The EMBO Journal vol.15 no.23 pp.6617–6628, 1996

Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes

Hironori Funabiki, Kazuki Kumada and Mitsuhiro Yanagida

Department of Biophysics, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-01, Japan

Fission yeast *Schizosaccharomyces pombe* temperature-sensitive (ts) *cut1* mutants fail to separate sister chromatids in anaphase but the cells continue to divide, leading to bisection of the undivided nucleus (the cut phenotype). If cytokinesis is blocked, replication continues, forming a giant nucleus with polyploid chromosomes. We show here that the phenotype of ts *cut2-364* is highly similar to that of *cut1* and that the functions of the gene products of *cut1*$^+$ and *cut2*$^+$ are closely interrelated. The *cut1*$^+$ and *cut2*$^+$ genes are essential for viability and interact genetically. Cut1 protein concentrates along the short spindle in metaphase as does Cut2. Cut1 (~200 kDa) and Cut2 (42 kDa) associate, as shown by immunoprecipitation, and co-sediment as large complexes (30 and 40S) in sucrose gradient centrifugation. Their behavior in the cell cycle is strikingly different, however: Cut2 is degraded in anaphase by the same proteolytic machinery used for the destruction of cyclin B, whereas Cut1 exists throughout the cell cycle. The essential function of the Cut1–Cut2 complex which ensures sister chromatid separation may be regulated by Cut2 proteolysis. The C-terminal region of Cut1 is evolutionarily conserved and similar to that of budding yeast Esp1, filamentous fungi BimB and a human protein.

Keywords: anaphase/checkpoint control/cyclosome–APC/spindle/ubiquitin-dependent proteolysis

Introduction

Chromosome duplication during the S phase and sister chromatid separation in anaphase are crucial events for the passage of genetic information from mother to daughter cells. To separate chromosomes accurately, the linkage between replicated sister chromatids must be maintained from the completion of DNA replication until the metaphase–anaphase transition (for reviews, see Holm, 1994; Miyazaki and Orr-Weaver, 1994; Holloway, 1995; Yanagida, 1995). The mechanism that ensures the simultaneous separation of sister chromatids is a long-standing enigma in cell biology.

A number of events, such as DNA replication, chromosome condensation, spindle formation and microtubule–chromosome interactions must be orchestrated for the formation of a metaphase plate (Earnshaw and Pluta, 1994; Koshland, 1994; Rieder and Salmon, 1994; Hyman and Karsenti, 1996). All these mitotic events are under cell cycle control, driven by the rise and fall of Cdc2 protein kinase activity (Nurse, 1990; King *et al.*, 1994). In mitosis, Cdc2 protein (designated Cdc2p hereafter) forms complexes with mitotic cyclins B, and this active complex is required for cells to enter M phase. After the formation of the metaphase plate, the mitotic cyclins are degraded by ubiquitin-mediated proteolysis at the metaphase–anaphase transition and this cyclin B destruction is responsible for the exit from mitosis (Murray *et al.*, 1989; Glotzer *et al.*, 1991; Hershko *et al.*, 1991). Sister chromatid separation can occur, however, in the presence of non-destructive cyclin B (Holloway *et al.*, 1993; Surana *et al.*, 1993; Rimmington *et al.*, 1994; Sigrist *et al.*, 1995). Since ubiquitin-mediated proteolysis has been shown to be required for sister chromatid separation (Ghislain *et al.*, 1993; Gordon *et al.*, 1993; Holloway *et al.*, 1993; Irniger *et al.*, 1995; King *et al.*, 1995; Tugendreich *et al.*, 1995), a protein(s) other than cyclin must be degraded in order for this separation to occur.

Several proteins have been reported to be degraded in anaphase (Brown *et al.*, 1994; Ye *et al.*, 1995; Funabiki *et al.*, 1996; Stratmann and Lehner, 1996). Fission yeast Cut2p is degraded in anaphase and its degradation depends on Cut9p (Samejima and Yanagida, 1994) which is one of the components of the 20S complex required for cyclin ubiquitination, called the cyclosome or anaphase-promoting complex (APC) (King *et al.*, 1995; Sudakin *et al.*, 1995). Cut2p is stabilized by deleting the N-terminal 80 amino acids which contain two sequences similar to the cyclin destruction box (Glotzer *et al.*, 1991). A non-degradable mutant of Cut2p blocks sister chromatid separation, but not other cell cycle events (Funabiki *et al.*, 1996).

The *cut2-364* strain was isolated originally as one of many temperature-sensitive (ts) *cut* mutants in which cytokinesis occurs without proper chromosome segregation (Hirano *et al.*, 1986; Samejima *et al.*, 1993). The *cut2*$^+$ gene encodes a protein of 301 amino acids and shows genetic interaction with the *cut1*$^+$ gene which codes for a protein of 1828 amino acids that is also required for sister chromatid separation (Uzawa *et al.*, 1990). In a *cut1* mutant, multiple rounds of the cell cycle proceed in the absence of chromosome separation (Creanor and Mitchison, 1990; Uzawa *et al.*, 1990). Hence, a polyploid nucleus, together with multiple SPBs (spindle pole bodies) and spindles, is observed in *cut1* mutant cells when cytokinesis is blocked. The C-terminal 400 amino residues of Cut1p show significant homology to those of *Saccharomyces cerevisiae* Esp1 (McGrew *et al.*, 1992), *Aspergillus nidulans* BimB (May *et al.*, 1992) and human KIAA0165 protein (Nagase *et al.*, 1996). The ts mutant *esp1* and *bimB* strains both showed defects in chromosome segregation (Baum *et al.*, 1988; May *et al.*, 1992; McGrew *et al.*, 1992).

We report here that the *cut1*$^+$ and *cut2*$^+$ genes are

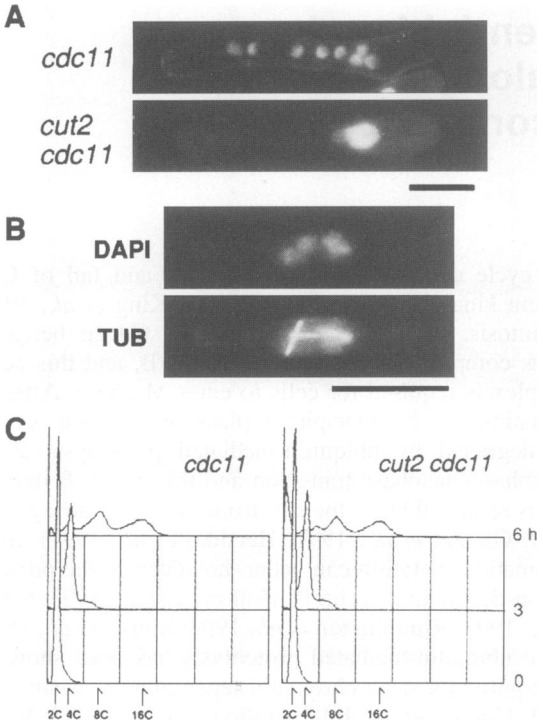

Fig. 1. The phenotype of *cut2-cdc11*. (**A**) DAPI-stained ts *cdc11* defective in septation (top) and the double ts mutant *cut2-cdc11* (bottom) cultured at 36°C for 6 h. *cdc11* cells were multinucleate, whereas the double mutant produced the single giant nucleus. Bar, 10 μm. (**B**) *cut2-cdc11* incubated at 36°C for 6 h was stained with DAPI and anti-tubulin antibody (TAT1). The M phase cell contains condensed chromosomes and multiple spindles. Bar, 10 μm. (**C**) FACScan showed rounds of DNA replication in *cdc11* and *cut2-cdc11* leading to the increase of DNA contents up to 16C after 6 h.

functionally related. They are not only similar in their mutant phenotypes but also their gene products appear to be present in the same large complexes: thus, the $cut1^+$ and $cut2^+$ gene products probably act together to mediate sister chromatid separation. This possibility is corroborated by our finding that the Cut1 and Cut2 proteins are both enriched along the metaphase spindle. The $cut1^+$ and $cut2^+$ genes may be required specifically for chromosome separation, but not for several other cell cycle events, including DNA replication, spindle formation, chromosome condensation and cytokinesis.

