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Polo boxes and Cut23 (Apc8) mediate an interaction between polo kinase and the anaphase-promoting complex for fission yeast mitosis

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The fission yeast *plo1*$^+$ gene encodes a polo-like kinase, a member of a conserved family of kinases which play multiple roles during the cell cycle. We show that Plo1 kinase physically interacts with the anaphase-promoting complex (APC)/cyclosome through the noncatalytic domain of Plo1 and the tetratricopeptide repeat domain of the subunit, Cut23. A new *cut23* mutation, which specifically disrupts the interaction with Plo1, results in a metaphase arrest. This arrest can be rescued by high expression of Plo1 kinase. We suggest that this physical interaction is crucial for mitotic progression by targeting polo kinase activity toward the APC.

Introduction

Proteolysis plays a crucial role in cell cycle progression, enabling rapid irreversible progression from one event to the next. During mitosis, several proteins are specifically targeted for destruction (for review see Morgan, 1999; Zachariae and Nasmyth, 1999). With the exception of cohesins (Ciosk et al., 1998; Uhlmann et al., 1999), these mitotic proteins are degraded by ubiquitin-mediated proteolysis. The anaphase-promoting complex (APC)*/cyclosome, which acts as a ubiquitin ligase (E3 enzyme) is considered to determine the timing and specificity with which proteins are degraded during mitosis.

The APC is activated during mitosis and remains active into G1 (Amon et al., 1994; Brandeis and Hunt, 1996; Fang et al., 1998). However, the substrate specificity of the APC changes, leading to degradation of different proteins sequentially throughout mitosis. This is thought to be mediated through a transient physical association of the different cofactors, Cdc20 and Cdh1/Hct1, to the APC (Schwab et al., 1997; Visintin et al., 1997; Fang et al., 1998; Kramer et al., 2000).

The APC is a multiprotein complex. In vertebrates and fission yeast, the APC is a 20S complex containing 10 or more core subunits (Peters, 1999). So far studies have been focused on understanding the roles and regulation of the APC as a whole complex. There have been few reports defining the roles of individual APC subunits, with the exception of Apc11, which displays catalytic activity in vitro (Gmachl et al., 2000; Leverson et al., 2000). Further defining the interactions between specific subunits of the APC with its many regulators and substrates would be crucial for molecular dissection of regulation and substrate recognition of the APC.

A balance between activating and inhibitory phosphorylation of the APC itself and its regulatory cofactors is also thought to be important for regulation of the APC (King et al., 1995; Lahav-Baratz et al., 1995; Kotani et al., 1999; Morgan, 1999; Kramer et al., 2000). There is a body of evidence suggesting that polo kinase is involved in APC activation (Charles et al., 1998; Descombes and Nigg, 1998; Kotani et al., 1998, 1999; Shirayama et al., 1998). Polo kinases are conserved kinases which are implicated in multiple steps of the cell cycle. The fission yeast polo kinase Plo1 has been shown to be required for multiple mitotic processes in vivo (Ohkura et al., 1995; Bähler et al., 1998).

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*Abbreviations used in this paper: APC, anaphase-promoting complex; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; SPB, spindle pole body; TPR, tetratricopeptide repeat.

Key words: polo kinase; APC; anaphase-promoting complex; mitosis; fission yeast

To dissect the multiple functions of polo kinase, we developed a genetic screen to isolate high *plo1*$^+$-dependent (*pld)* mutants whose viability is dependent on high levels of *plo1*$^+$ expression (Cullen et al., 2000).

Here we show that one of the *pld* mutants, *pld9*, is allelic to *cut23*, which encodes a subunit of the APC. In addition to the genetic interaction, we have also found that Plo1 physically interacts with Cut23 and that this interaction is compromised by the *pld9* mutation. Our results provide evidence for a vital role of Cut23/Apc8 in the regulation of mitotic progression by polo kinase.

