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Fission yeast Cut2 required for anaphase has two destruction boxes

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The fission yeast *Schizosaccharomyces pombe cut2*$^+$ gene is essential for sister chromatid separation. Cut2 protein, which locates in the interphase nucleus and along the metaphase spindle, disappears in anaphase with the same timing as mitotic cyclin destruction. This proteolysis depends on the APC (Anaphase-Promoting Complex)–cyclosome which contains ubiquitin ligase activity. The N-terminus of Cut2 contains two stretches similar to the mitotic cyclin destruction box. We show that both sequences (33RAPLGSTKQ and 52RTV-LGGKST) serve as destruction boxes and are required for *in vitro* polyubiquitination and proteolysis. Cut2 with doubly mutated destruction boxes inhibits anaphase, whereas Cut2 with singly mutated boxes can suppress *cut2* mutations. Strong expression of the N-terminal 73 residues containing the destruction boxes leads to the accumulation of endogenous cyclin and Cut2, and arrests cells in metaphase, whereas the same fragment with the mutated boxes does not. Cut2 proteolysis occurs *in vitro* using *Xenopus* mitotic extracts in the presence of functional destruction boxes. Furthermore, Cut2 is polyubiquitinated in an *in vitro* system using HeLa extracts, and this polyubiquitination requires the destruction boxes.

Keywords: anaphase/APC–cyclosome/cyclin/destruction box/mitosis

Introduction

Ubiquitin-mediated proteolysis is involved in several important biological events, such as cell cycle progression, signal transduction and the stress response (e.g. Hershko, 1996). Ubiquitin is conjugated to lysine residues of a target protein by ubiquitinating machinery which consists of E1, E2 and E3. Polyubiquitinated proteins are then degraded by the 26S proteasome in an ATP-dependent manner. The budding and fission yeast mutants of the 26S proteasome are blocked at metaphase (Ghisiain *et al.*, 1993; Gordon *et al.*, 1993), consistent with the notion that ubiquitin-mediated proteolysis is required for both sister chromatid separation and inactivation of mitotic cyclin-dependent kinases (CDKs) in order for cells to undergo anaphase and to exit from the M phase (Holloway *et al.*, 1993; Surana *et al.*, 1993). Mitotic exit and sister chromatid separation also fail to occur in mutants of the 20S anaphase-promoting complex (APC)–cyclosome, which contains the activity for ubiquitin ligase (Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995; Tugendreich *et al.*, 1995).

The best studied substrates of ubiquitin- and APC–cyclosome-mediated proteolysis are mitotic cyclins. The N-terminal portion of mitotic cyclins is dispensable for binding to and activation of $p34^{cdc2}$, and is solely required for mitotic destruction (Murray *et al.*, 1989). When non-degradable B-type cyclins carrying deletions in the destruction boxes were expressed, the cell cycle was blocked in late anaphase with high H1 kinase activity (Murray *et al.*, 1989; Holloway *et al.*, 1993; Surana *et al.*, 1993; Sigrist *et al.*, 1995; Rimmington *et al.*, 1996; Yamano *et al.*, 1996). The onset of sister chromatid separation (anaphase) can occur in the presence of non-degradable B-type cyclin. Final exit from mitosis requires inactivation of the Cdc2 kinase–cyclin B complex.

To degrade mitotic cyclins, the 20S APC–cyclosome is necessary as it has the E3 ubiquitin ligase activity for cyclins (King *et al.*, 1995; Sudakin *et al.*, 1995). The APC–cyclosome in budding yeast contains several (perhaps eight or more) subunits including Cdc16 and Cdc27 (Peters *et al.*, 1996; Zachariae *et al.*, 1996), which are known to be essential for mitotic progression. Similar proteins have been found in fission yeast and mammals, and shown to be required for the exit from mitosis and sister chromatid separation (Hirano *et al.*, 1988; O'Donnell *et al.*, 1991; Samejima and Yanagida, 1994; Tugendreich *et al.*, 1995; Yamashita *et al.*, 1996; Yamada *et al.*, 1997). The 20S APC–cyclosome probably exists in all eukaryotes, and was thought to be required for the destruction of mitotic cyclins and other unidentified proteins (e.g. Holloway *et al.*, 1993).

The region in mitotic cyclins required for proteolysis contains an amino acid motif called the destruction box which is necessary for regulated proteolysis (Glotzer *et al.*, 1991). The consensus for this motif is RXALGXIXN. While R and L residues are conserved in all destruction boxes of A- and B-type cyclins, N residues are only conserved in B-type cyclins (Glotzer *et al.*, 1991). The destruction box of fission yeast cyclin B Cdc13 is RHALDDVSN (Yamano *et al.*, 1996). The destruction box sequences thus appear to be quite variable except for the minimal RXXL. The exact role of the destruction box is unknown, although it is needed for ubiquitination of B-type cyclin *in vitro* (Glotzer *et al.*, 1991; King *et al.*,

1996). Frog B-type cyclins whose destruction boxes were substituted by A-type ones were ubiquitinated in frog egg extract; however, they were not destroyed efficiently (Klotzbücher *et al.*, 1996). This suggests that ubiquitination is a necessary step for programmed cyclin destruction, but is not by itself sufficient. Triggering proteolysis probably involves a mechanism still to be identifed.

The budding yeast Pds1 and the fission yeast Cut2 show cell cycle-regulated proteolysis, and are the targets of the 20S APC–cyclosome complex (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996b). Their destruction is required for the progression of anaphase, and is dependent upon the presence of functional APC–cyclosome complex. In the mutants *pds1* and *cut2*, cyclin destruction takes place, while sister chromatid separation fails to occur. It is of considerable interest how simultaneous proteolysis of cyclins and Pds1 or Cut2 can occur, as their proteolysis seems to be a key regulatory aspect for coordinated progression of anaphase (sister chromatid separation) and mitotic exit (cyclin destruction).

The fission yeast *cut2*$^+$ gene is required for sister chromatid separation in anaphase (Hirano *et al.*, 1986; Uzawa *et al.*, 1990). In temperature-sensitive (ts) *cut2-364*, sister chromatids are only partly separated (Funabiki *et al.*, 1996a). Most cell cycle events, except for sister chromatid separation, can occur, as evidenced in ts *cut2-cdc11* double mutant cells where cytokinesis is blocked by *cdc11*, leading to a huge polyploid nucleus. The *cut2*$^+$ gene is essential for viability and encodes a protein of 301 amino acids which interacts with Cut1 to form the large complex (Uzawa *et al.*, 1990; Funabiki *et al.*, 1996a). Cut2 is degraded in anaphase and becomes highly unstable in G_1-arrested cells (Funabiki *et al.*, 1996b). Its level recovers rapidly in S phase.

The degradation of Cut2 is dependent on the presence of the *cut9*$^+$ gene required for the formation of the 20S APC–cyclosome complex (Yamada *et al.*, 1997). Furthermore, Cut2 appears to contain the sequences essential for proteolysis in the first 80 amino acids, since truncation of this region leads to stabilization of Cut2 in G_1-arrested cells (Funabiki *et al.*, 1996b). Expression of the non-degradable truncated mutant (Cut2Δ80) blocks sister chromatid separation but not other cell cycle events. Furthermore, grafting the N-terminal region of the fission yeast cyclin Cdc13 gene to that of N-truncated Cut2 allows suppression of the ts phenotype of *cut2-364* (Funabiki *et al.*, 1996b). Cut2 proteolysis is hence essential for the onset of anaphase. Cut2 may not be a glue-like protein postulated to link sister chromatids until anaphase (Holloway *et al.*, 1993; Irniger *et al.*, 1995). Deletion of the glue protein would result in premature sister chromatid separation. However, *cut2* null mutant cells failed to separate sister chromatids (Funabiki *et al.*, 1996a,b). Cut2 is thought to have a positive role in bringing cells to normal metaphase, but it must be degraded to enter anaphase.

