for the wild-type. The nucleus becomes already elongated while the nuclear signal of GFP-Cdc13 is still intense, indicating that spindle extension occurs in the abundant nuclear signal of Cdc13. Similar analysis was performed for GFP-Cut2 in *cut8* mutant. The nuclear signal first disappears, followed by the loss of spindle signal. Spindle extension initiates before a significant loss of Cut2 (H. Tatebe and M. Yanagida, unpublished data). A major role of Cut8 hence may recruit the ubiquitinated substrates for efficient proteolysis. Alternatively, ubiquitination of the cyclosome substrates is delayed. Cut8 appears to play a role in coordinating the onset of spindle extension with anaphase proteolysis. The loss of Cut8 results in the triggering of spindle extension before the completion of anaphase proteolysis.

(f) *Scn1, Scn2*

Mutations in Scn1 and Scn2 genetically interact with the cyclosome subunits, but Scn1 is not the stable component of the cyclosome (the $scn2^+$ gene has not been isolated). The $scn1^+$ and $scn2^+$ genes were initially identified as extragenic suppresser mutations of ts *cut9-665* (Samejima & Yanagida 1994*a*). *scn1* and *scn2* mutants are cs, and *scn1* is a gain-of-function mutant. Contrary to the interaction with *cut9-665*, both mutations are synthetically lethal with *nuc2-663*. This is another example of the fact that Cut9 and Nuc2 are functionally distinct. The $scn1^+$ gene encodes an unknown protein conserved from bacteria to human (K. Kumada, unpublished data). Interestingly, single *scn1* mutation causes an anaphase-defective phenotype. Scn1 may be a chaperone-like protein interacting with the TPR-containing subunits. Alternatively, Scn1 may be implicated in the dissociation of sister chromatid cohesion, either by interacting with the Cut1–Cut2 complex (Kumada *et al.* 1998) or with cohesion molecules such as cohesin or adherin (Furuya *et al.* 1998). These possibilities remain to be examined.

(g) *Sti1*

High-dosage expression of $sti1^+$, a stress-inducible gene, suppresses the ts phenotype of *cut4-533* (Yamashita *et al.* 1996); the defect of cyclosome assembly is partly restored. As Sti1 is an activator of chaperones Hsp70 and Hsp90, suppression may be explained by renaturing mutant Cut4 protein, leading to the assembly of the cyclosome. Additionally, reduced cyclosome function in ts *cut4* mutant cells at the restrictive temperature may be compensated for by increasing Sti1 as the level of stress-inducible denatured proteins, the substrates of 26S proteasome, should be reduced by increasing the chaperone activity.

5. TARGET SUBSTRATES OF THE CYCLOSOME

Two gene products, Cdc13 and Cut2, are known to be the substrates of the cyclosome (figure 6). They contain the DB sequences required for polyubiquitination and proteolysis (Funabiki *et al.* 1996*a*; Yamano *et al.* 1996). Those deleting DB, if expressed, become stable and cause a strong negative-dominant effect on growth. Even moderate overexpression of Cut2, deleting or substituting DB, blocks sister chromatid separation and spindle elongation (Funabiki *et al.* 1996*a*). DB-dependent polyubiquitination of Cut2 has been demonstrated *in vitro* using HeLa cell anaphase extracts (Funabiki *et al.* 1997). DB-dependent proteolysis of Cdc13 and Cut2 is also shown *in vitro* in $Ca2^+$-induced frog egg mitotic extracts (Yamano *et al.* 1996; Funabiki *et al.* 1997). The recognition system of the substrates for cyclosome-dependent proteolysis seems to be evolutionarily conserved.

Proteolysis of Cdc13 results in the inactivation of mitotic Cdc2 kinase, while proteolysis of Cut2 leads to the onset of anaphase. Cut2 binds to a large (200 kDa), evolutionarily conserved protein, Cut1 (Uzawa *et al.* 1990; Funabiki *et al.* 1996*b*). Cut1 is in the cytoplasm during interphase, and is loaded into the SPB and along the spindle with the assistance of Cut2 (Kumada *et al.* 1998).