Results and discussion

A high *plo1*$^+$-dependent mutation, *pld9*, is allelic to *cut23* encoding a subunit of the APC

The *pld9-PD26* mutation was identified through a genetic screen for mutants whose viability is dependent on elevated expression of *plo1*$^+$ (Cullen et al., 2000). To determine the identity of the *pld9* gene, we screened a genomic library for complementation of the mutation. Analysis of one of the isolates indicated that the *cut23*$^+$ gene which encodes an APC/cyclosome subunit is responsible for the complementation. Random spore analysis showed that *pld9-PD26* is tightly linked to *cut23*. To confirm the allelism, the *cut23* gene was amplified from the *pld9-PD26* mutant by PCR. Sequencing of the PCR product revealed a single nucleotide substitution, which results in the change of a conserved amino acid (see below). Based on these results, we concluded that *pld9* is allelic to *cut23*. We therefore renamed the *pld9-PD26* mutation *cut23-PD26*.

The *cut23-PD26* mutation leads to metaphase arrest which can be relieved by elevated expression of *plo1*$^+$

The *cut23-PD26* mutant exhibits a mitotic arrest phenotype in the absence of elevated expression of *plo1*$^+$ (Cullen et al., 2000). To identify the exact stage of the arrest, we performed immunostaining of α-tubulin and the spindle pole body (SPB) component Sad1. Two types of cells accumulated after repression of high Plo1 expression. The first category of cells arrested in a metaphase-like stage with hypercondensed chromosomes which were unseparated and associated with a short bipolar spindle (~3 μm; Fig. 1 B). Some of these cells were highly elongated (up to 27 μm). In addition, a significant number of the elongated cells had an interphase nucleus and microtubule array (Fig. 1 A).

To confirm that this metaphase-like arrest is caused by a failure of degradation of APC substrates, cyclin B (Cdc13) and securin (Cut2) were examined using green fluorescent protein (GFP)-tagged proteins. In wild-type, both proteins accumulate in the nucleus during G2 and localize to the mitotic spindle before their degradation at the metaphase/anaphase transition (Alfa et al., 1990; Funabiki et al., 1996). In *cut23-PD26*, which expresses Cdc13-GFP or Cut2-GFP, most mitotically arrested cells have a GFP signal on the spindle (Fig. 1, C and D). Even in cases where spindle elongation has been initiated presumably due to prolonged arrest, the GFP signals were still retained. These results indicate that the degradation of APC substrates is defective in the *cut23-PD26* mutant.

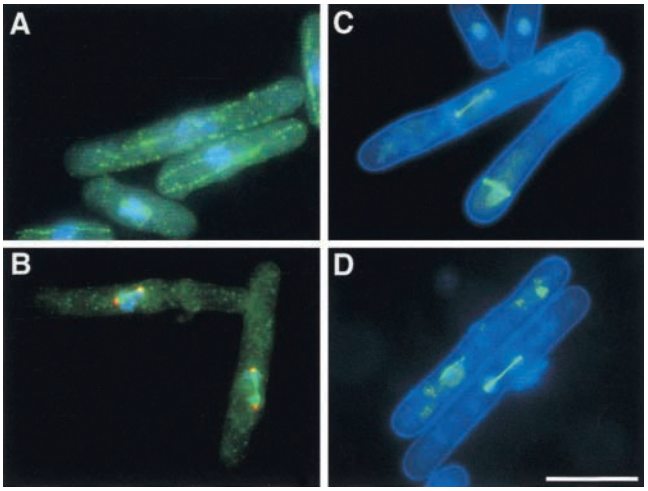

Figure 1. ***cut23-PD26* arrests at metaphase with APC substrates not degraded.** *cut23-PD26 nmt1-plo1*$^+$ was cultured in the presence of thiamine to lower the level of Plo1 to wild-type. (A) Immunostaining using antibodies against α-tubulin (green) with DAPI (blue) counterstaining. Long cells which have an interphase nucleus and an interphase microtubule array, and one cell with overcondensed chromosomes which has a metaphase-like short spindle. (B) Immunostaining of an SPB component, Sad1 (red), confirmed that the spindles are bipolar. (C and D) *cut23-PD26* cells expressing GFP-tagged Cdc13 (C) or Cut2 (D). Both of the APC substrates remain on mitotic spindles associated with overcondensed chromosomes. Bar, 10 μm.