Results

Aberrant mitosis in cut2 mutants

When ts *cut2-364* cells were incubated at the restrictive temperature (36°C), the phenotype observed resembled that previously described for *cut1* mutants, which exhibited multiple rounds of entry into the cell cycle in the absence of nuclear division (Creanor and Mitchison, 1990; Uzawa *et al.*, 1990). This was represented most clearly in *cut2-cdc11* double mutant cells where cytokinesis was blocked by *cdc11* (Nurse *et al.*, 1976). A single giant nucleus stained by 4′,6-diamidino-2-phenylindole (DAPI) was seen in the double mutant cells after 6 h at 36°C, whereas a number of nuclei were produced in single *cdc11* cells (Figure 1A). FACScan analysis confirmed the occurrence of rounds of DNA replication in *cut2-cdc11*, leading to DNA contents of up to 16C (Figure 1C). Multiple spindles observed by anti-tubulin staining formed within the large nucleus during mitosis, but the chromosomes failed to separate (Figure 1B). These phenotypes were identical to those obtained for *cut1-cdc11* (Creanor and Mitchison, 1990; Uzawa *et al.*, 1990).

The phenotypes of single *cut2-364* cells were studied in synchronous cultures at 36°C using early G_2 cells collected by elutriation (Creanor and Mitchison, 1990) of the mutant culture grown at 26°C. The cell viability of the synchronous culture at 36°C decreased sharply to 55% during the first mitosis and then further to 10% after the second mitosis (Figure 2A). No obvious defect was observed in the timing of chromosome condensation or spindle formation (Figure 2B). However, mutant cells displayed aberrantly extended chromosomes with an elongating spindle (Figure 2C), known as the 'archery-bow' phenotype (Hirano *et al.*, 1986; Uzawa *et al.*, 1990). These were most abundant in the first and second anaphase (at 100 and 220 min). Fluorescence *in situ* hybridization (FISH) probed with a centromere DNA (Funabiki *et al.*, 1993) revealed that the centromeres were separated in cells showing the 'archery-bow', whereas the distal regions of the chromosomes remained stuck together (Figure 2D) as previously described for *cut1* mutants (Funabiki *et al.*, 1993). These results indicate that *cut2-364* cells enter mitosis but are defective in sister chromatid separation. The histone H1 kinase activity that was used as the marker for the activity of $p34^{cdc2}$ peaked as the cells passed through mitosis (Figure 2E). Thus most cell cycle events proceeded in *cut2-364* with normal timing, except that nuclear division failed even though sister centromeres were temporarily pulled apart.

The $cut2^+$ gene is essential for viability

The $cut2^+$ gene was disrupted by one-step gene replacement. Briefly, a part of the $cut2^+$ gene in a plasmid was replaced with the *Schizosaccharomyces pombe* $ura4^+$ gene, and the disrupted gene was integrated onto the chromosomes of a Ura^- diploid (CM6/HK6; Materials and methods) by homologous recombination. Gene disruption in the resulting Ura^+ heterozygous diploid cells was verified by Southern hybridization (data not shown). Tetrad dissection showed that only two spores were viable, and all of the viable spores were Ura^-, indicating that $cut2^+$ is an essential gene for viability.

The disruption phenotype of $cut2^+$ was investigated. Germinated spores were cultured at 26°C for 36 h on a thin agarose (1.5%) film overlaid on a YPD plate, followed by staining with DAPI. Gene-disrupted spores were germinated and divided once or at most twice (Figure 3A). Nuclear division apparently failed as only one nucleus was present in cells after division. Despite the absence of nuclear division, cytokinesis occurred producing the cut phenotype (inset) or anucleate cells. Non-gene-disrupted spores incubated at 26°C for 36 h produced microcolonies on the YPD plate.

To obtain a more detailed phenotype, antibodies against tubulin and the SPB protein Sad1 (Hagan and Yanagida, 1995) were employed. Gene-disrupted spores were cultured at 36°C for 10, 16 and 19 h in minimal EMM2 liquid medium (Figure 3B); non-gene-disrupted spores failed to grow in this medium. Although the spindle

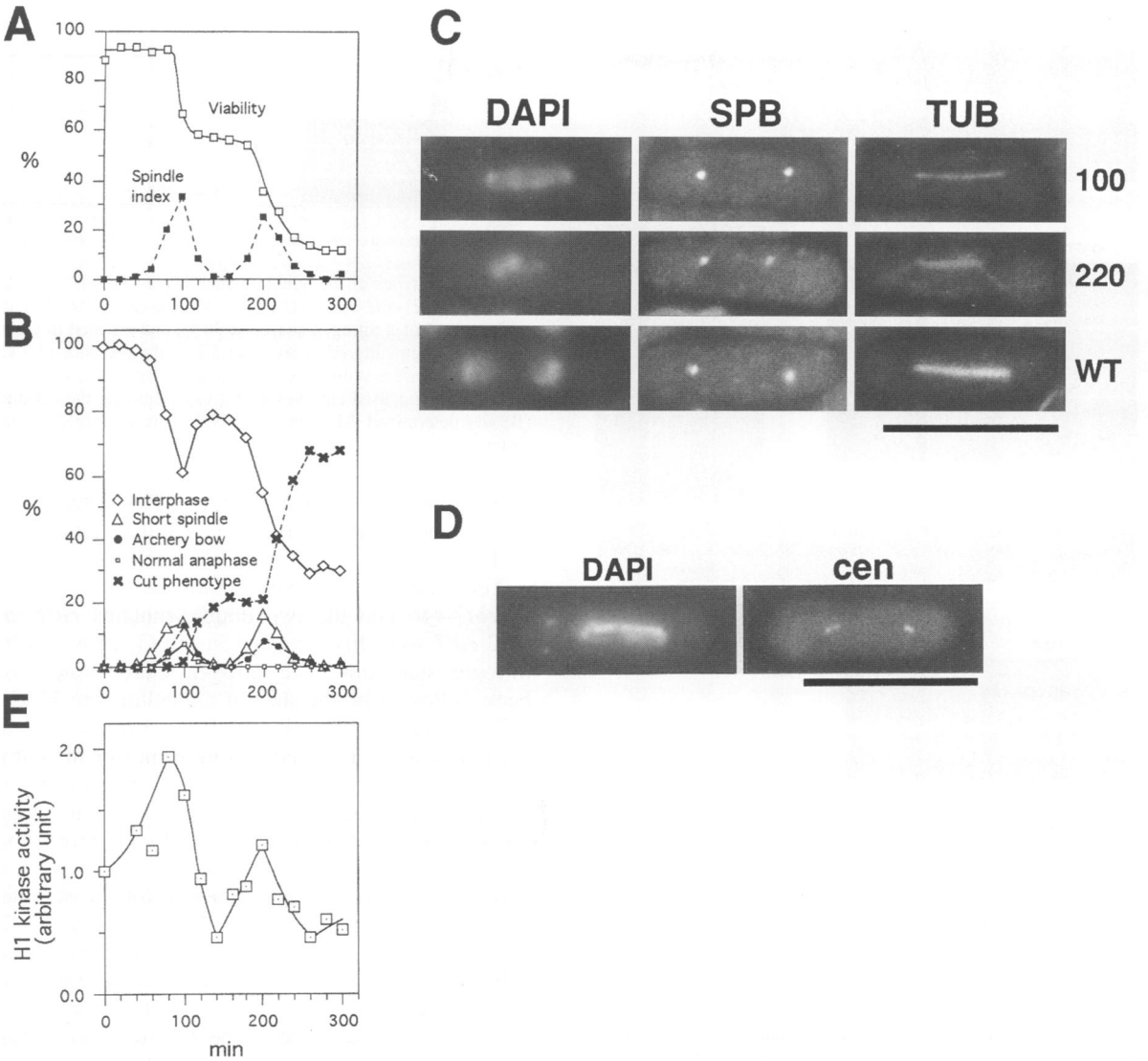

Fig. 2. Synchronous culture of ts *cut2-364* at 36°C. Small early G_2 cells were collected from the mutant culture grown at 26°C by elutriation, followed by incubation at 36°C. (**A**) Percentage of viable cells and percentage of cells containing the spindle are shown. (**B**) Percentage frequencies of interphase cells, mitotic cells with the short spindle, cells displaying normal anaphase or abnormal archery-bow chromosomes, and cells displaying the cut phenotype were determined by DAPI and anti-tubulin staining. (**C**) Mutant cells taken at 100 and 220 min were stained with DAPI, anti-tubulin and anti-Sad1 (SPB) antibodies. The wild-type (WT) anaphase B cell is shown as control. (**D**) FISH using pRS140 (Funabiki *et al.*, 1993) as the centromere probe was applied to *cut2-364* at 36°C for 120 min. Bars, 10 μm. (**E**) Histone H1 kinase was assayed by incubation of extracts with histone H1 and [γ-^{32}P]ATP.

formed and elongated, condensed chromosomes neither segregated nor formed the archery-bow phenotype (Figure 3Ba–c). Ten hours after germination, a significant fraction (~10%) of germinated cells showed the condensed chromosomes with the spindle: such cells were negligible after 16 and 19 h. Half of those cells at 10 h showed the elongating spindle, suggesting that the spindle failed to pull the sister chromatids apart. The frequency of the cut phenotype followed was ~30 and 70% after 10 and 16 h, respectively, and divided cells without a nucleus were also common. Large cells showing condensed chromosomes, and multiple spindles (1–1.5% after 16 and 19 h; Figure 3Be) were also observed as well as those containing a single interphase nucleus with the aggregated SPBs (8 and 12% after 16 and 19 h, respectively; Figure 3Bd). The phenotype of gene disruption was thus similar to that of ts *cut2-364*, except that it is more severe than that of the ts mutant in relation to the extent of the failure in sister chromatid separation.