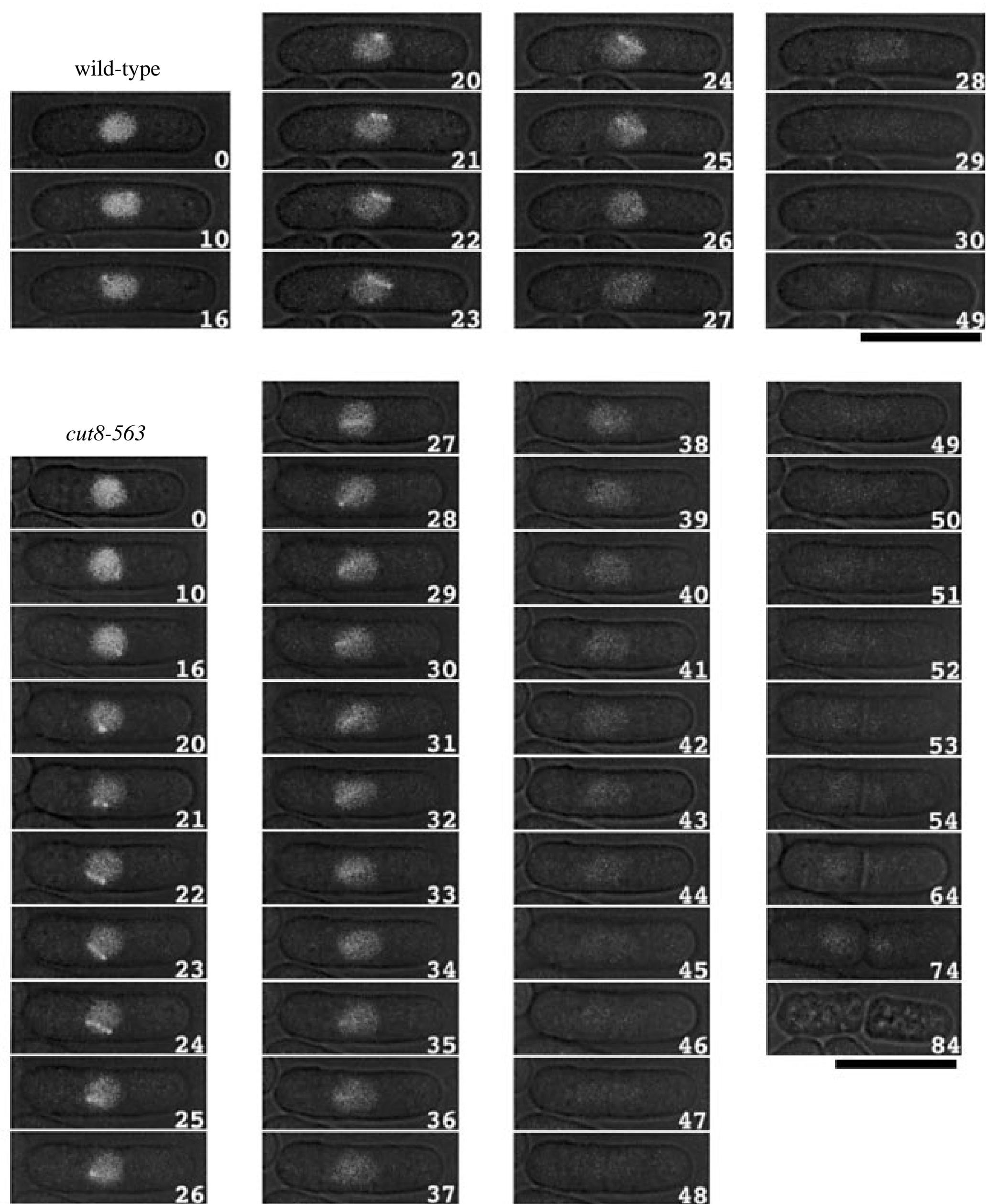

Figure 5. Mitotic cyclin destruction in wild-type and *cut8* mutant cells. The gene of Cdc13-GFP was integrated onto the chromosome of the wild-type and the *cut8-563* mutant. Living cells cultured in the synthetic medium at 36 °C were observed with a confocal microscope (H. Tatebe and M. Yanagida, unpublished data). The number indicates minutes (the initial 0 min is in G2, approximately 10 min before the SPB signal was seen). In a wild-type G2 cell (0 min) at 36 °C, the signal is entirely nuclear, then in late G2 and early mitosis (10–20 min), an additional signal was seen around the SPB. In the mitotic stage (20–25 min), the intense signal was seen along the short spindle. The spindle signal disappeared when its length reached the size of the nucleus (length *ca*. 2.5 μm), followed by the destruction of the nuclear signal (27–28 min). Some signal still remained when the nucleus was elongated at 27 min, suggesting that a fraction of Cdc13 remained in the nucleus of anaphase B. See Nabeshima *et al*. (1998) for detailed analyses of the distinct stages of spindle dynamics in *S. pombe*. In *cut8-563* mutant cells at 36 °C, the single SPB signal was seen at 10 min, while SPB duplication was at 21 min, indicating that the timing of SPB duplication in *cut8* mutants is normal, and similar to that in wild-type. However, destruction of the nuclear and the spindle signal was greatly delayed, suggesting that Cut8 might be needed for efficient degradation of Cdc13. The spindle signal disappeared while the nuclear signal was intense as in wild-type cells.

Upon Cut2 proteolysis, Cut1 still remains on the early anaphase spindle, and may play an essential role in executing anaphase spindle function (Kumada *et al*. 1998). In ts *cut1* mutant cells defective in sister chromatid segregation, spindle elongation is greatly diminished (Nabeshima *et al*. 1998). The force generation of the anaphase spindle might be reduced in the *cut1* mutant. Alternatively, the link between sister chromatids is still present in *cut1* mutant cells, so that spindle elongation is physically restrained.

Nasmyth and his associates (Ciosk *et al*. 1998) argue that Esp1 (homologue of Cut1) is required for removing