All of the defective phenotypes described above are eliminated by elevated levels of Plo1 either through expression of *plo1*$^+$ from the *nmt1* promoter in the absence of thiamine or by the introduction of wild-type *plo1* gene under the native promoter on a multicopy vector.

Specific rescue of *cut23-PD26* by high Plo1

As Cut23 is a core subunit of the APC/cyclosome, it is possible that complementation of *cut23-PD26* by high levels of Plo1 is due to a general enhancement of APC activity by excess Plo1 kinase. To test this hypothesis, we asked if elevated levels of Plo1 could rescue the mitotic defects of other core APC subunit mutants. Multicopy expression of *plo1*$^+$ is sufficient to complement *cut23-PD26* well, but did not improve the growth of any of the other APC mutants tested (*cut9–665*, *nuc2–663*, *cut4–533* and *cut23–194*), even at semirestrictive temperatures.

We also tested whether overexpression of *plo1*$^+$ is sufficient to induce degradation of APC substrates. Overexpression of *plo1*$^+$ from the *nmt1* promoter on a multicopy plasmid can induce septation in interphase-arrested *cdc25–22* cells (Ohkura et al., 1995). Although 80% of cells underwent septum formation, immunoblots showed no changes in the levels of two APC substrates, cyclin B and securin.

These results indicate that elevated levels of Plo1 kinase cannot compensate for low APC activity in general, but are able to rescue the specific defect caused by the *cut23-PD26* mutation.

APC formation is unaffected in the *cut23-PD26* mutant

To identify the molecular defects of the APC in the *cut23-PD26* mutant, we tested whether APC formation is dis-

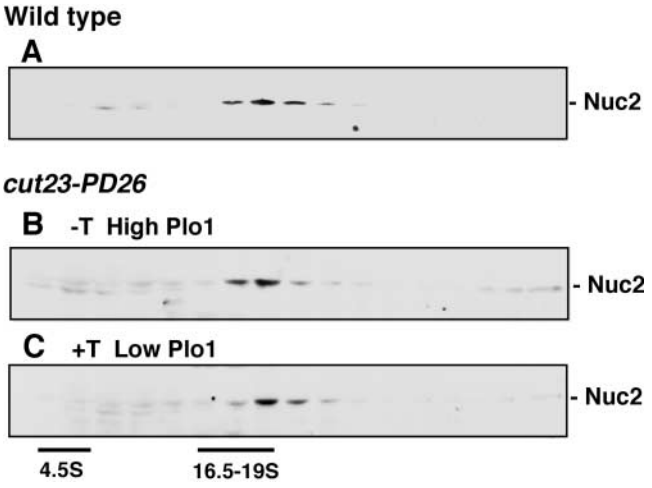

Figure 2. ***cut23-PD26* has an intact APC.** Protein extracts prepared from wild-type (A) and the *cut23-PD26 nmt1-plo1*$^+$ mutant without thiamine (B) and with thiamine (C) were centrifuged through a sucrose gradient. Each fraction was analyzed by immunoblotting using an antibody against Nuc2, a core APC subunit. In the presence of thiamine (C), *cut23-PD26* showed the defective phenotype and the APC sedimented marginally faster than the wild-type APC as observed in other mitotically arrested cells. Sedimentation values were determined from protein markers, BSA (4.5S), and thyroglobulin (16.5–19S).

rupted in the *cut23-PD26* mutant. All of the existing mutants of core APC subunits examined so far disrupt the formation of the 20S APC (Yamada et al., 1997; Yamashita et al., 1999). In extracts prepared from wild-type asynchronous cultures, a core APC subunit, Nuc2, sediments primarily in the 20S fractions (Fig. 2 A). In the presence of high Plo1, when the *cut23-PD26* mutant grows normally, the Nuc2 sedimentation profile was comparable to wild-type with a single peak at 20S (Fig. 2 B). Without high Plo1, when cells show mitotic arrest, Nuc2 still formed a single peak which was shifted slightly to the more rapidly sedimenting fractions as described in other mitotically arrested cells (Fig. 2 C; Yamada et al., 1997). In conclusion, the *cut23-PD26* mutation does not disrupt overall complex formation of the APC, unlike other mutants of core subunits.