The cut1$^+$ gene is essential for viability

To determine whether *cut1$^+$* is essential for viability, a null allele was created by replacing a large part of the *cut1$^+$* open reading frame (ORF) by the *ura4$^+$* gene. This deleted gene was used to replace one copy of the *cut1$^+$* gene in the genome of a Ura$^-$ diploid CHP428/CHP429. Dissection of 37 tetrads indicated that only two spores from each tetrad gave rise to colonies. All viable progeny were Ura$^-$, indicating that *cut1$^+$* is essential for cell viability. The phenotype of the gene disruptant (*cut1*Δ) after germination was investigated by staining of cells with DAPI, anti-tubulin and anti-Sad1 antibodies. The *cut1*Δ germinated cells entered mitosis, showing condensed chromosomes and an elongating mitotic spindle.

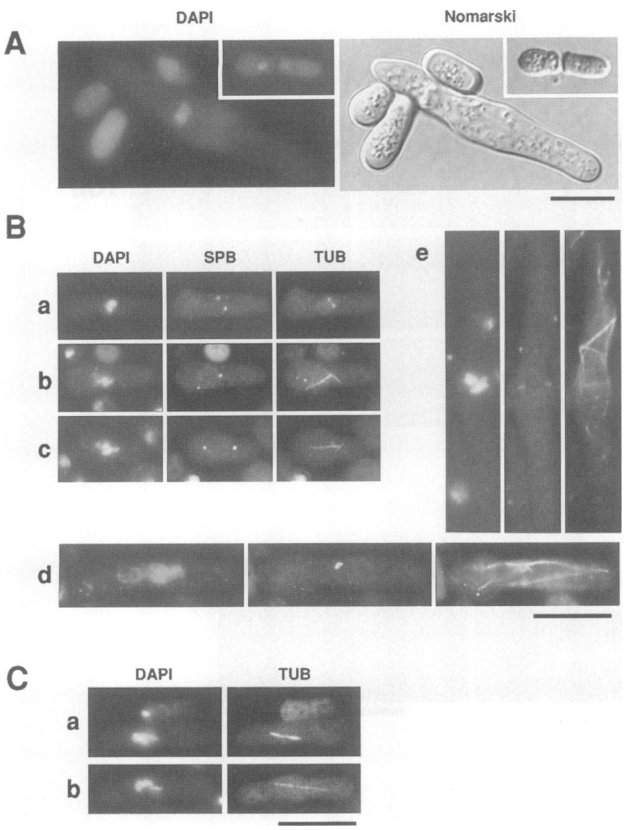

Fig. 3. Gene disruption of *cut1*$^+$ and *cut2*$^+$. (**A**) *cut2*Δ spores were incubated on solid agarose at 26°C for 36 h and stained by DAPI (left) and Nomarski (right). Germinated cells divided once (the cut phenotype, inset) or twice (one large and three small cells). Bar, 10 μm. (**B**) *cut2*Δ spores were cultured at 26°C in the liquid media, and cells were taken after 10 or 19 h, and triple stained with DAPI, anti-Sad1 (SPB) and anti-tubulin (TUB) antibodies. (a–c) Mitotic cells with condensed chromosomes and the short or elongating spindle (taken after 10 h). Condensed chromosomes were pulled in b–c but not separated. The chromosomes observed in b and c were scattered along a spindle longer (4–5 μm) than the metaphase spindle (2.0–2.5 μm), but shorter than the wild-type anaphase B spindle (10–12 μm). (d) A large interphase cell containing multiple SPBs (taken after 19 h). (e) A large mitotic cell containing multiple spindles and condensed chromosomes (19 h). Bar, 10 μm. (**C**) *cut1*Δ spores were incubated at 26°C in the liquid medium. Cells were taken after 10 h and double stained with DAPI and anti-tubulin (TUB) antibody. Bar, 10 μm.

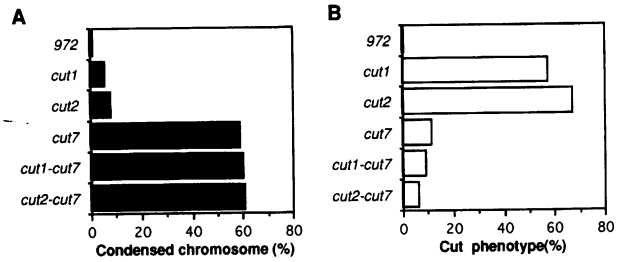

Fig. 4. Phenotypes of the double mutants *cut1-cut7* and *cut2-cut7*. Wild-type *972*, *cut1-206*, *cut2-364*, *cut7-446*, *cut1-cut7* and *cut2-cut7* were starved of a nitrogen source at 26°C, and arrested in G_1 phase. They were then cultured at 36°C for 5 h in the presence of a nitrogen source. Cells were stained by DAPI and the frequency of cells containing condensed chromosomes (**A**) or showing the cut phenotype (**B**) was determined. More than 200 cells were examined for each strain.

However, chromosome separation was not observed even in cells showing the elongating spindle (Figure 3C). Twenty percent of the germinated cells showed the cut phenotype (a portion of the bisected cell is shown in Figure 3Ca, above).

Mitotic arrest in the absence of proper spindle formation

The *cut1* and *cut2* mutant cells performed mitotic and subsequent cell cycle events without nuclear division. This kind of phenotype might have arisen due to the failure in spindle assembly checkpoint control (Hoyte *et al.*, 1991; Li and Murray, 1991). We therefore carried out an experiment to address the question of whether the checkpoint control for spindle assembly was defective in the *cut1* or *cut2* mutant. We used *cut7-446*, a ts mutant of the kinesin-like motor gene, in which proper spindle formation was inhibited (Hagan and Yanagida, 1990, 1992), but cytokinesis occurred in only a small fraction of cells (<15%).

The wild-type *972*, single mutants *cut1-206*, *cut2-364* or *cut7-446* and the two double mutants *cut1-cut7* and *cut2-cut7* were first arrested in the G_1 phase at 26°C by nitrogen starvation. The nitrogen source was then added back, followed by the shift of the cultures to 36°C. When cells passed the first mitosis after 5.5 h, the frequency of cells showing condensed chromosomes or the cut phenotype was estimated (Figure 4). In *cut1* and *cut2* mutants, the frequency of mitotically arrested cells containing condensed chromosomes was <10% (Figure 4A), while that of the cut phenotype was much higher (~60%) (Figure 4B). In contrast, a high frequency (60%) of mitotically arrested cells was observed in the *cut7* mutant, indicating that the spindle assembly checkpoint is activated in these cells. In *cut1-cut7* or *cut2-cut7* double mutants, 60% of the cells showed condensed chromosomes and the mitotically arrested phenotype (Figure 4A), while cells with the cut phenotype were infrequent (Figure 4B), demonstrating that a spindle assembly checkpoint function is not defective in either *cut1* or *cut2* mutants.

Determination of mutation sites

Ten *cut1* alleles and one *cut2* allele were isolated previously (Hirano *et al.*, 1986, Samejima *et al.*, 1993). All were recessive and showed genetic interactions. *cut1-21* was the only allele whose temperature sensitivity was partially rescued by multicopy plasmid pCUT2-4, while *cut2-364* was suppressed by multicopy plasmid pCUT1-1 (Hirano *et al.*, 1986). The double mutant *cut1-cut2* was synthetically lethal (Uzawa *et al.*, 1990).