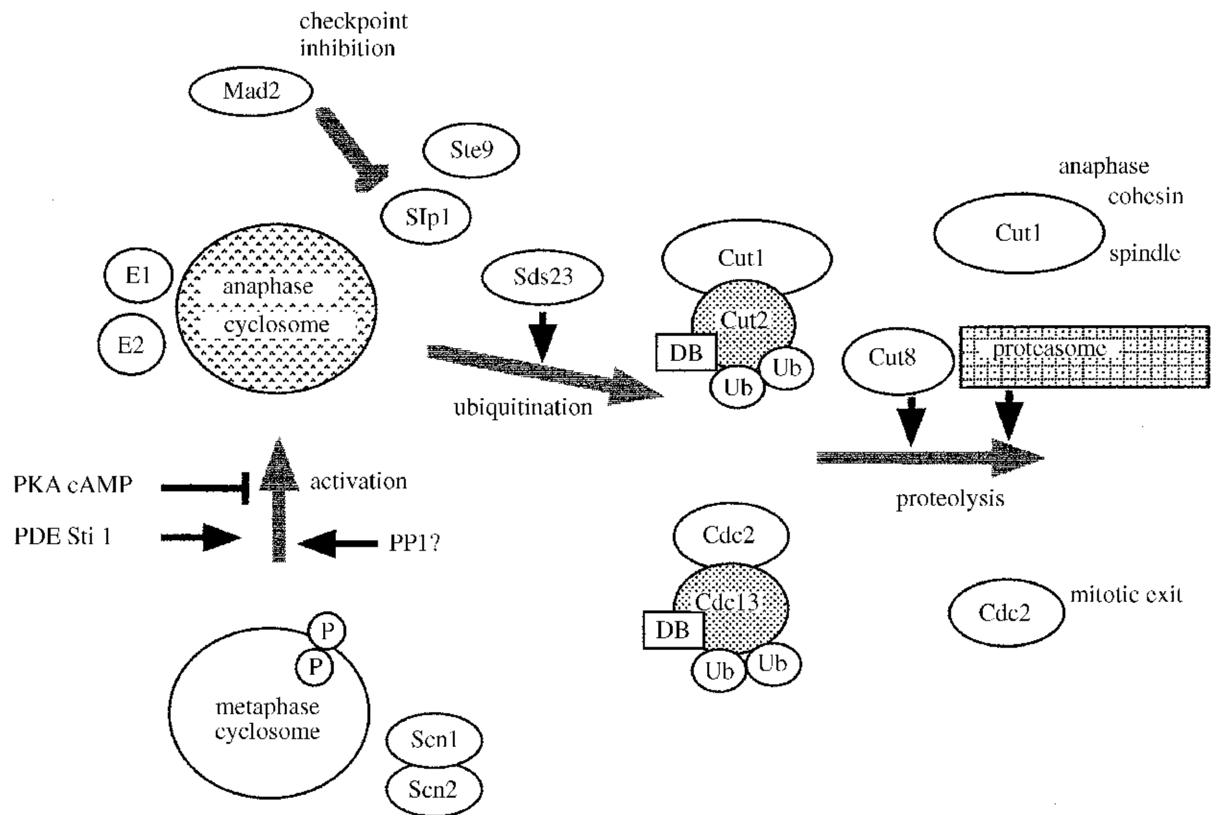

Figure 6. Regulation of the cyclosome complex for ubiquitination of the target substrates (Cut2 and Cdc13). The gene products are placed at the positions according to their roles discussed in the present articles. The major regulatory steps are activation, ubiquitination and proteolysis. See §§ 4 and 5.

cohesin, the sister cohesion complex, from anaphase chromosomes. Their model is apparently not compatible with our localization data if Esp1 directly interacts with the cohesion protein: the full-length Cut1–GFP signal is not seen in the chromatin region. However, the N-terminal fragment of Cut1 is localized in the nucleus throughout the cell cycle, and this region can bind to Cut2 (Kumada *et al.* 1998). Cut1 might thus briefly interact with cohesin and bring it with the bound chromatin region close to the metaphase spindle, as the C-terminal region can bind to the spindle microtubules. These interactions could be greatly altered by Cut2 proteolysis. The N-terminus is the site regulated by proteolysis, while the C-terminus is needed for altering the structures of spindle microtubules required for anaphase. The central domain of Cut1 is needed for cytoplasmic retention (Kumada *et al.* 1998). Cut1 is thus fundamentally important for understanding the mechanism to initiate anaphase.