Cut23 physically interacts with Plo1

In an attempt to identify genes that interact with *plo1*$^+$, we performed a two-hybrid screen of a fission yeast cDNA library using the full-length *plo1*$^+$ as bait. From ~2.5 million cDNAs screened, the *cut23* gene was isolated three times as at least two independent clones. The known interactor *dmf1*$^+$/*mid1*$^+$ was also isolated but no genes encoding other APC subunits were isolated. The plasmids were recovered and the two-hybrid interaction with *plo1*$^+$ confirmed. Neither *cut23*$^+$ nor *plo1*$^+$ alone could activate the reporter genes.

Next, we asked whether the two-hybrid interaction between Plo1 and Cut23 reflects a physical association in fission yeast cells. We immunoprecipitated Cut23 or Plo1 protein from extracts of fission yeast in which the endogenous *cut23*$^+$ was COOH terminally tagged with the hemagglutinin (HA) epitope (Yamashita et al., 1999). Western blotting shows that

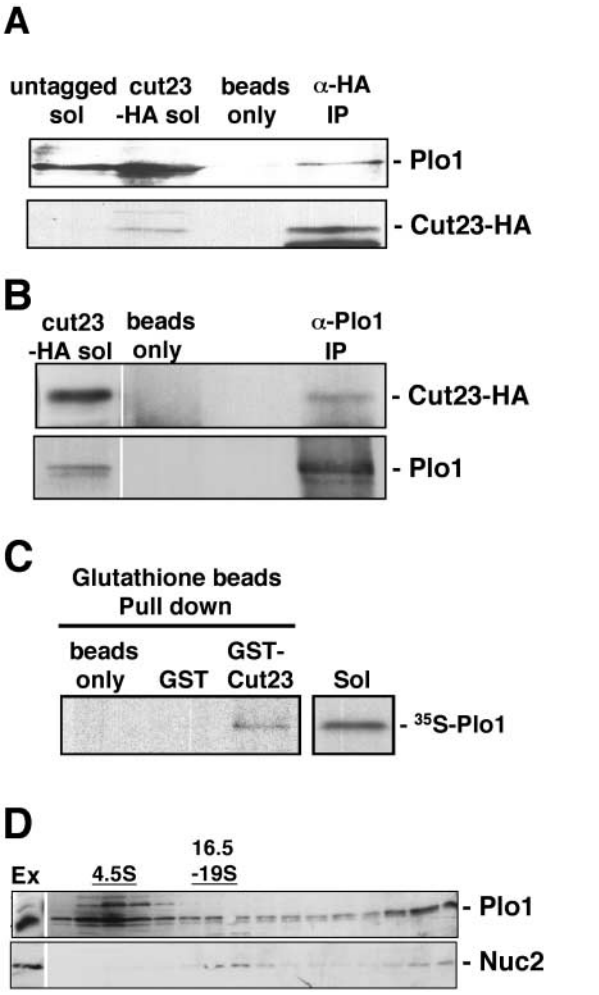

Figure 3. **Cut23 physically interacts with Plo1.** (A and B) Plo1 is coimmunoprecipitated with Cut23 from fission yeast extract. Cut23-HA (A) or Plo1 (B) was immunoprecipitated from soluble extracts of fission yeast expressing HA-tagged Cut23 from the endogenous promoter. The soluble extracts (Sol) and immunoprecipitates (IP) were analyzed by immunoblots probed with antibodies against HA and Plo1. As controls, soluble extracts from an untagged strain and precipitates without antibodies (beads only) from tagged strains were also included. Precipitates were loaded equivalent to ~7 times more than the soluble fractions. (C) Plo1 interacts directly with Cut23. Bacterially produced GST-Cut23 fusion protein or GST protein was incubated with in vitro–translated ^{35}S-labeled Plo1 protein (the soluble fraction; Sol), and pulled down by glutathione beads. (D) A wild-type extract was centrifuged through a sucrose gradient and fractions were blotted with Plo1 and Nuc2 antibodies.

fractions of Plo1 and Cut23-HA proteins can be coimmunoprecipitated from a fission yeast extract (Fig. 3, A and B).