Approximate locations of mutations were first estimated by integration rescue of temperature sensitivity using variously truncated *cut1* genes. DNAs of those regions in the mutant genes were then amplified by the PCR method and their nucleotide sequences were determined. Mutation sites were thus determined by comparison with the wild-type sequence (Figure 5A, upper panel). All the *cut1* mutations showed missense mutations. While two mutations (*679* and *693*) resided in the conserved C-terminal region, other mutations were located around the central region. Two *cut1* mutations residing in the C-terminal region are indicated in juxtaposition with the sequences of *S.cerevisiae* Esp1, *A.nidulans* BimB and

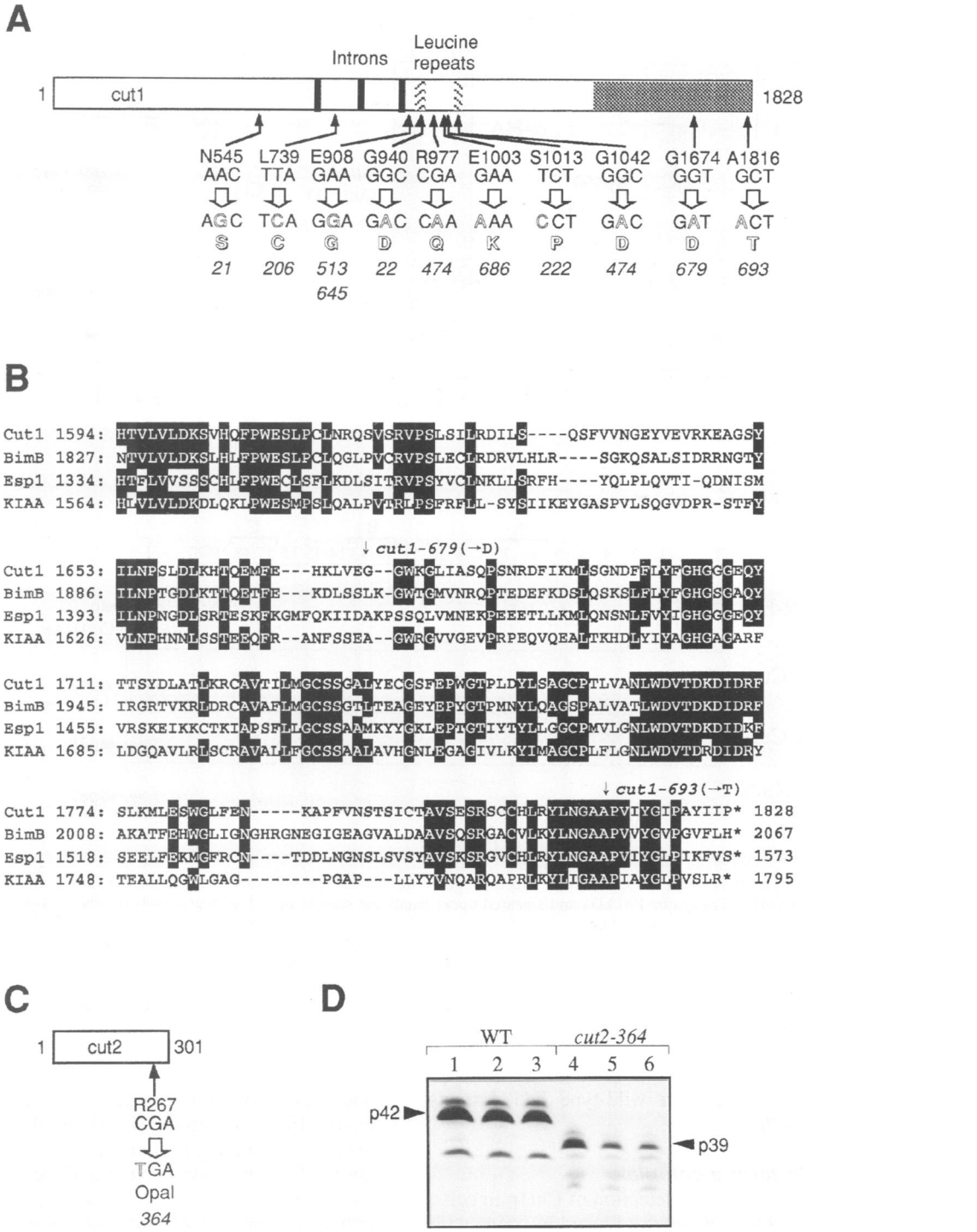

Fig. 5. Mutation sites of *cut1* and *cut2*. (**A**) Mutation sites of 10 ts *cut1* strains were determined (see text). *cut1-21*, for example, contained a mutation in the codon 544 from AAC to AGC, causing an amino acid change from N to S. *cut1-474* was found to contain mutations at codons 977 and 1042. (**B**) Amino acid sequence comparison of the C-terminal regions in Cut1p, BimB, Esp1 and KIAA0165 protein. Residues conserved in more than three sequences are boxed. The conserved A at codon 1816 was changed to T in *cut1-693*, while the non-conserved G was altered to D in *cut1-679*. (**C**) The *cut2-364* mutation site was determined by PCR cloning and sequencing. The 267th codon for R was changed to the opal stop codon. (**D**) An immunoblot of wild-type and *cut2-364* extracts using affinity-purified anti-Cut2 antibodies. Lanes 1–3, wild-type at 36°C for 0, 1.5 and 3 h, respectively. Lanes 4–6, *cut2-364* at 36°C for 0, 1.5 and 3 h, respectively.

human KIAA0165 (Figure 5B). These analyses show that both the central region and the C-terminus are important for Cut1p function.

The *cut2* mutant gene was isolated from *cut2-364* by PCR. Nucleotide sequencing of the whole mutant gene established that only one nucleotide was substituted, such that the arginine (R) codon (CGA) at position 267 is changed to an opal stop codon (TGA; Figure 5C). This mutant Cut2 protein thus must lack the C-terminal 35 amino acids. This was confirmed by immunoblotting of wild-type and mutant cell extracts (Figure 5D). Mutant cell extracts produced the 39 kDa protein band instead of