In the present study, we provide evidence that Cut2 contains two destruction boxes in its N-terminal region. Construction of Cut2 with mutagenized boxes clearly indicated that both boxes were implicated in proteolysis of Cut2. They also served as the essential destruction boxes *in vitro* for the *Xenopus* mitotic extracts. Therefore, the two destruction boxes, though their consensus sequences are only loosely conserved, can be recognized by an *in vitro* proteolytic system of an evolutionarily distant organism. We further show that Cut2 is polyubiquitinated in a manner depending on the presence of destruction boxes in an *in vitro* system using HeLa cell extracts prepared 1 h after the release from colcemid block. In this *in vitro* system, the N-terminal 73 amino acid-containing fragment can also be polyubiquitinated. These results indicate that the destruction boxes are required for polyubiquitination of Cut2.

Results

Failure of sister chromatid separation by a Cut2 destruction box mutant

The amino acid sequence of Cut2 (Uzawa *et al.*, 1990; Funabiki *et al.*, 1996b) reveals two stretches in the N-terminal region (33RAPLGSTKQ and 52RTVLGGKST) that are similar to the consensus for the destruction box of mitotic cyclins (Glotzer *et al.*, 1991; Yamano *et al.*, 1996; Figure 1A). To examine whether these sequences are required for regulated instability of Cut2, two arginine (33R and 52R) and two leucine (36L and 55L) residues conserved in all the destruction boxes of mitotic cyclins were mutated to alanine (A). The resulting mutant genes having substitutions in the first destruction box (33AAPAGSTKQ), in the second box (52ATVAGGKST) or in both boxes are designated *cut2dm1*, *cut2dm2* and *cut2ddm*, respectively. The *cut2* mutant gene, *cut2Δ80*, with deletions of the first 80 residues, was described previously by Funabiki *et al.* (1996b).

The wild-type and mutant genes were placed downstream of the weak inducible *nmt1* promoter (REP81) and overexpressed in wild-type *Schizosaccharomyces pombe* cells in the absence of thiamine (Basi *et al.*, 1993). While the wild-type Cut2 protein overproduced under the control of the promoter REP81 in wild-type cells did not inhibit colony formation at all, overproduced mutant proteins did so strongly (Figure 1B). The degree of inhibition by *cut2dm1* was the lowest among the mutants examined, whereas both *cut2Δ80* and *cut2ddm* showed a very strong inhibitory effect.

Fluorescence microscopic observation of liquid cultures of wild-type cells carrying plasmids with the REP81-*cut2Δ80*, -*cut2dm1*, -*cut2dm2* or -*cut2ddm* gene in the absence of thiamine indicated that sister chromatid separation failed to occur, followed by the cut phenotype where cytokinesis took place in the absence of nuclear division (Figure 1C, micrograph taken from *cut2ddm*). This cytological phenotype greatly resembled that of *cut2Δ80* (Funabiki *et al.*, 1996b).

Inability of the double destruction box mutant to suppress cut2 null and ts mutant cells

To test whether Cut2 with the destruction box mutations was functional, a ts *cut2* mutant was transformed by plasmids carrying the wild-type gene (*cut2*$^+$), *cut2dm1*, *cut2dm2*, *cut2ddm* or *cut2Δ80* under the control of the REP81 promoter. Colonies were produced at 26°C by all transformants in the repressed condition (+ thiamine, data not shown). Transformants were then plated at 36°C in the presence or absence of thiamine (Figure 2). Temperature-sensitive mutant cells carrying a plasmid with the wild-

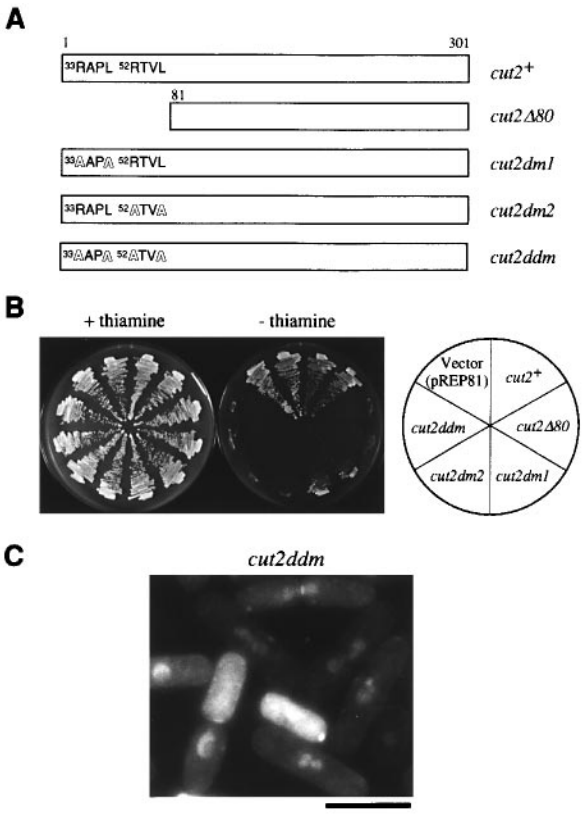


Fig. 1. Induced overexpression of wild-type and mutant Cut2. (**A**) This diagram indicates genes encoding the full-length Cut2 (*cut2*$^+$), N-terminal 80 amino acid deletion (*cut2Δ80*), substitution mutations in the first destruction box (*cut2dm1*), the second destruction box (*cut2dm2*) or both destruction boxes (*cut2ddm*). In the substituted destruction box mutants, 33R, 52R, 36L and 55L are replaced by A as indicated in the figure. These genes were placed downstream of the weak inducible promoter REP81. (**B**) The wild-type strain carrying one of the above plasmids was plated in the absence or presence (2 μM) of thiamine at 33°C. Overexpression took place in the absence of thiamine. Colonies were made in cells carrying a plasmid with the wild-type *cut2*$^+$ gene or a vector plasmid, but colony formation was inhibited in cells carrying the mutant *cut2* genes. The growth inhibition effects of *cut2Δ80* and *cut2ddm* were the strongest, whereas *cut2dm1* showed the least effect. (**C**) Wild-type cells carrying plasmid with the *cut2ddm* mutant gene were cultured in liquid EMM2 medium at 33°C for 12 h in the absence of thiamine and stained by DAPI. Cells displayed a failure of sister chromatid separation. The frequent cut phenotype was observed. This phenotype was identical to that obtained by overproducing the deletion *cut2Δ80* (Funabiki *et al.*, 1996b). Bar, 10 μm.

type *cut2*$^+$ or the single destruction box mutant genes (*cut2dm1* and *cut2dm2*) placed downstream of REP81 were found to be viable in the repressed condition, indicating that reduced production of single mutants *cut2dm1* and *cut2dm2* could suppress the ts phenotype of *cut2-364*. However, the double destruction box mutant *cut2ddm* and the N-terminal deletion *cut2Δ80* did not suppress this phenotype. Although single destruction box mutant proteins (Cut2dm1 and Cut2dm2) might be destroyed less efficiently than the wild-type Cut2, they could support colony formation if expressed at a very low level in the *cut2* mutant. However, Cut2ddm was found to be no longer functional, and inhibited colony formation as did Cut2Δ80. An unexpected finding was that, in the derepressed condition (–thiamine), Cut2dm1 did not block colony formation and could suppress the ts phenotype

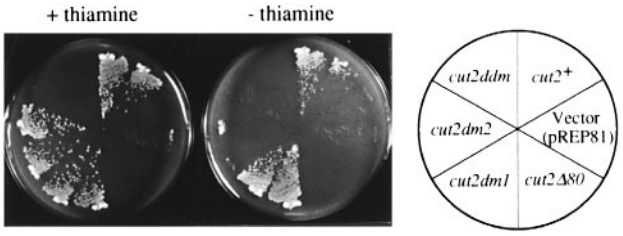


Fig. 2. The substitution mutant *cut2ddm* fails to rescue *cut2-364*. Mutant *cut2-364* was transformed with plasmid REP81 carrying the wild-type *cut2*$^+$ or *cut2* mutant genes. Transformants obtained at 26°C in the presence of thiamine were plated at 36°C in the presence or absence of thiamine. Plasmids carrying the wild-type *cut2*$^+$, mutant *cut2dm1* or mutant *cut2dm2* gene suppressed the ts phenotype of *cut2-364* in the presence of thiamine. However, plasmids carrying *cut2Δ80* or *cut2ddm* failed to suppress the ts phenotype of *cut2-364* in the presence or absence of thiamine.