To test whether the interaction between Plo1 and Cut23 is direct, Cut23 protein was expressed in bacteria as a glutathione *S*-transferase (GST) fusion protein. The purified GST-Cut23 was incubated with ^{35}S-labeled Plo1 protein produced in an in vitro translation system, and then pulled down using glutathione beads. Autoradiography revealed that a fraction of ^{35}S-labeled Plo1 protein was coprecipitated with GST-Cut23 (Fig. 3 C). Plo1 protein was not coprecipitated with GST alone. These results indicate that Plo1 and Cut23 are able to associate directly in vitro.

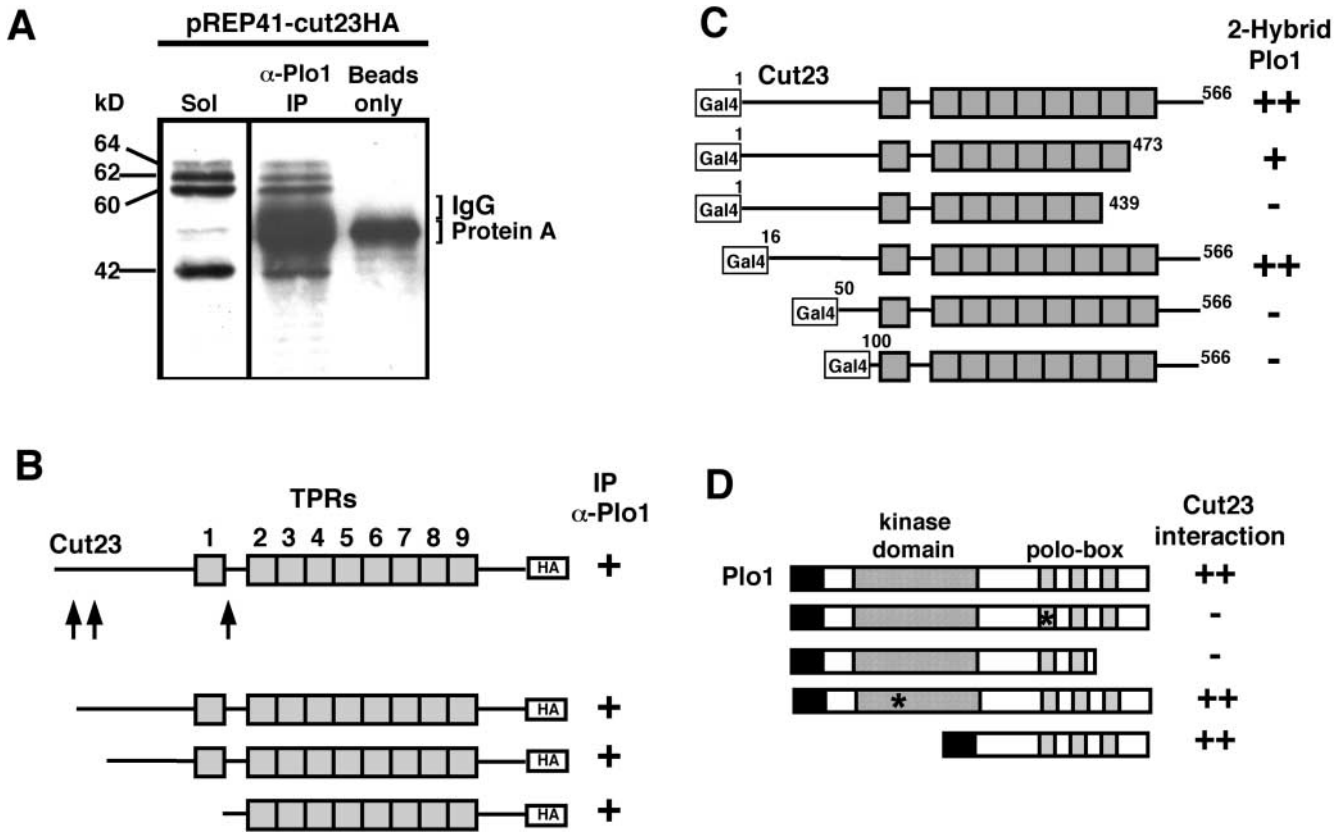

Figure 4. **Interaction between Cut23 and Plo1 is mediated by the TPR domain of Cut23 and polo boxes of Plo1.** (A) Three degradation products of Cut23 caused by moderate overexpression were coimmunoprecipitated with Plo1 from a soluble extract prepared from a strain containing pREP41(cut23-HA). The extract and the immunoprecipitates were analyzed by immunoblotting with an anti-HA antibody. (B) A rough estimate of cleavage sites (arrows) and degradation products detected by the anti-HA antibody. The COOH-terminal TPR domain resistant to protein degradation is sufficient for the association. (C) Truncations of Cut23 were tested for the interaction with Plo1 in the two-hybrid assay. ++, strong interaction; +, weak interaction; −, no interaction. (D) Various mutants of Plo1 were tested for interaction with Cut23 using the two-hybrid assay. The truncations and mutations are, from the top, the full-length wild-type Plo1, YQL508AAA, 1–583 amino acids, K69R, and 313–683 amino acids.

We examined whether Plo1 protein in fission yeast extract cosediments with 20S APC through a sucrose gradient. Plo1 sediments broadly from the top to the bottom fractions without an obvious peak at 20S (Fig. 3 D), indicating that Plo1 associates with a variety of complexes in fission yeast extract.

The interaction is mediated by the noncatalytic region of Plo1 and the TPR domain of Cut23