Overproduced Cut1 and Cut2 are purified as a large complex, which runs at a peak of 40S in sucrose-gradient centrifugations (Funabiki *et al.* 1996*b*; K. Kumada and M. Yanagida, unpublished data). It is unknown whether the large complex formation of Cut1 is functionally relevant. Mammalian Cut1-like protein (KIAA0165) has been detected in cytoplasm, but it does not locate along the mitotic spindle (K. Takahashi and M. Yanagida, unpublished data). However, the N-terminal region of human Cut1 is present in the nucleus although its involvement in anaphase remains to be determined.

6. SPINDLE DYNAMICS DURING THE METAPHASE–ANAPHASE TRANSITION AND PROTEOLYSIS

Exactly when does proteolysis occur in fission yeast mitotic cells? A most sensitive assay for anaphase proteolysis is to measure the signal intensity of GFP-tagged Cut2 or Cdc13 (Kumada *et al.* 1998; H. Tatebe *et al.*, unpublished data). The GFP signals rapidly vanish in mitotic cells, showing that proteolysis is completed within a few minutes in wild-type cells. At which stage of this does proteolysis takes place? In fission yeast, the spindle dynamics consist of three phases (Nabeshima *et al.* 1998): spindle formation (phase 1, the short spindle increases its length up to 2.5 μm), constant spindle length (phase 2, the spindle length is *ca.* 2.5 μm) and spindle elongation (phase 3, the spindle length increases from 2.5 to *ca.* 15 μm). The time of phase 1, 2 and 3 is 2.5, 7.0 and 10.5 min respectively at 26 °C. Sister chromatid separation rapidly occurs at the end of phase 2. This was demonstrated by GFP tagging on the centromere-1-linked DNA sequence. Prior to anaphase A in phase 2, the centromere signal moves back and forth along the spindle. These distinct phases are strongly reminiscent of metazoan mitotic processes.

