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An ESP1/PDS1 Complex Regulates Loss of Sister Chromatid Cohesion at the Metaphase to Anaphase Transition in Yeast

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Summary

Cohesion between sister chromatids during G2 and M phases depends on the "cohesin" protein Scc1p (Mcd1p). Loss of cohesion at the metaphase to anaphase transition is accompanied by Scc1p's dissociation from chromatids, which depends on proteolysis of Pds1p mediated by a ubiquitin protein ligase called the anaphase promoting complex (APC). We show that destruction of Pds1p is the APC's sole role in triggering Scc1p's dissociation from chromatids and that Pds1p forms a stable complex with a 180 kDa protein called Esp1p, which is essential for the dissociation of Scc1p from sister chromatids and for their separation. We propose that the APC promotes sister separation not by destroying cohesins but instead by liberating the "sister-separating" Esp1 protein from its inhibitor Pds1p.

Introduction

Chromosome duplication during S phase produces sister chromatids that are held together by specific chromosomal proteins called cohesins (Guacci et al., 1997; Michaelis et al., 1997). Sister chromatids are later pulled to opposite poles of the cell during anaphase by microtubules that connect sister kinetochores to opposite poles of the mitotic spindle. The "splitting" force exerted by these microtubules is initially counteracted by cohesion between sisters, and the balance of these two forces results in chromosome alignment in the middle of the cell during metaphase (Nicklas, 1988). Loss of cohesion rather than an increase in the splitting force exerted by microtubules is thought to trigger disjunction of sisters at the metaphase to anaphase transition (Miyazaki and Orr-Weaver, 1994).

The properties of a cohesin called Scc1p or Mcd1p in *Saccharomyces cerevisiae* suggest how cohesion might be lost. Scc1p binds to chromosomes during S phase, it prevents premature separation of sister chromatids during G2/M, and disappears from chromosomes at the metaphase to anaphase transition (Michaelis et al., 1997). Thus, Scc1p's disappearance from chromosomes could be responsible for the separation of sister chromatids during anaphase. A key question is what causes the sudden disappearance of Scc1p and the loss of sister chromatid cohesion.

Cyclin proteolysis coincides with the metaphase to anaphase transition and depends on a large protein complex called the anaphase promoting complex (APC), which mediates ubiquitination of cyclins via destruction boxes close to their N termini (King et al., 1995; Sudakin et al., 1995; Zachariae and Nasmyth, 1996a). Inactivation of any one of at least ten APC subunits prevents not only cyclin destruction but also sister chromatid separation (Irniger et al., 1995; Zachariae et al., 1996b, 1998). Because cyclin destruction per se is not required for sister separation (Holloway et al., 1993; Surana et al., 1993), it has been proposed that the APC also mediates the destruction of proteins that act as inhibitors of anaphase. What might these proteins be?

One possibility is that degradation of cohesins facilitates loss of sister chromatid cohesion. Scc1p is an APC substrate that is destroyed during anaphase. Furthermore, loss of Scc1 function allows sister chromatids to separate in the absence of the APC activity. However, proteolysis of Scc1p commences at the onset of anaphase and is not completed until after chromosomes have reached opposite poles of the cell, which might be too late to trigger separation (Michaelis et al., 1997).

Another candidate is a protein called Pds1p, which is required for preventing anaphase when DNA or spindles are damaged (Yamamoto et al., 1996b). Pds1p is normally destroyed by the APC around the metaphase to anaphase transition (Cohen-Fix et al., 1996). Furthermore, mutant variants of Pds1p, which cannot be degraded due to mutations in their destruction boxes, block the separation of sister chromatids. This strongly suggests that destruction of Pds1p is necessary for the separation of sister chromatids.

Results described here and by Yamamoto et al. (1996b) imply that the APC promotes sister separation and dissociaton of Scc1p solely through destruction of Pds1p. We purified Pds1p and found it tightly associated with a 180 kDa protein, which was identified by mass spectrometric sequencing as the product of the *ESP1* gene (McGrew et al., 1992). We show that Esp1p is essential for the separation of sister chromatids and for the dissociation of Scc1p from all regions of chromosomes. Our data imply that the APC mediates sister chromatid separation not by degrading cohesins but by liberating the "sister-separating" Esp1 protein from an inhibitory embrace by its guardian Pds1p. Esp1p is related to fission yeast Cut1p, which is required for chromosome segregation, and associates with a protein destroyed by the APC called Cut2p (Uzawa et al., 1990; Funabiki et al., 1993, 1996a, 1996b). These parallels suggest that sister separation may be triggered by a similar mechanism in all eukaryotic cells.

Results

The APC's Sole Role in Separating Sisters Is to Destroy Pds1p

Temperature-sensitive (ts) *apc* mutant cells arrest uniformly in metaphase when shifted to 37°C. However,

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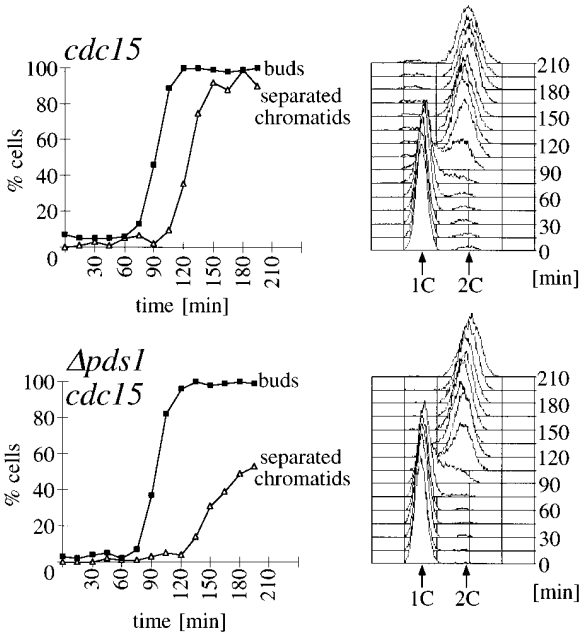