To limit the region of Cut23 interacting with Plo1, we took advantage of the partial degradation of Cut23-HA that occurs when it is moderately overproduced (Fig. 4 A). As Cut23 is tagged with HA at the COOH-terminal end, faster migrating bands revealed by an anti-HA antibody represent a series of NH_2-terminal truncations (Fig. 4 B). All three of the main degradation products recognized by the anti-HA antibody were coimmunoprecipitated with Plo1 (Fig. 4 A). The smallest (42 kD) band is estimated to roughly correspond to a peptide containing a block of eight tandem TPRs (the 2nd to the 9th) and the COOH-terminal tail (Fig. 4 B). These results indicate that the TPR domain, which is resistant to degradation, is sufficient for association with Plo1.

The two-hybrid assay was also used to further define the interaction domain of Cut23 (Fig. 4 C). The interaction with Plo1 was still detected in a truncation lacking the last TPR and the COOH-terminal tail, although the interaction was weaker. Deletion of a further 34 amino acids completely abolished the interaction. On the other hand, a truncation lacking the first 50 amino acids failed to interact. This was somewhat unexpected as it contains the Plo1 interaction region identified by immunoprecipitation of degradation products. One possible explanation is the failure of the protein to fold correctly during synthesis without the NH_2-terminal region in the two-hybrid assay.

We also determined the region of Plo1 which interacts with Cut23. Various point mutations and truncations of Plo1 were tested for interaction with Cut23 in a two-hybrid assay (Fig. 4 D). Plo1 kinase has an NH_2-terminal catalytic domain and a COOH-terminal noncatalytic domain containing motifs (polo boxes) conserved among the polo kinase family. Mutations introduced in the polo boxes abolished the interaction with Cut23, whereas point mutations, or indeed complete deletion of the kinase domain, had no effect on the interaction. The noncatalytic domain of Plo1 was sufficient for interaction with Cut23, suggesting that the interaction observed between Plo1 and Cut23 is not simply a kinase–substrate interaction.