The degradation of Cut2 along the spindle occurs at the end of phase 2, that is, probably during anaphase A. However, the Cut2 nuclear signal degrades at a slightly earlier stage, perhaps still in metaphase. Similarly, the decay of Cdc13 along the spindle occurs in metaphase,

but the nuclear signal disappears during anaphase. These results show that all of Cdc13 and Cut2 do not degrade at the same instance. Their degradation timing seems to be dependent on localization. It is tempting to speculate that the timing of Cdc13 degradation on the spindle is determined by the completion of phase 1 (spindle formation), while Cdc13 in the nucleus fully degrades after phase 2 (sister chromatid separation).

7. CONCLUDING REMARKS

A scheme illustrating regulating elements of cyclosome-dependent proteolysis is presented in figure 6. Involvement of cAMP in sister chromatid separation was first reported in the studies of *nuc2* and *dis2* mutants (Hirano *et al*. 1988*b*), as a transient decrease of cAMP concentration during mitosis appeared to enhance the segregation of chromosomes in anaphase. Genetic and biochemical evidence has been accumulated showing that PKA directly regulates structure and function of the cyclosome complex. However, it is completely unknown how the PKA pathway is negatively regulated during anaphase. PP1 or polo-like kinase may be opposing to PKA, but their actual involvement in the metaphase–anaphase progression is little understood. It is important to identify genes that regulate the PKA activity during mitosis.

The same target substrates differ in their destruction timing dependent on localization. Two cyclosome-directed substrates, Cut2 and Cdc13, degrade on the spindle at different times, suggesting that the substrates rather than the cyclosome may determine the precise timing of destruction. The substrates of the cyclosome at a particular location might not become fully ubiquitinated until specified cell cycle timing. Alternatively, the substrates fully ubiquitinated might be protected from proteolysis by another modification (such as phosphorylation) or a binding protein. For example, Cut2 locating at the spindle might be phosphorylated at a special site or bound to a spindle protein until anaphase. In any case, the substrates are likely to have built-in structures other than DB determining their destruction pattern. Such structures could be considered as additional regulatory elements for anaphase proteolysis, and their identification is important for future studies.

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Discussion

P. D. Andrews (*University of Dundee, Dundee, UK*). What is the subcellular localization of Sds23p?

M. Yanagida. Sds23 appears to be everywhere in the cell. But this hasn't yet been done by integration of the Sds23–GFP fusion.

A. Hershko (*Technion—Israel Institute of Technology, Haifa, Israel*). What is the evidence that Sds23p is an isopeptidase inhibitor?

M. Yanagida. Genetics! Geneticist thinking! There is no biochemical data yet but this would explain all of the phenotypes we have.

T. Toda (*Imperial Cancer Research Fund, London, UK*). You showed the degradation of Cut2p and Cdc13p in relation to localization. If you examine this by immunoblotting, do you see any differences in the kinetics of degradation?

M. Yanagida. Within one minute all of the mitotic cyclin and Cut2p disappears in the wild-type, so it's impossible to do any biochemistry. It is only with the GFP fusions that we can see a difference. But if we use *cut8* mutant cells for biochemistry, we might be able to see the difference.

J. Raff (*Wellcome—CRC Institute, Cambridge, UK*). It looks like the destruction of Cdc13p occurs in two phases. Can you relate this to fizzy and fizzy-related or Cdc20 and Cdh1?

M. Yanagida. That is certainly the currently fashionable way of thinking about this. But I think that besides the destruction box, these substrates contain additional structural information which affect the timing of their destruction.