(Figure 2, –thiamine). A possible explanation for the phenotypic difference between wild-type and ts *cut2* mutant is that, while Cut2dm1 would become toxic in the wild-type, because cells containing the wild-type Cut2 should move through anaphase rapidly, it would not become toxic in the absence of wild-type Cut2, because anaphase might be slowed down by or 'adapted' to the mutant Cut2dm1.

A similar test was carried out for the gene-disrupted strain (*cut2*Δ), which carried these plasmids. To this end, heterozygous diploid cells in which one of the chromosomal *cut2*$^+$ genes was disrupted by *S.pombe ura4*$^+$ (Grimm *et al.*, 1988) were transformed by plasmids. When haploid segregants obtained by sporulation of these transformed diploid cells were tested for their viability in the medium lacking uracil at 33°C, only suppressed *cut2*Δ spores could grow. Results of colony formation were basically identical to those for ts *cut2* mutants (data not shown), indicating that Cut2 protein with one destruction box was capable of supporting colony formation.

Metaphase block by overproduction of the N-terminal fragment

In *Xenopus* extracts, the N-terminal fragment of cyclin B acts as a competitive substrate for degradation of the endogenous full-length cyclin B (Holloway *et al.*, 1993). Yamano *et al.* (1996) recently showed that overproducing the N-terminal fragment of Cdc13 (*S.pombe* cyclin B) led to inhibition of the metaphase–anaphase transition and also to the degradation of endogenous Cdc13. If Cut2 is a substrate for the same proteolytic machinery employed for destroying mitotic cyclins, massive overproduction of the N-terminal fragment of Cut2 might also prevent the destruction of endogenous Cut2 and cyclin B, thus preventing both sister chromatid separation and the inactivation of p34^{cdc2}.

We examined the phenotype of wild-type cells carrying a plasmid with the N-terminal 73 residues of Cut2 (the gene designated *cut2N73*; Figure 3A) placed under the strong REP1 promoter in the absence of thiamine (Maundrell, 1990). Overproduction of Cut2N73 and Cut2N73dm1 fragments blocked colony formation in the absence of thiamine (Figure 3B). In contrast, overproduction of Cut2N73dm2 and Cut2N73ddm allowed the formation of colonies in the absence of thiamine (Figure 3B). Thus the growth-inhibitory effect of the N-terminal

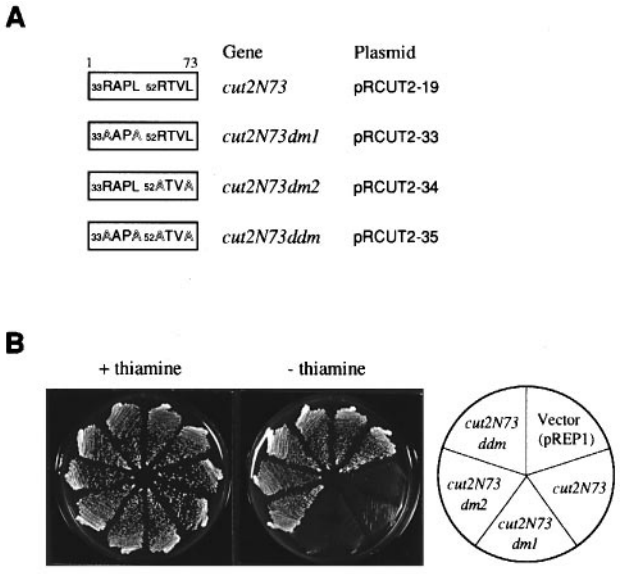


Fig. 3. Overproduction of the N-terminal fragments. (**A**) Construction of clones coding for the N-terminal 73 amino acid fragment of Cut2 (*cut2N73*), with mutations in the first destruction box (*cut2N73dm1*), in the second destruction box (*cut2N73dm2*) and in both destruction boxes (*cut2N73ddm*). These fragments were placed under the control of the strong *nmt* promoter REP1 (Maundrell, 1990). The names of the resulting plasmids are indicated. (**B**) Wild-type strains carrying the plasmids described above were plated in the presence or absence of thiamine (2 μM) at 33°C. No colony was produced by a plasmid carrying the N73 fragment without a mutation in the absence of thiamine, but colonies were made by cells overproducing double destruction box mutant fragments.

fragment requires the presence of the second destruction box sequence. We interpret these results as showing that the two destruction boxes are not equal, and that the second box is more important than the first one.

Cell cycle phenotypes of cells overproducing the wild-type (Cut2N73) and mutant (Cut2N73ddm) N-terminal fragments were then examined (Figure 4). At 14 h after the removal of thiamine, the level of the N73 fragments (p11) increased sharply (Figure 4A). The level of Cdc13 (p63) also increased when Cut2N73 was overproduced. The upper band of endogenous Cut2, which appeared only in mitosis (Funabiki *et al.*, 1996b), was seen. When the mutant Cut2N73ddm fragment was overproduced, however, the level of Cdc13 (p63) did not increase. For the endogenous Cut2 (p42), the mitotic upper band did not appear, but the level of endogenous p42 slightly increased.

Cytological phenotypes were then examined (Figure 4B–D). About 45% of the cells overproducing the Cut2N73 fragment (left panel in B) displayed condensed chromosomes and a short spindle (TUB, anti-tubulin antibody stain; SPB, anti-sad1 stain). Cdc13-dependent histone H1 kinase activity was elevated 5-fold in cells overproducing Cut2N73 (Figure 4D), suggesting that overproduction of the N-terminal fragment arrested cells at metaphase. In sharp contrast, neither the frequencies of cells revealing condensed chromosome (and a short metaphase spindle) nor the level of histone H1 kinase activity increased in cells overproducing the same fragment with mutated destruction boxes (N73ddm). These results clearly demonstrated that overproduction of the Cut2N73 fragment led