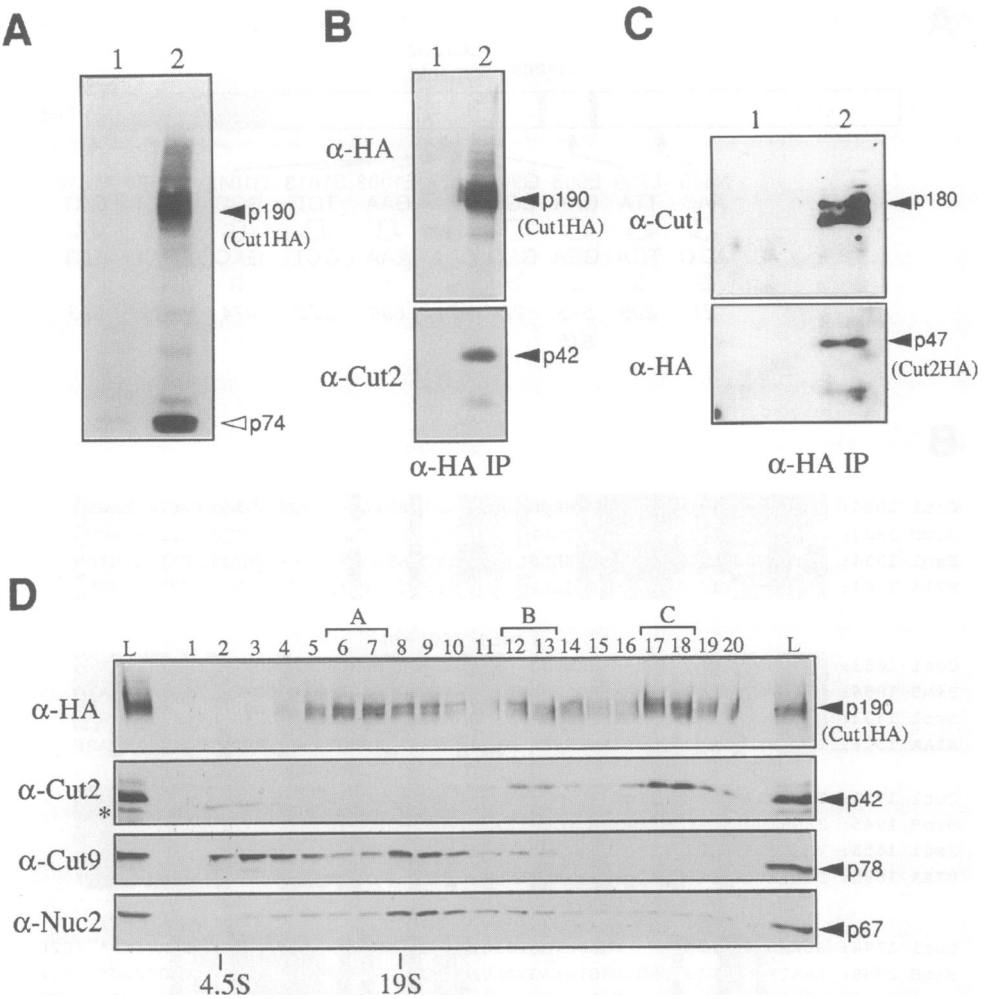

Fig. 6. Interaction between Cut1p and Cut2p. (**A**) Cell extracts prepared from wild-type *972* (lanes 1) and *cut1HA*-integrated HF173 (lanes 2) were immunoblotted using anti-HA. The major 190 kDa and smeared upper bands are seen in lane 2. p74 was probably the cleaved product of Cut1p. (**B**) Immunocomplexes were obtained from wild-type (lane 1) and *cut1HA*-integrated HF173 (lane 2) using anti-HA antibody, and immunoblotted using anti-HA (top) and anti-Cut2 (bottom) antibodies. Cut2p (p42) forms the immunocomplex with Cut1HAp. (**C**) Immunocomplexes were obtained from the wild-type (lane 1) and *cut2HA*-integrated HF178 (lane 2) using anti-HA antibody, and immunoblotted using anti-Cut1 (top) and anti-HA antibodies. Cut1p (p180) forms the immunocomplex with Cut2HAp. (**D**) Cell extracts from *cut1HA*-integrated HF173 (designated L) were run in sucrose gradient centrifugation and examined by immunoblot using anti-HA, anti-Cut2, anti-Cut9 and anti-Nuc2 antibodies. The band indicated by an asterisk is a proteolytic product of Cut2p.

the 42 kDa band produced in wild-type cell extracts (Funabiki *et al.*, 1996).

Cut1p and Cut2p form a complex

To allow sensitive and specific detection of Cut1p in cells, the hemagglutinin (HA) epitope was ligated in-frame with the C-terminus of the *cut1*$^+$ ORF, and the resulting HA-tagged *cut1*$^+$ gene was integrated onto the chromosome to replace the untagged *cut1* gene. This *cut1HA*-integrated strain was termed HF173. Using the 12CA5 monoclonal anti-HA antibody, multiple bands migrating at ~190 kDa with smeared upper bands in SDS–PAGE (Figure 6A, lane 2) were detected in HF173 extracts, while no band was detected by 12CA5 in the wild-type strain (lane 1). The 74 kDa band was probably the cleaved polypeptide of Cut1p.

Because the phenotypes of *cut1* and *cut2* mutants were so similar, we examined whether Cut2p was physically associated with Cut1p. Tagged Cut1HAp was immunoprecipitated by anti-HA antibody from extracts of HF173 and immunoblotted with anti-Cut2 antibodies (Funabiki *et al.*, 1996). A strong signal for the Cut2 protein was observed (Figure 6B, lane 2, lower panel), which was absent from wild-type extracts (lane 1). Similarly, when tagged Cut2HAp was immunoprecipitated by anti-HA antibody in the *cut2HA*-integrated strain HF178 (Funabiki *et al.*, 1996), Cut1p detected by anti-Cut1 antibodies (Uzawa *et al.*, 1990) was found in the immunoprecipitates (Figure 6C, lane 2, upper panel). These results established that Cut1p and Cut2p formed a complex.

Extracts from the *cut1HA*-integrated HF173 strain were run in a sucrose gradient by ultracentrifugation at 40 000 r.p.m. for 12 h. Figure 6D shows that three peaks of Cut1p detected by anti-HA were observed, sedimenting at ~15, 30 and 40S, designated A (fractions 6 and 7), B (fractions 12 and 13) and C (fractions 17 and18). These three peaks were reproduced in many runs of sucrose gradient centrifugation. Antibodies against Cut9p (Samejima and Yanagida, 1994) and Nuc2p (Hirano *et al.*, 1988) detected the 20S cyclosome/APC which was used as a reference

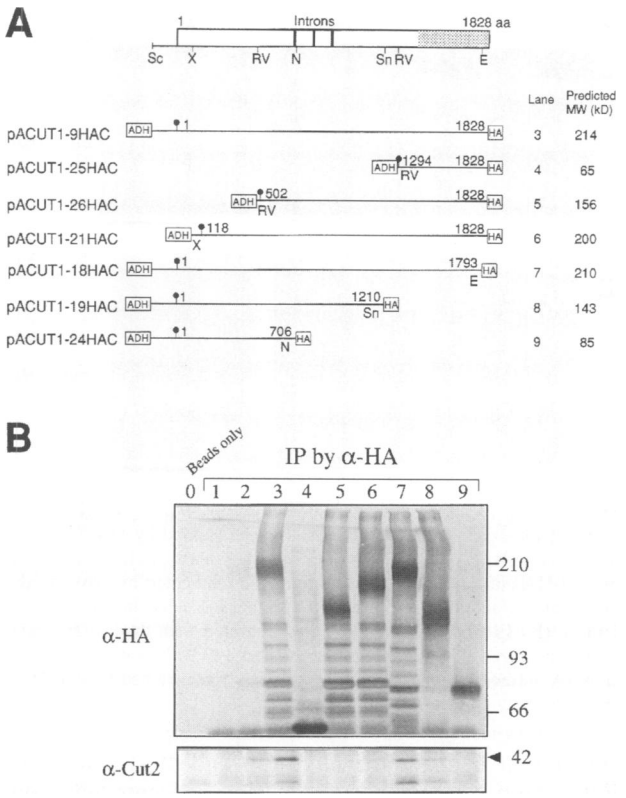

Fig. 7. Overproduction of truncated Cut1p and interaction with Cut2p. (**A**) Plasmids carrying truncated *cut1HA* genes under control of the *S.pombe* ADH promoter (Russell and Hall, 1983) were made. The positions of the first methionine codons are indicated by filled circles. The predicted molecular weight of each gene product is also shown. Sc, *Sca*I; X, *Xba*I; RV, *Eco*RV; N, *Nco*I; Sn, *Sna*BI; E, *Eco*RI. (**B**) Immunocomplexes were obtained from extracts of wild-type-carrying vector plasmid (lane 1), *cut1HA*-integrated HF173 strain (lane 2) and wild-type-carrying plasmid with the full-length *cut1HA* gene (lane 3) or variously truncated *cut1HA* genes (lane 4–9; see A) using anti-HA antibody, and immunoblotted using anti-HA (top) and anti-Cut2 (bottom) antibodies. The positions of the marker proteins (210, 93, 66 kDa) and $p42^{Cut2}$ are indicated.

in addition to serum albumin (4.5S) and thyroglobulin (19S). Cut2p co-sedimented with the 30 and 40S complexes of Cut1p, but not with the 15S complex. Most, if not all, of Cut2p was in the large complexes. Cut2p is enriched in the nucleus of interphase cells (Funabiki *et al.*, 1996), suggesting that these complexes are localized in the nucleus. When ts *cut2* mutant extracts containing integrated Cut1HAp and cultured at 36°C for 4 h were run in sucrose gradient centrifugation, mutant Cut2p and Cut1HAp detected by polyclonal anti-Cut2 and monoclonal anti-HA antibodies, respectively, were scarcely present in the 30S fractions (K.Kumada, T.Nakamura and M.Yanagida, unpublished result). Consistently, Cut2p in *cut1* mutant extracts was not present in the 30S fraction, suggesting that 30S complex formation might require the association of both functional Cut2p and Cut1p.

The region of Cut1p required to interact with Cut2p

To determine which region of Cut1p is required for interaction with Cut2p, we constructed several plasmids which produced truncated Cut1p tagged with HA under the fission yeast alcohol dehydrogenase (ADH) promoter (Figure 7A, Russell and Hall, 1983). Extracts were prepared from cells carrying one of these multicopy plasmids, and then truncated HA-tagged Cut1p was immunoprecipitated using anti-HA antibody, followed by immunoblotting using anti-HA and anti-Cut2 antibodies (Figure 7B). The full-length Cut1HAp as well as the N-terminal 1793 amino acid polypeptide was able to co-precipitate with Cut2p (lower panel, lanes 3 and 7). The N-terminal 1210 amino acids (lane 8) also co-precipitated with Cut2p, though the degree of co-precipitation was greatly reduced. The other versions of truncated Cut1 proteins co-precipitated little or not at all with Cut2p. These results suggest that the conserved C-terminal region of Cut1p might not be required for association with Cut2p.