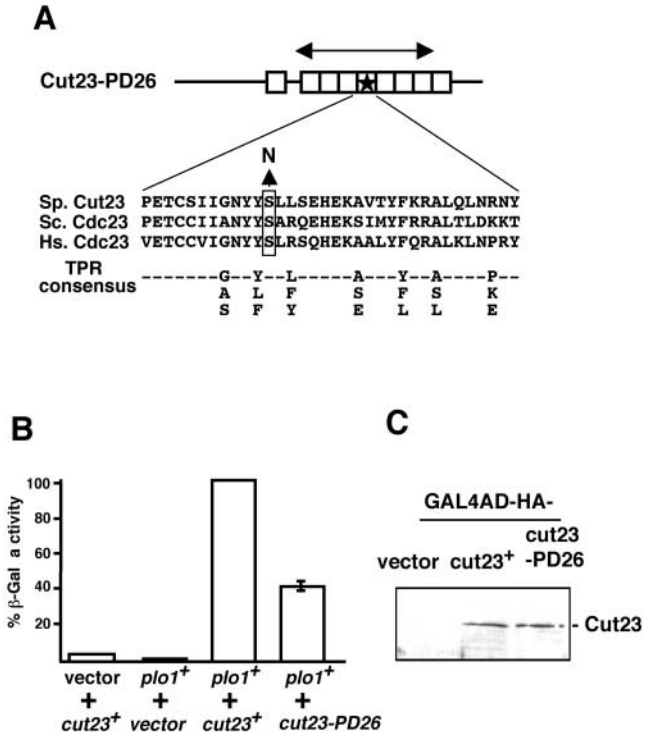

Figure 5. **The *cut23-PD26* mutation reduces the interaction between Cut23 and Plo1.** (A) *cut23-PD26* results in the substitution of the serine (S) residue at 349 (star) with an asparagine (N), which is located in the fifth of the nine TPRs (boxes). The residue is conserved among the Cdc23 family and situated in the TPR domain, which is responsible for interaction with Plo1 (horizontal arrow). The TPR consensus sequences is indicated below. (B) Interaction of wild-type and Cut23-PD26 with Plo1 was measured using the two-hybrid assay. The interaction was quantified by measuring the activity of β-galactosidase, the product of the *lacZ* reporter gene. (C) Immunoblots using an anti-HA antibody confirmed that the *cut23-PD26* mutation does not affect the abundance and size of GAL4AD-HA-Cut23 fusion proteins.

The *cut23-PD26* mutation reduces interaction with Plo1

Sequencing of the *cut23-PD26* gene revealed a single missense mutation resulting in the change of serine 349 to asparagine (Fig. 5 A). This residue is conserved among the Cut23/Cdc23/Apc8 family and is located in the middle of the TPR domain, which is responsible for the interaction with Plo1. As this mutation does not disrupt APC formation and is complemented by a high level of Plo1, we tested whether the mutation affects the ability of Cut23 to interact physically with Plo1. A quantitative two-hybrid assay indicates that the *cut23-PD26* mutation dramatically reduced the ability to activate a reporter gene, *lacZ*, compared with wild-type Cut23 (Fig. 5 B). Immunoblotting confirmed that the *cut23-PD26* mutation does not affect the amount and the size of the fusion protein (Fig. 5 C). Therefore, we conclude that the *cut23-PD26* mutation dramatically reduces the interaction between Cut23 and Plo1.

Significance of the interaction between polo kinase and Cut23

We have shown that the fission yeast polo kinase Plo1 interacts both genetically and physically with the APC. Plo1 kinase interacts directly with the APC subunit Cut23 through the noncatalytic domain of Plo1 and the TPR domain of Cut23. A new *cut23* mutation, *cut23-PD26*, specifically disrupts this interaction with Plo1 and causes a metaphase arrest. This arrest can be rescued by elevating levels of Plo1 kinase. These findings lead us to suggest the following working hypothesis: (a) in wild-type cells, polo kinase associates with Cut23 to phosphorylate and activate the APC allowing progression through mitosis. (b) In the *cut23-PD26* mutant, the affinity between polo kinase and Cut23 is greatly reduced, resulting in insufficient activation of the APC and metaphase arrest. (c) An elevated level of polo kinase can compensate for the reduction in affinity enabling activation of the APC.