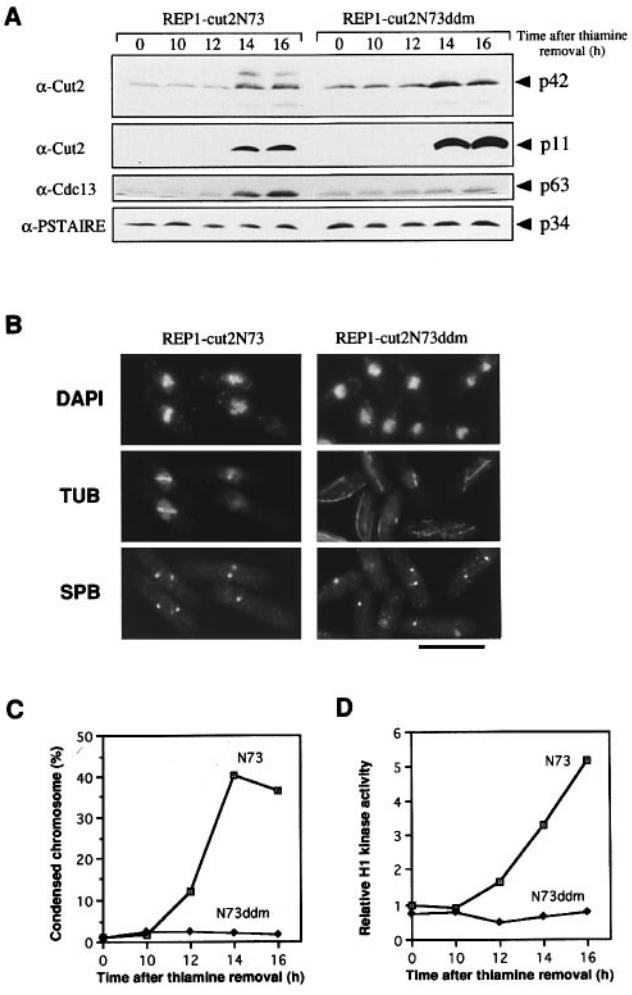


Fig. 4. Metaphase arrest by overproducing the Cut2N73 fragment. Wild-type cells carrying a plasmid with the wild-type N-terminal fragment Cut2N73 or the mutant fragment Cut2N73ddm under the control of the strong promoter REP1 were cultured at 33°C for 16 h in the absence of thiamine. (**A**) Overexpression of the N-terminal fragment (p11) was detected by anti-cut2 (α-Cut2) antibodies. The levels of full-length Cut2, Cdc13 and Cdc2 were determined by anti-cut2 (α-Cut2), anti-cdc13 (α-Cdc13) and anti-PSTAIRE (α-PSTAIRE) antibodies, respectively. (**B**) Cells overproducing Cut2N73 or Cut2N73ddm at 14 h after the removal of thiamine were stained by DAPI, anti-tubulin (TUB) and anti-sad1 (which stains spindle pole bodies) antibodies. Cells overproducing Cut2N73 were blocked at metaphase, whereas cells overexpressing Cut2N73ddm grew normally, showing interphase and mitotic cells. The bar indicates 10 μm. (**C**) The frequency of cells containing condensed chromosomes in the cultures expressing Cut2N73 (N73) or Cut2N73ddm (N73ddm) was determined by DAPI staining. (**D**) The H1 kinase activities dependent upon Cdc13 in the cultures overproducing Cut2N73 or Cut2N73ddm were measured using immunoprecipitation by anti-cdc13 antibodies.

to the metaphase arrest and that this arrest required the presence of functional destruction boxes.

Stability of Cut2ddm in G_1-arrested cells

Cut2 was shown previously to be highly unstable in G_1-arrested *cdc10* cells, whereas the N-terminal deletion Cut2Δ80 was stable (Funabiki *et al.*, 1996b). To examine the stability of the mutant Cut2 proteins under these conditions, wild-type Cut2, mutant Cut2ddm and mutant Cut2Δ80 were expressed in G_1-arrested *cdc10* cells under

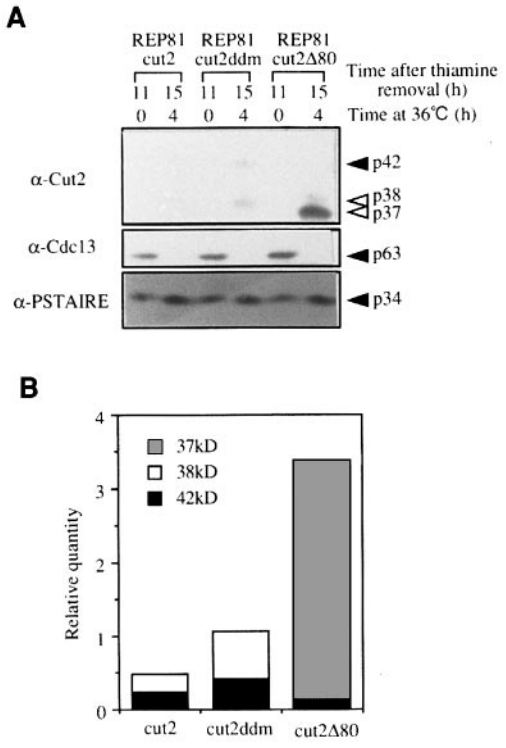


Fig. 5. Accumulation of Cut2ddm protein in *cdc10* mutant cells. (**A**) Wild-type *cut2*$^{+}$, mutant *cut2ddm* and mutant *cut2Δ80* placed under the control of the weak promoter REP81 were overexpressed in G_1-arrested *cdc10* mutant cells in which mitotic cyclin Cdc13 and Cut2 were unstable (Funabiki *et al.*, 1996b; Yamano *et al.*, 1996). The cultures of the *cdc10* mutant carrying a plasmid with the wild-type *cut2*$^{+}$ or mutant *cut2* genes were shifted to 36°C at the time (11 h) that overproduction began. Cells were collected at 0 (11) and 4 (15) h after the shift to 36°C (hours after the removal of thiamine), and assayed for the levels of Cut2, Cdc13 and Cdc2 proteins using antibodies α-Cut2, α-Cdc13 and αPSTAIRE. The levels of wild-type Cdc13 and Cut2 were negligible in G_1-arrested cell extracts (after 4 h), but the level of mutant Cut2Δ80 was high. The level of *cut2ddm* was higher than that of wild-type but significantly lower than that of *cut2Δ80*. (**B**) Cut2ddm seemed to be cleaved to produce the 38 kDa band. The intensities of these bands were summed and are shown.

the control of the weak promoter REP81 and their abundance measured at the indicated times (see Figure 5A). Overproduction started after 11 h in the absence of thiamine, and then *cdc10* cells were shifted to 36°C. Cut2ddm did not accumulate as Cut2Δ80. The level of Cut2ddm (the total of native p42 and cleaved p38) was twice that of wild-type Cut2 and one-quarter that of p37 Cut2Δ80 (Figure 5B). Thus Cut2ddm mutant protein was not as stable as Cut2Δ80, and was only slightly more stable than the wild-type in G_1-arrested cells (Figure 5A and B). The single destruction box mutants proteins (Cut2dm1 and Cut2dm2) did not accumulate at all in G_1-arrested *cdc10* cells (data not shown). The level of Cdc13 shown as control was greatly reduced in G_1-arrested cells (4 h at 36°C). These results suggested that sequences other than the two destruction boxes might cause the instability in G_1-arrested cells.

Overproduction of the N-terminal fragment in G_1-arrested cells blocks proteolysis of mitotic cyclin

The wild-type and mutant N-terminal fragments were overproduced under the control of the strong promoter