Cut1p exists throughout the cell cycle

Cut2p is degraded in anaphase and remains unstable during G_1 (Funabiki *et al.*, 1996): Cut2p is scarce in G_1-arrested *cdc10* mutant cells. We examined the level of Cut1p in mutant cells blocked at different stages of the cell cycle. The wild-type strain and *cdc10-129*, *cdc25-22* and *nuc2-663* strains each having an integrated *cut1HA* gene were shifted from 26 to 36°C. The *cdc10*, *cdc25* and *nuc2* strains were arrested in G_1, G_2 and metaphase, respectively (Nurse and Bissett, 1981; Hirano *et al.*, 1988; Moreno *et al.*, 1989). Tagged Cut1HAp existed in all the cell cycle-arrested cells examined (Figure 8A, upper panel), except that its level was reduced significantly in M-arrested *nuc2* mutant cells. The reason for this reduction is not understood. In contrast, Cut2p and Cdc13p were specifically absent from the extracts of G_1-arrested *cdc10* cells (Figure 8A, middle two panels). Thus Cut1p does not behave in the same way as Cut2p in G_1-arrested *cdc10* cells.

We addressed the question of whether the level of Cut1p oscillates during the cell cycle. The level of Cut1HAp was also roughly constant in the synchronized *cdc25* mutant culture made by a block–release experiment in which the culture was shifted from 36 to 25°C (Figure 8B): no obvious reduction was found in synchronized mitotic cells (20–30 min after release). The percentage of anaphase cells was determined by anti-tubulin and DAPI staining. We thus conclude that the level of Cut1p does not oscillate greatly in the cell cycle of *S.pombe*. The intensity of the 74 kDa cleaved product also did not fluctuate during the cell cycle. These results suggest that Cut1p, although susceptible to proteolysis, does not degrade in anaphase simultaneously with Cut2p.

Cut1p localizes along the metaphase spindle

Cut2p localizes in the nucleus during G_2 phase, along the short spindle in metaphase, and then disappears at anaphase (Funabiki *et al.*, 1996). We examined the immunolocalization of Cut1p using the *cut1HA*-integrated strain HF173. While weak signals were seen throughout the G_2 phase cells, strong signals were localized along the short mitotic spindle (indicated by arrowheads in the left panel of Figure 9A). Cut1HAp was highly concentrated along the short spindle produced in the metaphase-arrested ts *nuc2* cells (Figure 9B, left panel). However, in the anaphase spindle (inset in Figure 9A), the Cut1HAp signal was hardly observed.

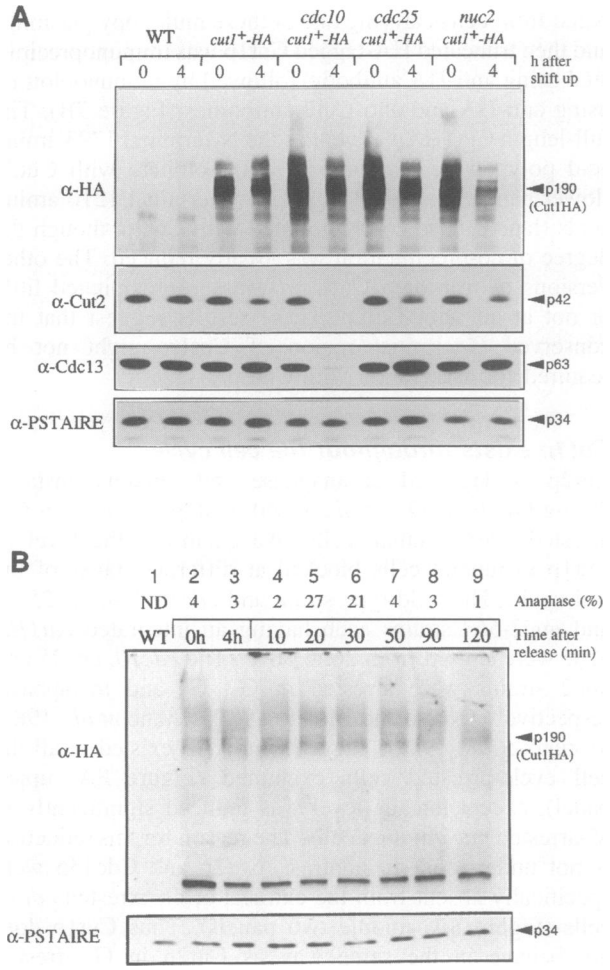

Fig. 8. The constant level of Cut1p during the cell cycle. (**A**) Cell extracts were prepared from wild-type (WT), HF173 (*cut1HA* integrant) and ts mutant strains *cdc10*, *cdc25* and *nuc2* integrated with the *cut1HA* gene. The level of Cut1HAp was estimated by immunoblot using anti-HA antibodies. The levels of Cut2p, Cdc13p and Cdc2p were also determined by immunoblotting against whole cell extracts using anti-Cut2, anti-Cdc13 and anti-PSTAIRE antibodies, respectively. (**B**) Cell extracts were prepared from wild-type (lane 1) and ts *cdc25-22* mutant integrated with the *cut1HA* gene, grown at 25°C (lane 2), blocked in G_2 phase at 36°C for 4 h (lane 3), then released at 25°C (lane 4–9). The level of Cut1HAp was determined by immunoblotting using anti-HA antibodies. Anti-PSTAIRE antibody served as a loading control. The frequency (%) of anaphase cells determined by DAPI and anti-tubulin staining is also shown.

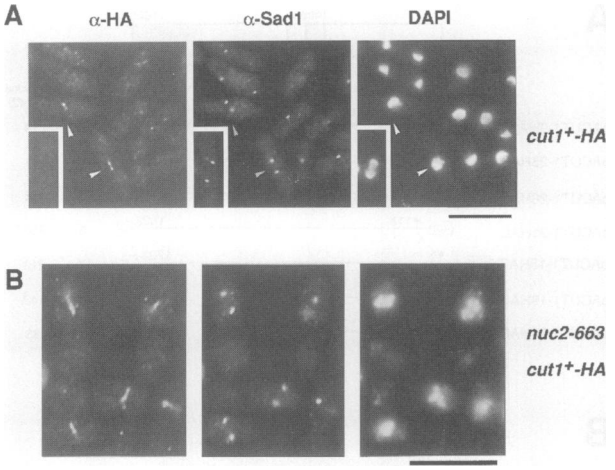

Fig. 9. Cut1p localizes along the short mitotic spindle. (**A**) HF173 (*cut1HA* integrant) cells grown at 33°C were stained by DAPI, anti-Sad1 (for SPB) and anti-HA antibodies. The short spindles are stained by anti-HA antibody in the mid-mitotic cells indicated by arrowheads. Few anti-HA signals were observed in anaphase cells (inset). Bar, 10 μm. (**B**) Ts *nuc2-663* mutant cells integrated with the *cut1HA* gene were incubated at 36°C for 4 h and stained by DAPI, anti-Sad1 and anti-HA antibodies. The metaphase spindles were stained by anti-HA antibody. Bar, 10 μm.

Discussion

We have shown in this and previous studies (Hirano *et al.*, 1986; Uzawa *et al.*, 1990; Funabiki *et al.*, 1996) that the *cut1*$^+$ and *cut2*$^+$ genes are essential for sister chromatid separation and that the functions of their gene products are closely interrelated. Several lines of evidence suggest that the two gene products collaborate in ensuring sister chromatid separation. First, *cut1* and *cut2* mutant phenotypes are very similar. Second, *cut2-364* is suppressed by the multicopy plasmid pCUT1-1 (Hirano *et al.*, 1986), whereas a single allele of *cut1-21* was suppressed by plasmid pCUT2-4. Third, the double mutant *cut1-cut2* is inviable at the permissive temperature (Uzawa *et al.*, 1990). Fourth, both immunochemical and sucrose gradient centrifugation experiments indicate that Cut1p and Cut2p are present in the same large (30 and 40S) complexes. Fifth, both Cut1p and Cut2p are concentrated along the short spindle in metaphase but disappear from the elongating spindle in anaphase.

The identification of the components in the 30 and 40S complexes other than Cut1p and Cut2p is of obvious interest. If the complex contains only Cut1p and Cut2p, they may be polymerized into a higher order structure. If the complexes contain many different subunits, they may have intricate functions. What makes up these complexes and how their composition and activity are regulated during the cell cycle are obvious targets for further investigation. The complex structures seem to be functionally relevant, as Cut1p and Cut2p are not detected in the 30S fraction of *cut2* mutant extracts in sucrose gradient centrifugation.