Although there is a substantial body of evidence to support a role for polo kinase in APC activation (Charles et al., 1998; Descombes and Nigg, 1998; Shirayama et al., 1998), only one study has provided evidence for a direct interaction between polo kinase and the APC (Kotani et al., 1998). Our findings demonstrate that this interaction is conserved among eukaryotes. In addition, we have identified the APC subunit mediating the interaction, defined the interacting domains, and isolated a mutation which compromises the interaction. Although Cut23 is an attractive candidate for a polo kinase substrate, currently there is no evidence to support this possibility (Kotani et al., 1998; Rudner and Murray, 2000; unpublished data). The role of Cut23 may be to recruit polo kinase to the APC in order to facilitate phosphorylation of other subunits.

Materials and methods

Fission yeast techniques

Analysis of fission yeast were performed as described in Moreno et al. (1991), Alfa et al. (1993), and Ohkura et al. (1995). To examine the cytological phenotype, *h⁻ leu1 cut23-PD26 int[nmt1-plo1⁺, LEU2]* (Cullen et al., 2000) was first cultured without thiamine, and then in the presence of thiamine for a further 21–28 h at 25°C. To follow Cut2 or Cdc13, the chromosomal gene was tagged with GFP in the *cut23-PD26* strain. Samples were observed using an Axioplan2 microscope (ZEISS). Images were captured using a CCD camera (Hamamatsu) and processed using Openlab2 (Improvision) and Photoshop (Adobe).

To isolate the *pld9* genes, *h⁻* leu1-*32 ura4-d18 pld9-PD26 int[nmt1-plo1⁺, LEU2]* was transformed with a genomic library (Barbet et al., 1992). Plasmids containing *plo1⁺* and *cut23⁺* were isolated from Pld⁺ transformants. The inserts were further subcloned and tested for complementation. Tight genetic linkage between *cut23* (or adjacent *cut9*) and *pld9* was shown by random spore analysis between *cut9–665* and *pld9-PD26*.

Molecular techniques

General molecular analysis were performed according to Sambrook et al. (1989). Soluble extracts from fission yeast were prepared by centrifugation at 14,000 rpm for 20 min after disrupting cells in HB buffer (Moreno et al., 1991) with glass beads (Sigma-Aldrich) in a Ribolyser (Hybaid). For immunoprecipitation, soluble extracts were incubated with anti-Plo1 (Ohkura et al., 1995) or anti-HA (12A5, Boehringer) antibodies followed by incubation with protein A beads (Amersham Pharmacia Biotech). Beads were washed four times in HB buffer before analysis by SDS-PAGE. Coimmunoprecipitation of Cut23 and Plo1 was observed in two strains, one in which the endogenous Cut23 is tagged with the HA epitope and another which moderately overexpresses *cut23-HA* from pREP41 in the presence of thiamine (Yamashita et al., 1999). Sucrose density gradient centrifugation was performed as described in Yamashita et al. (1999) and Yamada et al. (1997).

The *cut23⁺* coding sequence was cloned into pGEX4T-2 (Amersham Pharmacia Biotech) and expressed as a GST fusion protein in *Escherichia coli* BL21(DE3). The fusion protein was purified using glutathione beads (Amersham Pharmacia Biotech) and then dialyzed in TXB buffer (Reynolds et al., 2000). *plo1⁺* was transcribed and translated in vitro in the presence

of ^{35}S-methionine using T7 Quick coupled reticulocyte system (Promega), and then incubated with GST-Cut23 or GST before addition of glutathione beads. After four washes in TXB buffer, the beads were analyzed by SDS-PAGE and autoradiograph.

Two-hybrid screening and quantitative assays

Two-hybrid screening was performed in strain Y190 (CLONTECH Laboratories, Inc.) using *plo1*$^{+}$ in pGBT9 (CLONTECH Laboratories, Inc.) and an *Schizosaccharomyces pombe* cDNA library in pGADGH (CLONTECH Laboratories, Inc.). Various mutations were made by site-directed mutagenesis and tested for interaction using pACT2 and pBTM116 vectors in the strain CTY10-5d (MacNeill et al., 1996). Quantitative two-hybrid assays were performed as described (MacNeill et al., 1996). Results were obtained in each case for two independent transformants assayed in triplicate.

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