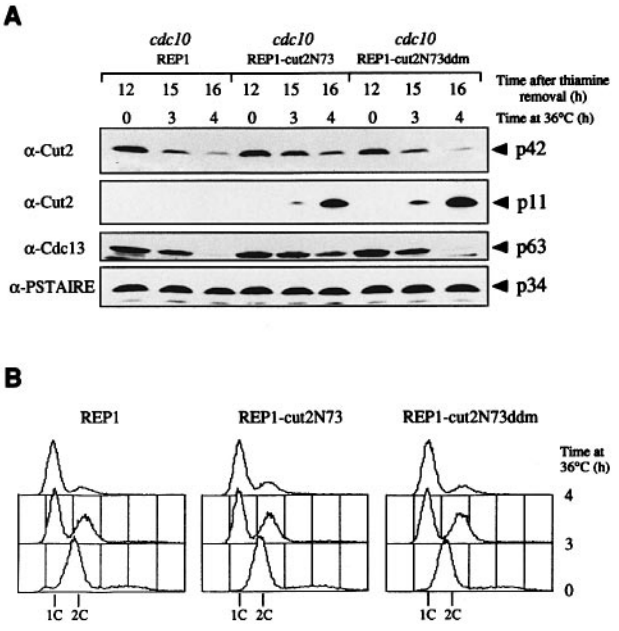
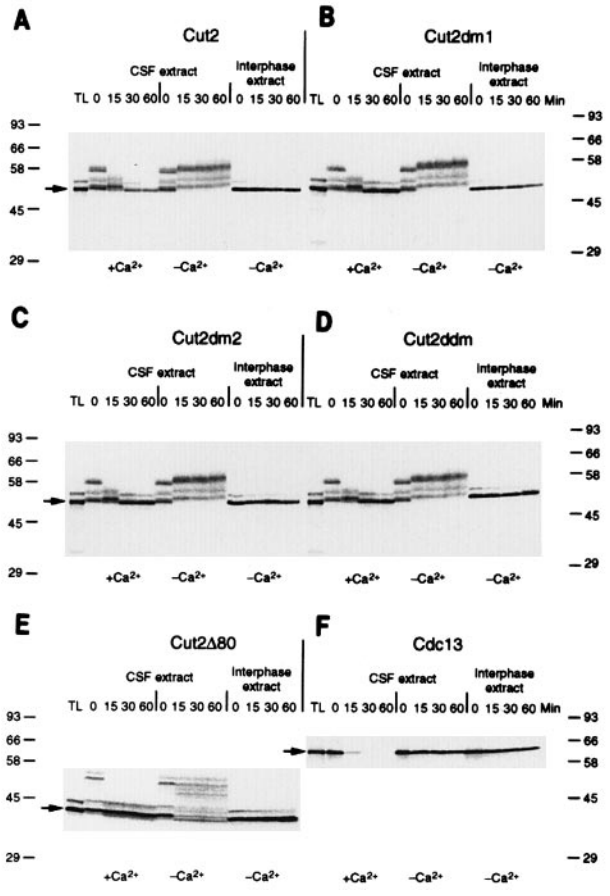


Fig. 6. Overproduction of Cut2N73 and Cut2N73ddm in G_1-arrested *cdc10* mutant cells. *cdc10-129* strains carrying the vector plasmid pREP1 or plasmid with the *cut2N73* or the *cut2N73ddm* sequence under the control of the REP1 promoter were cultured in the absence of thiamine for 12 h at 26°C, and then transferred to 36°C. Cultures were taken at 0, 3 and 4 h, and extracts were subjected to SDS–PAGE for immunoblotting. (**A**) Immunoblot using anti-cut2, anti-cdc13 and anti-PSTAIRE. The p11 band represents the polypeptide produced by a plasmid carrying the *cut2N73* or *cut2N73ddm* gene. After 4 h, p42 (full-length Cut2) and p63 (Cdc13) were degraded in *cdc10* carrying the vector plasmid, and considerably degraded in *cdc10* producing the mutant p11 fragment Cut2N73ddm, but a significant fraction of p42 and p63 remained in *cdc10* producing the wild-type p11 fragment Cut2N73. (**B**) The DNA contents of the above cells were examined by FACScan. Most *cdc10* cells contained 1C DNA after 4 h at 36°C.

REP1 in *cdc10*-arrested cells (Figure 6A). When the mutant fragment Cut2N73ddm (p11) was overproduced, the levels of both p42 (Cut2) and p63 (Cdc13) were lowered in G_1-arrested *cdc10* mutant cells. In contrast, the degradation of p42 (Cut2) and p63 (Cdc13) was suppressed significantly in *cdc10*-arrested cells which overproduced the wild-type N-terminal fragment Cut2N73 (p11). *cdc10* cells were verified to be in the G_1 state by fluorescence-activated cell sorting (FACS) analysis (4 h at 36°C after overproduction; Figure 6B). These results demonstrated that excess N-terminal fragment with the destruction boxes was capable of inhibiting proteolysis of endogenous cyclin and Cut2 in G_1-arrested cells.

Proteolysis of Cut2 in vitro using Xenopus egg extracts

The *S.pombe* B-type cyclin, Cdc13, was degraded in *Xenopus* egg extract with kinetics similar to endogenous frog B-type cyclins, and the proteolysis was destruction box-dependent (Yamano *et al.*, 1996). Using the same *Xenopus* cell-free system, we tested whether Cut2 destruction box mutant proteins were degraded (Figure 7). ^{35}S-labelled substrates for destruction assays were prepared by translation in a reticulocyte lysate (indicated by 'TL' in Figure 7A–F). As shown in a control experiment (Figure 7F), Cdc13 was degraded in the CSF extracts in the presence of Ca^{2+}, but not in the absence of Ca^{2+} (cyclin destruction is triggered in meiosis II metaphase-arrested



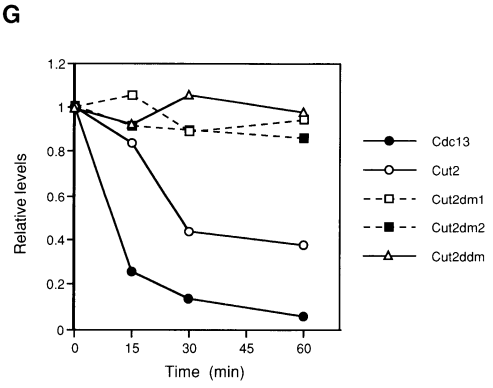


Fig. 7. Proteolysis of Cut2 *in vitro* using *Xenopus* extracts. Wild-type and mutant Cut2 proteins were ^{35}S-translabelled in reticulocyte lysates and assayed for destruction in *Xenopus* CSF extracts (in the presence or the absence of Ca^{2+}) or interphase extracts. TL; *in vitro* translated products as substrate. (**A**) Cut2; (**B**) Cut2dm1; (**C**) Cut2dm2; (**D**) Cut2ddm; (**E**) Cut2Δ80; (**F**) Cdc13. Cdc13 was employed as the control substrate for proteolysis in the *Xenopus* extracts, and was completely degraded in CSF extracts in the presence of Ca^{2+}. Proteolysis did not take place if Ca^{2+} was not added or if the interphase extract was used. The destructability of wild-type Cut2 and mutant Cut2 proteins was examined in the same extracts (A–E). The initial protein bands were greatly shifted to the upper positions when mixed with the CSF extracts, but not with the interphase extracts. The upper bands were shown to be due to phosphorylation (see Figure 8). (**G**) Quantitative estimation of the band intensities for wild-type and mutant Cut2 proteins after the addition of Ca^{2+} to the CSF extracts by scanning densitometry; normalized with reference to the intensities found in the absence of Ca^{2+} at each time point. Approximately 60% of the wild-type Cut2 was degraded after 60 min, whereas all mutant Cut2 proteins were stable.

CSF extract by the addition of Ca^{2+}). No proteolysis occurred in the interphase extracts.

Cut2 showed multiple upper bands (due to protein phosphorylation; see following section) immediately after being mixed with CSF extract, and ~60% of Cut2 was clearly degraded in the presence of Ca^{2+} after 60 min (while >90% of Cdc13 was lost). In the absence of Ca^{2+}, the upper bands were maintained and not degraded at all. If incubated with interphase extract, no upper bands were obtained and Cut2 was not degraded. The total intensities of the bands were quantified by scanning densitometry, and are plotted in Figure 7G. Consistently, ~50% of Cut2 was degraded during anaphase in *S.pombe* synchronous culture (Funabiki *et al.*, 1996b).

We next tested the mutant Cut2 proteins in the *Xenopus* system. As expected, Cut2Δ80 and Cut2ddm were stable and they showed no proteolysis. Surprisingly, neither single destruction box mutant protein (Cut2dm1, Cut2dm2) was degraded (Figure 7B–E and G), even though the upper phosphorylation bands were shifted downwards in the presence of Ca^{2+}. There was also a transient rise of the upper bands. Thus the destruction box in Cut2 seems to be functional in the *Xenopus* cell-free system, and both boxes have to be intact for proteolysis in this *in vitro* system.