Checkpoint control for anaphase progression appears to be retained in *cut1* or *cut2* mutant cells. Proper spindle formation and subsequent cell cycle progression are inhibited in cells containing the double mutation *cut1-cut7* or *cut2-cut7*: *cut7*, which is essential for spindle formation (Hagan and Yanagida, 1990, 1992), is epistatic to *cut1* and *cut2*. Neither *cut1* nor *cut2* is hypersensitive to UV or hydroxyurea (Y.Saka and M.Yanagida, unpublished result). The *cut1*$^+$ and *cut2*$^+$ genes are unlikely to be a part of the cell cycle control which promotes cells to enter anaphase, and may be involved specifically in sister chromatid separation. This is consistent with the phenotypes of *cut1* and *cut2* mutants in which H1 kinase activation and inactivation, spindle dynamics, cell separation and DNA replication occur with normal cell cycle timing.

The C-terminal region of Cut1p is evolutionarily conserved, revealing considerable similarity (30–40% identity) to that of *S.cerevisiae* Esp1 (McGrew *et al.*, 1992), *A.nidulans* BimB (May *et al.*, 1992) and human KIAA0165 protein (Nagase *et al.*, 1996). Mutant phenotypes of *cut1*, *esp1* and *bimB* are similar to each other

with regard to the occurrence of multiple rounds of cell cycle in the absence of nuclear division (Baum *et al.*, 1988; Creanor and Mitchison, 1990; Uzawa *et al.*, 1990; May *et al.*, 1992; McGrew *et al.*, 1992; Surana *et al.*, 1993). The N-terminal to central region of Cut1p consisting of >1200 amino acids, however, does not show strong similarity to known proteins. This non-homologous region appears to perform an essential function as well, because in it reside many ts mutation sites. One possibility is that the genome might have several proteins with the same conserved C-terminal region. However, analysis of the whole *S.cerevisiae* genome indicates that Esp1 is the sole protein having the conserved C-terminal region. Non-conserved regions of Cut1p and related proteins may interact with divergent but essential portions of spindle and/or chromosome structures. Alternatively, regions displaying little amino acid sequence similarity may have similar three-dimensional structures.

The level of Cut1p does not oscillate during the cell cycle. By analogy to a complex composed of stable Cdc2p and unstable cyclin B, Cut2p may regulate the activity of Cut1p by degradation in anaphase. It is difficult, however, to predict whether the complex containing Cut1p and Cut2p is functionally activated or inactivated in the progression from metaphase to anaphase, as little is known about the molecular functions of Cut1p and Cut2p except that they are essential for sister chromatid separation. Cut2p is phosphorylated, producing a slowly migrating 44 kDa band specifically in mitosis (Funabiki *et al.*, 1996) and a cdc2 consensus site (S109PRR) is actually phosphorylated (H.Funabiki and M.Yanagida, unpublished data). However, the role of this phosphorylation is unknown. Cut1p may also be phosphorylated or post-translationally modified, as upper smear bands are observed throughout the cell cycle. Cut1p is highly susceptible to proteolysis: a number of cleaved protein bands were produced in cell extracts.

As reported previously, the anaphase degradation of Cut2p is essential for sister chromatid separation, and it depends upon the same machinery required for proteolysis of cyclin B (Funabiki *et al.*, 1996). Simultaneous destruction of Cut2p and cyclin B may ensure the occurrence of sister chromatid separation and metaphase–anaphase transition with the same timing. Then what triggers proteolysis of cyclin B and Cut2p? Perhaps elements that regulate the 20S cyclosome/APC (King *et al.*, 1995; Sudakin *et al.*, 1995), which contains the activity of ubiquitin ligase for mitotic cyclins, are crucial for understanding the onset of anaphase proteolysis. We are investigating the role of protein phosphatase 1 (PP1) and a novel gene product Sds23 in regulatingon of the activity of 20S cyclosome/APC (Ishii *et al.*, 1996). PP1 in fission yeast is known to be required for the exit from mitosis (Ohkura *et al.*, 1989; Stone *et al.*, 1993) and interacts strongly with the components of the 20S cyclosome/APC. Sds23 interacts with both PP1 and 20S cyclosome/APC (Ishii *et al.*, 1996).

How does the Cut1p–Cut2p complex act in sister chromatid separation? Holloway *et al.* (1993) described two possible roles of proteins whose degradation would be required for sister chromatid separation. The degradable proteins might physically 'glue' sister chromatids together, making the dissolution of this glue essential for sister chromatid separation. Alternatively, a degradable protein might inhibit the activity of the protein(s) which brings about the dissociation of the linkage between chromatids, so that protein destruction would activate a sister separation activity. One would expect that loss of a chromosome glue protein through deletion ought to cause premature sister chromatid separation, which does not seem to occur in *cut2* null mutant cells. We thus think that Cut2p is not a glue. The Cut2p–Cut1p complex appears to act negatively on sister chromatid separation, and the destruction of Cut2p would activate sister chromatid separation.A number of possibilities exist for the actual functions of the complex. For instance, the complex may be an inhibitor of the protease responsible for degradation of the chromosome glue or spindle components: a proteolytic cascade initiated by proteolysis of Cut2p might be required for successful sister chromatid separation. Another possibility is that the complex regulates a set of enzymes implicated in protein modifications such as acetylation or deacetylation which might profoundly affect some of the steps of sister chromatid separation, as tubulin and histones are well known targets of protein acetylation.

Phenotypic differences between ts *cut2-364* and *cut2* null mutants are intriguing. They are similar with regard to phenotype in that both mutants show spindle elongation followed by the cut phenotype. Rounds of DNA replication and mitotic events including chromosome condensation and spindle elongation can take place in them, with the exception of sister chromatid separation. However, while centromere-proximal portions of the chromosomes are separated in ts *cut2-364* (the archery-bow phenotype), no portion of the chromosomes appears to be separated in *cut2* null mutant cells in spite of spindle elongation. Temperature-sensitive Cut2p is probably partly functional at the restrictive temperature, resulting in the leaky phenotype in sister chromatid separation. Mitotic chromosomes in *cut2* null might be condensed abnormally in such a way that they cannot be separated by the elongating spindle. Alternatively, chromosome condensation is normal in *cut2* null cells, but the chromosome–kinetochore attachment is impaired so that the condensed chromosomes remain in the middle of the elongating spindle. We consider that the involvement of Cut1p and Cut2p in formation of the normal mitotic chromosomes and/or spindle kinetochore structures is equally possible.

DNA topoisomerase II has been the only known essential component directly involved in separating sister chromatid DNAs (Holm, 1994; Holloway, 1995). Cut2p and its associating partner Cut1p can be added to those factors specifically required for sister chromatid separation, but further work is required to determine whether the Cut1p–Cut2p complex acts directly on sister chromatid separation or indirectly as a regulator. As DNA topoisomerase II plays an additional essential role for chromosome condensation in prometaphase (Uemura *et al.*, 1987; Ishida *et al.*, 1994), it is likely that the Cut2p–Cut1p complex also acts in bringing cells to the metaphase state. We hope that identification of biochemical activities of the Cut1p–Cut2p complex will solve these questions and provide us with new insights into the mechanisms of sister chromatid separation.

Materials and methods

Strains and media

An *S.pombe* haploid wild-type strain h^- and its derivative strains were used: *cdc11-123*, *cdc10-129*, *cdc25-22* (Nurse *et al.*, 1976; Moreno *et al.*,

1989), *nuc2-663* (Hirano *et al.*, 1988), *cut1-206*, *cut1-513*, *cut1-645*, *cut1-679*, *cut2-364*, *cut7-446*, *cut10-222* (Hirano *et al.*, 1986; Uzawa *et al.*, 1990), *cut1-474*, *cut1-686*, *cut1-693* (Samejima *et al.*, 1993), *cut1-21* and *cut1-22* (generous gifts of R.Bartlett and P.Nurse). Minimal EMM2 and rich YPD media were employed for liquid cultures and plates (Mitchison, 1970). Cells were sporulated on SPA plates.

Determination of the mutation sites

pYC11 (Takahashi *et al.*, 1992) carrying the *S.cerevisiae LEU2* gene as the marker was used as the vector for integration rescue of the *cut1* mutant. The following plasmids containing the truncated *cut1*$^+$ alleles were made: pDC10 (containing 3.2 kb *Sca*I–*Sac*I), pDC11 (2.2 kb *Sca*I–*Kpn*I), pDC12 (3.9 kb *Kpn*I–*Sca*I), pDC13 (4.3 kb *Sca*I–*Bam*HI), pDC14 (1.8 kb *Bam*HI–*Sca*I), pDC15 (5.9 kb *Sca*I–*Cla*I), pDC16 (3.8 kb *Sca*I–*Pvu*II) and pDC17 (2.4 kb *Pvu*II–*Sca*I). These were used for transformation of various *cut1* mutant strains at 26°C. The approximate locations of *cut1* mutation sites were determined by examining the ratios of Ts$^+$ among Leu$^+$ transformants. In the course of this mapping analysis, it was learned that the *cut10-222* mutant (Hirano *et al.*, 1986; Uzawa *et al.*, 1990) was allelic to *cut1*. DNA sequences which encompassed the mutation sites were isolated by the PCR method, followed by nucleotide sequencing. In this sequencing, several errors were found in the nucleotide sequence (Uzawa *et al.*, 1990), and corrected sequences have been deposited (accession No. M36179). The genomic DNA of *cut2-364* was isolated by PCR using the primers CUT2-1 (5′-CCGGATCCATGTTGCCCAGAACCATGT-3′) and CUT2-2 (5′-GCGGATCCTGAAAATTCGGAATCT-3′). The mutant DNA was then cloned into the *Bam*HI site of pBluescript KS(+), and its nucleotide sequence was determined.