Protein phosphorylation causes the appearance of upper bands

To examine the nature of the upper bands of Cut2, we immunoprecipitated ^{35}S-labelled Cut2 after 30 min incubation with CSF extract in the absence of Ca^{2+} using anti-cut2 antibodies (see Figure 7A). The immunoprecipitates (Figure 8, lane 1) showed diffuse upper bands and were treated with alkaline phosphatase. After phosphatase treatment, all the upper bands disappeared and concentrated in a single band with faster mobility (AP, lane 3), whereas no change was seen if alkaline phosphatase was omitted (mock, lane 2) or phosphatase inhibitors were included in the incubation (AP + inhibitor, lane 4). These results demonstrated unambiguously that the upper bands were due to phosphorylation.

To test if the phosphorylation was due to protein kinases in the mitotic extract, we tried to re-phosphorylate the dephosphorylated Cut2 protein (lane 3) by incubation with CSF extract, interphase extract or reticulocyte lysate. The multiple upper bands of Cut2 reappeared after incubation with CSF extract, whereas no such bands were produced by interphase extracts or reticulocyte lysate (lanes 6–8). Cut2 in *S.pombe* cells was also phosphorylated at mitosis prior to proteolysis (Funabiki *et al.*, 1996b). The identity of the kinase(s) remains to be established.

Polyubiquitination of Cut2 in vitro

To determine whether polyubiquitination of Cut2 was dependent upon the presence of destruction box sequences, we attempted to immunoprecipitate wild-type and mutant Cut2 proteins in *S.pombe* mitotic extracts of mutant cells lacking a functional proteasome (Gordon *et al.*, 1993). Probably due to the inefficiency of immunoprecipitation and the limited sensitivity of the anti-polyubiquitin antibodies used, polyubiquitinated bands of Cut2 have not been demonstrated convincingly in *S.pombe* extracts thus far. However,

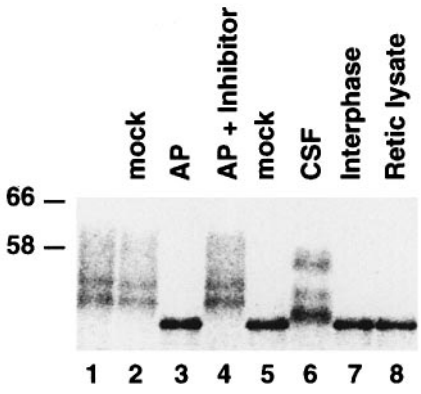


Fig. 8. Phosphorylation of Cut2. The upper bands observed after incubation with the CSF extract were due to phosphorylation. ^{35}S-translabeled Cut2 was incubated with CSF in the absence of Ca^{2+} and immunoprecipitated with anti-cut2 antibodies. The immunoprecipitates were used as the substrate for phosphatase treatment at 37°C for 30 min. The substrate immunoprecipitated without incubation (lane 1); mock, incubated without alkaline phosphatase (lane 2); treated with alkaline phsophatase (lane 3); phosphatase inhibitor and alkaline phosphatase (lane 4). Lanes 5–8, Cut2 immunoprecipitates after phosphatase treatment were incubated in either mock (lane 5), CSF (lane 6), interphase (lane 7) or reticulocyte lysate (lane 8). Only the CSF extract produced the upper bands.

we were able to detect polyubiquitinated Cut2 protein in an *in vitro* system developed for HeLa cell extracts (see Materials and methods). In this system, cyclin B is polyubiquitinated highly efficiently using human hE2-C ubiquitin-conjugating enzyme added exogenously (H.Tanaka and H.Yasuda, in preparation). hE2-C is similar to those enzymes previously identified in clam and frog (E2-C, Aristarkhov *et al.*, 1996; UBCx, Yu *et al.*, 1996). Ubiquitin is biotinylated and detected by the ECL avidin–peroxidase method. Various GST–Cut2 proteins were prepared by overproduction in bacterial cells and were purified.

Polyubiquitination of wild-type GST–Cut2 occurred efficiently in HeLa cell extracts prepared 1 h after release from a colcemid block (Figure 9A, lane 4), but was scarce in extracts prepared from asynchronous cells (lane 3). Polyubiquitinated bands (Ub) appeared at the positions expected from those of non-ubiquitinated bands stained by Coomasie brilliant blue (CBB, lanes 1 and 2). Mono-ubiquitination of Cut2 could take place in asynchronous extracts. In contrast, polyubiquitination of GST–Cut2Δ73 (lacking the N-terminal 73 amino acids) was scarcely detected (lane 8), in spite of using an amount of GST–Cut2Δ73 which was much higher than that of GST–Cut2 (compare lanes 5 and 6 with lanes 1 and 2). GST–Cut2Δ73 was, however, mono-ubiquitinated significantly.

We then examined whether mutant GST–Cut2ddm could be polyubiquitinated using the same colcemid block release extracts (Figure 9B). Wild-type GST–Cut2 (lanes 1 and 7), mutant GST–Cut2ddm (lanes 2–5 and lanes 8–11) and mutant GST–Cut2Δ73 (lanes 6 and 12) were used (lanes 1–6, Cut2 stained by CBB; lanes 7–12, Cut2 containing biotinylated ubiquitin). A series of increasing concentrations of GST–Cut2ddm were employed (see the legend to Figure 9). Cut2ddm was partly proteolysed during the preparation, resulting in production of two CBB-staining bands (and corresponding mono-ubiquitinated bands). GST–Cut2 was highly polyubiquitinated but GST–

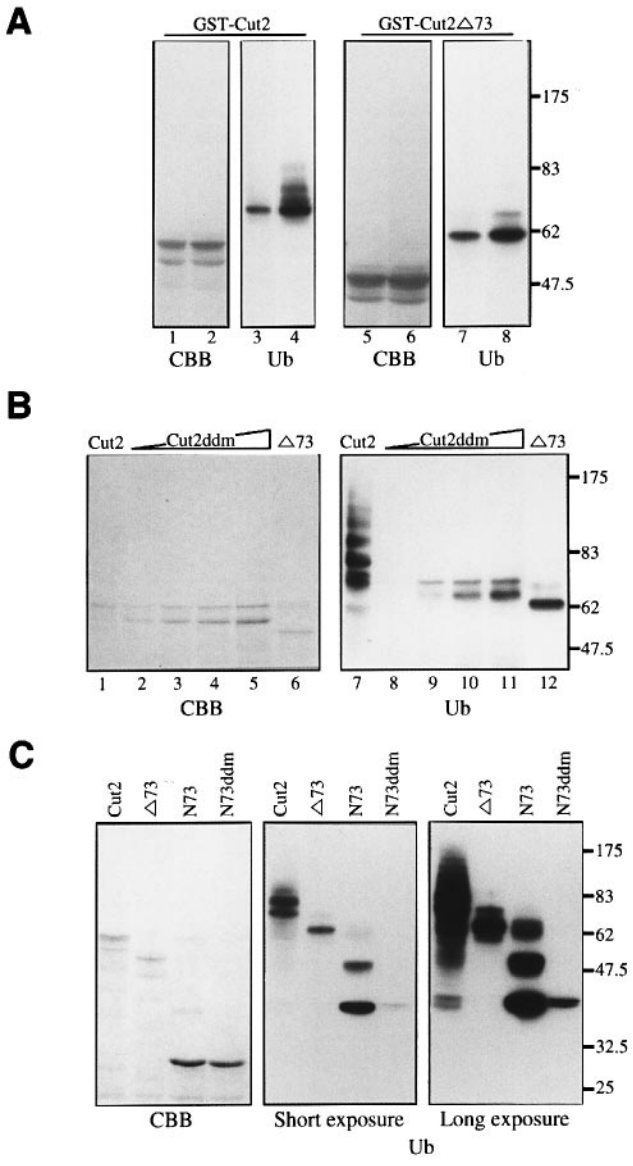


Fig. 9. Polyubiquitination of GST–Cut2. (**A**) GST–Cut2 and GST–Cut2Δ73 bound to beads were added to HeLa cell extracts and incubated in the presence of biotinylated ubiquitin and human hE2-C ubiquitin-conjugating enzyme (Materials and methods). Proteins bound to beads were stained with Coomassie brilliant blue (CBB), and ubiquitinated proteins were detected by the ECL avidin–peroxidase method. Lanes 1, 3, 5 and 7, asynchronous HeLa cell extracts; lanes 2, 4, 6 and 8, HeLa extracts prepared 1 h after the release from colcemid block. Lanes 1, 2, 5 and 6, CBB stain; lanes 3, 4, 7 and 8, Cut2 containing biotinylated ubiquitin. Molecular weight markers are indicated. (**B**) Ubiquitination of wild-type GST–Cut2 (lanes 1 and 7), mutant GST–Cut2ddm (lanes 2–5 and lanes 8–11) and GST–Cut2Δ73 (lanes 6 and 12) was examined using the HeLa cell extracts described in (A). CBB staining (lanes 1–6) and Cut2 containing biotinylated ubiquitin (lanes 7–12) are shown. Increasing concentrations of Cut2ddm were used; the relative ratios are 1 (lanes 2 and 8), 2 (lanes 3 and 9), 3 (lanes 4 and 10) and 4 (lanes 5 and 11). (**C**) The *in vitro* ubiquitination reaction was applied to the GST–N73 fragments containing the wild-type destruction boxes (N73) or the double mutant boxes (N73ddm). These fragments bound to beads were stained by CBB, and those containing biotinylated ubiquitin were detected (the short and long exposures). The mutant fragment GST–N73ddm was barely polyubiquitinated.

Cut2ddm and GST–Cut2Δ73 were only mono-ubiquitinated. These results demonstrated that polyubiquitination of Cut2 needed the presence of functional destruction boxes.

We then prepared the GST–N73 fragments with the wild-type (indicated as N73 in Figure 9C) and the mutant destruction boxes (N73ddm). Polyubiquitinatin of the GST–N73 fragment was found to occur in a destruction box-dependent manner (Ub, Figure 9C). Polyubiquination was hardly detected in GST–N73ddm; however, slight mono-ubiquitination occurred even in GST–N73ddm. These results indicated that the N-terminal N73 fragment contained a lysine residue(s) which could be polyubiquitinated depending upon the presence of destruction boxes.

Discussion

The results of the present study indicated that two destruction box-like sequences present in the N-terminal region of Cut2 are indeed the signals for polyubiquitination and proteolysis, and that the same proteolytic machinery is likely to be applied to the degradation of Cut2 and B-type cyclin Cdc13. The first five amino acids of the destruction boxes are similar to the consensus given by a number of mitotic cyclins (Glotzer *et al.*, 1991; Yamano *et al.*, 1996). We provided *in vivo* and *in vitro* evidence that these N-terminal sequences are utilized as destruction signals.

Mild overexpression of full-length wild-type Cut2 using the REP81 promoter showed no inhibitory effect on colony formation, but the Cut2 protein containing mutations in a single or both destruction boxes produced an inhibitory effect; sister chromatid separation in the cells overproducing Cut2dm1, Cut2dm2 or Cut2ddm failed. Both destruction boxes seem to be involved in programmed proteolysis of Cut2. However, single destruction box mutants Cut2dm1 or Cut2dm2 are functional since they could suppress ts *cut2* and *cut2* null mutants, if present not greatly in excess. Neither Cut2ddm nor Cut2Δ80 which lack both destruction boxes could rescue the ts *cut2* strain, probably due to the lack of a functional destruction box.

Overproduction of the N-terminal fragment by the strong REP1 promoter blocked cell cycle progression at metaphase, whereas overexpression of the mutant N-terminal fragment was not able to arrest cells. This inhibitory effect presumably is due to the sequestration of hypothetical destruction box-binding proteins or production of massive amounts of ubiquitinated substrates, resulting in the inhibition of the proteolytic machinery which is required for the progression from metaphase to anaphase. However, since mutation in the first destruction box (Cut2N73dm1) maintains the inhibitory effect, and overproduction of Cut2dm1 could suppress ts *cut2* at 36°C, the two destruction boxes may not function equally. The second destruction box may be more important than the first one for programmed proteolysis in anaphase.

Comparison of the *in vivo* levels of Cut2Δ80 deletion and Cut2ddm substitution mutant proteins clearly demonstrated that the substitution mutant protein was far more sensitive to proteolysis than the deletion mutant. The levels of deletion Cut2Δ80 and substitution Cut2ddm proteins measured in G_1-arrested *cdc10* were very different. The level of Cut2ddm protein was low and only twice more than that of wild-type Cut2. The N-terminal region of Cut2 possibly contains other signals for proteolysis. If this is the case, Cut2 could be destroyed in G_1-arrested cells through another proteolytic pathway which would not utilize the destruction boxes. Alternatively, the substitution mutations for the destruction boxes may not fully abolish their ability to be destruction signals in G_1-arrested *S.pombe* cells.

The *Xenopus* mitotic extracts were used for destruction of Cut2 *in vitro*. Using the same extracts, the fission yeast cyclin Cdc13 was degraded by an expected mechanism dependent upon the destruction box (Yamano *et al.*, 1996). The level of Cut2Δ80 protein was not affected, whereas approximately half of the wild-type Cut2 protein was degraded. The same level of wild-type Cut2 protein persisted even after a prolonged incubation, suggesting that some populations of Cut2 are resistant to proteolysis. In sharp contrast, Cdc13 was fully degraded by the same extracts (Yamano *et al.*, 1996; also see Figure 7F). Single destruction box mutant proteins, Cut2dm1 and Cut2dm2, were also stable in this cell-free destruction system. Mutation in one destruction box already reduced the efficiency for *in vitro* proteolysis. Thus the *Xenopus* anaphase proteolytic system requires Cut2 containing two intact destruction boxes. This differs from the *in vivo* result in *S.pombe*, which requires only one destruction box for viability.

We demonstrated in a HeLa anaphase cell extract system, using biotinylated ubiquitin and human hE2-C ubiquitin-conjugating enzyme, that Cut2 was polyubiquitinated in a manner dependent on the presence of functional destruction box sequences. Polyubiquitination was abolished completely if the double destruction boxes were mutated. Thus not only ubiquitin-dependent proteolysis but also polyubiquitination can take place in extracts of evolutionarily distant organisms, at least *in vitro*, strongly suggesting that the destruction boxes are functionally universal for anaphase proteolysis, though their consensus sequences are quite variable. Mono-ubiquitination was apparently not relevant for anaphase proteolysis, as it occurred in Cut2 lacking a functional destruction box or in wild-type Cut2 incubated in extracts derived from asynchronous culture. The destruction boxes of Cut2 are hence required for polyubiquitination, and identification of a destruction box-interacting protein(s) is of obvious interest. A specific question is whether such protein(s) also interact with the destruction boxes of mitotic cyclins. It is noteworthy that the N-terminal fragment (N73) is an excellent model for polyubiquitination in a destruction box sequence-dependent manner. We are currently trying to identify which lysine residue(s) in the fragment is polyubiquitinated.

We showed that Cut2 was highly phosphorylated prior to proteolysis in the *Xenopus* mitotic extracts. The phosphorylation occurred specifically in CSF-arrested mitotic extracts, but not in interphase extracts. No upper band was produced for Cdc13 under the same conditions (Yamano *et al.*, 1996). Phosphorylation of Cut2 also occurred *in vivo*, producing the upper band specifically during an early mitotic stage (Funabiki *et al.*, 1996b). Phosphopeptide mapping of overproduced Cut2 showed the presence of at least four phosphorylation sites (H.Funabiki and M.Yanagida, unpublished result). One of them was the S109 residue in the consensus for Cdc2 kinase, the alanine mutant of which abolished a phosphopeptide. Cut2S109A mutant protein, however, was completely functional and able to suppress the deletion mutant of $cut2^+$ (our unpublished result). Since Cut2 was multiply

phosphorylated, phosphorylation might be functionally redundant. Identification of the phosphorylation sites and construction of multiple substitution mutants at the phosphorylation sites are needed to examine the functional significance of Cut2 phosphorylation for anaphase proteolysis.