Epitope tagging of the cut1$^+$ gene

The C-terminus of the *cut1*$^+$ gene was ligated with a triple tandem HA1 epitope (Field *et al.*, 1988) for tagging. This epitope, having the *Not*I site at both ends, was amplified from pGTEP1 (Tyers *et al.*, 1992) by PCR using oligonucleotides HANSP (5′-GTGCGGCCGCATGCTT-TACCCATACG-3′) and HASTOP (5′-GAGCGGCCGCACTAAGCA-GCGTAATC-3′) as primers. The underlined sequences were changed from the original sequences to generate an *Sph*I site and a terminal codon, respectively. A *Not*I site was introduced at the C-terminus of the *cut1*$^+$ gene by PCR using the primers DC9 (5′-CCGTCGACTGAAGA-GAACAGGAGTCA-3′) and CONOT (5′-CCGCGGCCGCATGGAAT-AATATAAGCAGGT-3′). Plasmid pHAC15, a derivative of pBluescript KS(+), containing a part of the *cut1*$^+$ coding region (1.7 kb *Sac*I–*Not*I), the triple HA1 epitope and *S.cerevisiae LEU2*, was used for transformation of the strain *h*$^-$ *leu1-32 cut1-693*. Four stable Leu$^+$ transformants were obtained, and all were Ts$^+$. Southern hybridization verified that the *cut1-693* gene was disrupted by the insertion of the *cut1HA* gene in pHAC15. One transformant was designated HF173.

Gene disruption of cut2$^+$

The 350 bp long *Nde*I–*Eco*RV fragment in the coding region of *cut2*$^+$ was deleted and replaced with the *S.pombe ura4*$^+$ gene. The resulting linearized fragment (*Bgl*II–*Eco*RI) was used for transformation of the diploid CM6/HK6 (*h*$^-$/*h*$^+$ *ade6-M210/ade6-M216 leu1-32/leu1-32 lys1/+ ura4-d18/ura4-d18*). Genomic Southern hybridization confirmed that the stable *Ura*$^+$ heterozygous diploid contained the disrupted *cut2* gene (data not shown). These diploid cells were sporulated and tetrads were dissected. The phenotype of gene-disrupted spores was examined as follows. Dissected spores were cultured on a 1% agarose film (16 mm×40 mm, 1.5 mm thick) and placed on a YPD or EMM2 plate for 36 h at 26°C. Disrupted spores germinated and divided once or twice, whereas non-disrupted spores could be distinguished by their ability to form colonies. The agarose film was then placed on a drop of 50 μl DAPI solution (100 μg/ml) previously spotted on a slide glass. The staining solution was spread and soaked into the gel, and cells which existed on the other side of the agarose film were stained with DAPI. One minute later, a coverslip (24 mm×50 mm) was carefully placed on the cells, and cells were observed with a Zeiss Axiophot (Carl Zeiss, Inc., Oberkochen, Germany). Alternatively, the phenotype of gene-disrupted cells germinating in liquid culture was examined by the procedure described by Moreno *et al.* (1989). The spores were inoculated in the EMM2 liquid medium supplemented with adenine, leucine and lysine: only Ura$^+$ spores could germinate.

Gene disruption of cut1$^+$

Plasmid pBMCUT1-2 was made by inserting the 9.2 kb *Sph*I fragment of pCUT1-2 (Hirano *et al.*, 1986) into pBluescribe M13(+). The 5.1 kb *Spe*I fragment in the coding region of *cut1*$^+$ was deleted and replaced with the *S.pombe ura4*$^+$. The resulting plasmid pCOD13 was linearized by *Sph*I and used for transformation of the diploid CHP428/CHP429 (*h*$^-$/*h*$^+$ *ade6-M210/ade6-M216 leu1-32/leu1-32 his7-366/his7-366 ura4-d18/ura4-d18*; Apolinario *et al.*, 1993). Genomic Southern hybridization verified that the stable *Ura*$^+$ heterozygous diploid contained the disrupted gene (data not shown).

Indirect immunofluorescence microscopy and FISH

For indirect immunofluorescence, the procedures for *S.pombe* using glutaraldehyde and paraformaldehyde (Hagan and Hyams, 1988) were followed. For HA epitope staining, cells were incubated with 0.02% raw ascites fluid of mouse monoclonal antibody 12CA5 (BAbCO, Richmond, CA). They were then incubated with 0.2% CY3-conjugated affinity-purified goat anti-mouse IgG (Chemicon International, Inc., Temecula, CA). The SPBs were stained by rabbit polyclonal anti-Sad1 antibodies (Hagan and Yanagida, 1995) and fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit IgG antibodies (Cappel, Durham, NC) as described previously (Funabiki *et al.*, 1993). Microtubules were stained by mouse anti-α-tubulin monoclonal anti-serum TAT1 (Woods *et al.*, 1989) (a gift of Dr K.Gull) and rhodamine-conjugated goat anti-mouse Igs (G+L) (TAGO, Inc., Burlingame, CA). Chromosomal DNA was stained with 0.2 μg/ml DAPI. For FISH, the procedures described in Funabiki *et al.* (1993) were followed.

Preparation of cell extracts

The procedures previously described (Simanis and Nurse, 1986; Shiozaki and Yanagida, 1992) were followed with modifications. *Schizosaccharomyces pombe* cells were collected on filter paper and suspended in the STOP buffer (0.9% NaCl, 10 mM EDTA, 50 mM NaF, 1 mM NaN_3). After centrifugation, pelleted cells were frozen by liquid nitrogen and kept at −80°C until use. For immunoblotting, frozen cells (1×10^9/ml) were thawed in buffer I [50 mM Tris–HCl at pH 7.5 containing 10 mM EDTA, 10% glycerol, 10 mM *N*-ethylmaledimide (NEM) and the protease inhibitors 1 mM phenylmethylsulfonyl fluoride (PMSF) 2 mg/ml pepstatin A and 10 mM E-64]. Cells were broken with glass beads (three times for 15 s) and an equal volume of buffer I was added. The supernatant after centrifugation at 5000 r.p.m. for 5 min by TMA-S27II rotor (Tomy, Japan) was used as the extract for SDS–PAGE and immunoblotting. An enhanced chemiluminescence detection system (ECL, Amersham) was used to detect proteins on the cellulose nitrate membrane (Toyo Roshi Kaisha, Inc.). Anti-PSTAIRE mouse monoclonal antibody used to detect Cdc2 protein was a gift from Dr Y.Nagahama (National Institute of Basic Biology). For immunoprecipitation, frozen cells (1×10^9/ml) were suspended in buffer II (containing 30 mM NaCl but otherwise identical to buffer I). Cells were disrupted with glass beads and centrifuged at 14 000 r.p.m. for 20 min by TMA-S27II rotor. NP-40 was added to the supernatant (final concentration, 0.05%) before immunoprecipitation, which was done as described previously (Shiozaki and Yanagida, 1992). For sucrose gradient centrifugation, cells were disrupted by glass beads in HB buffer (Moreno *et al.*, 1989) without $MgCl_2$. The supernatant of extracts after centrifugation at 14 000 r.p.m. for 20 min was fractionated by sucrose gradient (15–40%) for 12 h at 40 000 r.p.m. in an SW50.1 rotor (Beckman). The histone H1 kinase activity using crude extracts was assayed by the method described by Moreno *et al.* (1989).

Acknowledgements

We thank Keith Gull and Yoshitaka Nagahama for antibodies, and Rachel Bartlett and Paul Nurse for *cut1* mutant strains. This work was supported by grants from the Ministry of Education, Science and Culture of Japan (specially promoted research) and the Human Frontier Science Program Organization. H.F. is a Research Fellow of the Japan Society for the Promotion of Science.

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Received on June 10, 1996; revised on August 12, 1996