The variation in destruction box sequences may represent the difference in the nature of degradation. A-type cyclin is degraded during metaphase, whereas B-type cyclins are only degraded after metaphase–anaphase transition (Hunt *et al.*, 1992). In addition, while mitotic degradation of cyclin A1 and B2 requires binding of Cdc2, that of cyclin B1 does not (Stewart *et al.*, 1994; van der Verden and Lohka, 1994). King *et al.* (1996) demonstrated that an asparagine residue (N) at position nine in the destruction box of B-type cyclin is essential for its ubiquitination and degradation *in vitro*, while the same position in A-type cyclins is only loosely conserved. Unlike cyclins (Glotzer *et al.*, 1991) and Pds1 whose degradation is necessary for sister chromatid separation in budding yeast (Cohen-Fix *et al.*, 1996), Cut2 has two functional destruction boxes whose position nine is glutamine or threonine, neither of which is often found in the destruction boxes of cyclins. The *Saccharomyces cerevisiae* Ase1 has a destruction box located in the C-terminus and contains an unusual F rather than L in the fourth position of the RXXL consensus (Juang *et al.*, 1997). The use of the *in vitro* method shown in the present study may enable us to determine essential residues in the destruction boxes of Cut2.

Since Cut2 forms a complex with Cut1 (Funabiki *et al.*, 1996a), normally regulated proteolysis of Cut2 may require the binding of Cut1. Note that the level of Cut1, like that of Cdc2, does not fluctuate during the cell cycle; and that Cut1 and Cut2 proteins are in a large complex (30–40S). Proteolysis of Cut2 might be regulated by interactions with Cut1 and other proteins. Our preliminary data showed that the level of Cut2 decreased in a *cut1* mutant whereas it increased in the strain with overproduced Cut1, suggesting that Cut1 stabilized Cut2. Further studies on the regulation of Cut2 degradation by Cut1 may determine the role of the bipartite destruction boxes present in Cut2.

Materials and methods

Strains and media

An *S.pombe* haploid wild-type strain h^- and its derivative strains, h^- *leu1-32*, *cdc10-129 leu1-32*, *cut2-364 leu1-32* and a heterozygous *cut2*Δ diploid strain were used (Funabiki *et al.*, 1996a). Minimal EMM2 media were employed for liquid cultures and plates.

Plasmids

pRECUT2-20 contains the full-length $cut2^+$ gene in which an authentic *Nde*I site was mutagenized and a new *Nde*I site was created at the position of the first methionine codon and placed under the control of the REP81 promoter (Basi *et al.*, 1993). This was constructed by the PCR method using oligonucleotides 5′-GGACATATGTTGCCCAGAACCATGTTTTCTTATGGAAAAGAA-3′ and 5′-GGAGGATCCTTATAACAATCCTGTATCCAA-3′. Site-directed mutations in the destruction boxes were made using pRECUT2-20 and the sequential PCR steps. Oligonucleotides CUT212 (5′-AGACCCCGCGGGTGCTGCTTTGGAGCCC-3′) and CUT213 (5′-GCTCCAAAGCAGCACCCGCGGGGTCTAC-3′) were used to make pRECUT2-30 (encoding *cut2dm1*); and CUT2HY4 (5′-AACGGTTCCTGCAACGGTAGCGGGAGGTAAG-3′) and CUT2HY5 (5′-CTTACCTCCCGCTACCGTTGCAGGAACCGTT-3′) used to make pRECUT2-31 (*cut2dm2*). pRECUT2-32 (*cut2ddm*) was constructed by simultaneous ligation of a *Nde*I–*Bsm*I fragment of pRECUT2-30 and a *Bsm*I–*Bam*HI fragment of pRECUT2-31 into the *Nde*I–*Bam*HI sites of plasmid containing the REP81 promoter. pRCUT2-19 (*cut2N73*) was constructed by ligation of pREP1 and a PCR fragment amplified by oligonucleotides CUT210 (5′-CGCATATGTTGCCCAGAACCATG-3′) and CUT211 (5′-CGGGATCCTATTTCTTTGTACTGGGAGCAG-3′), using pRECUT2-20 as a template. The same oligonucleotides were used to make pRCUT2-33 (*cut2N73dm1*), pRCUT2-34 (*cut2N73dm2*) and pRCUT2-35 (*cut2N73ddm*), but the templates employed were pRECUT2-30, pRECUT2-31 and pRECUT2-32, respectively.

Indirect immunofluorescence microscopy

For indirect immunofluorescence, the procedures for *S.pombe* using glutaraldehyde and paraformaldehyde were followed. The spindle pole bodies 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). Microtubules were stained by mouse anti-α-tubulin monoclonal antiserum TAT1; a gift of Dr K.Gull) and CY3-conjugated affinity-purified goat anti-mouse IgG (Chemicon International, Inc., Temecula, CA). Chromosomal DNA was stained with 0.2 μg/ml 4′,6′-diamino-2-phenylindole (DAPI).

Preparation of cell extracts

The procedures previously described were followed with modifications. *Schizosaccharomyces pombe* cells were collected on filter paper and suspended in STOP buffer (0.9% NaCl, 10 mM EDTA, 50 mM NaF, 1 mM NaN_3). After centrifugation, pelleted cells were frozen in liquid nitrogen and kept at –80°C until use. For immunoblotting, frozen cells (1×10^9/ml) were thawed in TEEG [50 mM Tris–HCl at pH 7.5 containing 10 mM EDTA, 1 mM EGTA, 10% glycerol, 10 mM *N*-ethylmaledimide (NEM) and the protease inhibitors 1 mM phenylmethylsulfonyl fluoride (PMSF); 2 mg/ml pepstatin A; 10 mM E-64]. Cells were broken with glass beads (three times for 15 s). The supernatant after centrifugation at 5000 r.p.m. for 5 min using a 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. Anti-PSTAIRE mouse monoclonal antibody used to detect Cdc2 protein was a gift from Dr Y.Nagahama (National Institute of Basic Biology). The procedures for histone H1 kinase assay using anti-cdc13 immunoprecipitates were described by Stone *et al.* (1993).

FACScan analysis

A Becton-Dickinson FACScan was used, and the procedures described in Saka and Yanagida (1993) were followed.

Destruction assay in Xenopus egg extract

Substrates for destruction assays were prepared by coupled transcription–translation in the presence of [^{35}S]methionine (Dupont, NEG-709A) in a reticulocyte lysate. Destruction assays were carried out as previously described (Stewart *et al.*, 1994; Yamano *et al.*, 1996).

Phosphatase treatment

Immunoprecipitates were treated with calf intestine alkaline phosphatase (Boehringer Mannheim) at 37°C for 30 min. A cocktail of 10 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA and 0.1 mM sodium orthovanadate was used as phosphatase inhibitor (Lanker *et al.*, 1996).

Ubiquitination using HeLa cell extracts

Substrates for ubiquitination were produced with the GST sequences in bacterial cells and bound to glutathione–Sepharose 4B beads (Pharmacia Co.). Biotinylated ubiquitin was detected by the ECL avidin–peroxidase method. HeLa cell extracts were prepared by disrupting cells 1 h after the release from colcemid block (Honda *et al.*, 1995). Protein concentrations (final) of E1, hE2-C, biotinylated ubiquitin and HeLa cell extracts used were 20 μg/ml, 60 μg/ml, 0.3 mg/ml and 0.2 mg/ml, respectively. Detailed procedures for the assay method for ubiquitination will be described elsewhere (H.Tanaka and H.Yasuda, in preparation).

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