Figure 1. Pds1p Is Required for Efficient Separation of Sister Chromatids

Small G1 cells of *cdc15-2* (K7173) and *cdc15-2 pds1*Δ (K7191) strains, both having cenV marked by GFP (cenV-GFP), were inoculated into medium at 37°C. Left, the fraction of budded cells and cells with two separated GFP "dots" (i.e., separated sister chromatids at cenV). Right, cellular DNA content measured by flow cytometry (FACS). Comparing the kinetics of sister separation in different mutant strains is complicated by differences in their ability to undergo cytokinesis and rereplicate their genomes. It was to alleviate this problem that we employed a *cdc15* mutation (Culotti and Hartwell, 1971), which does not affect the kinetics of sister separation (data not shown) but prevents cytokinesis and DNA rereplication. It was not needed for analyzing *apc* mutants, which fail anyway to cytokinese and rereplicate.

deletion of *PDS1* allows between 33%–57% of ts *apc* mutant cells to undergo nuclear division at the restrictive temperature 3 hr after removal from hydroxyurea (HU) caused arrest (Yamamoto et al., 1996b). These data raise the possibility that destruction of Pds1p might be the APC's sole function in promoting sister chromatid separation. To test this, we compared the kinetics of sister chromatid separation in wild-type, *pds1*Δ, *apc*, and *pds1*Δ *apc* double mutants as unbudded G1 cells isolated by centrifugal elutriation were incubated at 37°C. We followed sister separation in these cultures by visualizing tetracycline repressor-GFP fusion proteins, bound to multiple tet operators integrated 30 kb from the centromere of chromosome V (Michaelis et al., 1997).

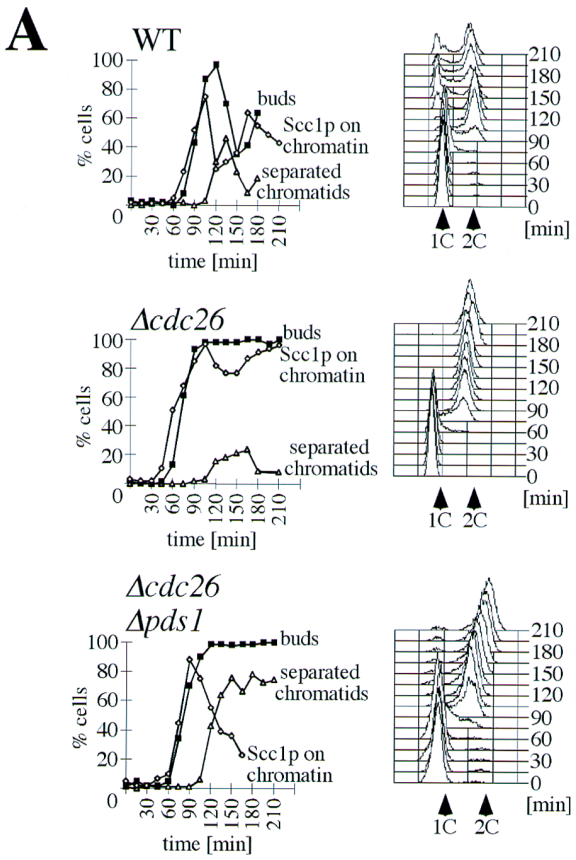
In *PDS1 cdc15-2* cells (i.e., wild type; see legend to Figure 1), sister separation occurs 30–35 min after cells have produced a bud. In *pds1*Δ *cdc15-2* cells, however, sister separation does not commence until 60 min after budding and is delayed for even longer in many cells (Figure 1). Mutations in genes encoding APC subunits (such as *CDC26* and *APC2*) have a much more severe effect. Little or no sister separation occurs in *apc2-1* or *cdc26*Δ mutants at 37°C (Zachariae et al., 1998). Remarkably, the kinetics of sister separation are very similar, if not identical, in *pds1*Δ *cdc15-2* and in *pds1*Δ *apc2-1* mutants (Figure 1; Zachariae et al., 1998). The implication is that deletion of *PDS1* causes the APC to become redundant for sister separation. Similar data were obtained with *pds1*Δ *cdc26*Δ double mutants (data not shown). Although *pds1*Δ *apc* double mutant cells separate sister chromatids, they fail to destroy the mitotic cyclin Clb2p, to disassemble their mitotic spindle, to undergo cytokinesis, and to rereplicate their genome (Yamamoto et al., 1996b; Zachariae et al., 1998).

Scc1p Dissociates from Chromosomes in *apc* Mutants Lacking Pds1p

To test whether APC-dependent destruction of Pds1p is also sufficient to trigger Scc1p's dissociation from chromosomes, we measured the association of Scc1myc18p with chromatin in "chromosome spreads" prepared from wild-type, *cdc26*Δ, and *pds1*Δ *cdc26*Δ double mutant cells as they progressed through the cell cycle at 37°C. Whereas Scc1p did not dissociate from chromatin in *cdc26*Δ single mutants, it dissociated from chromatin in *pds1*Δ *cdc26*Δ double mutants with kinetics that were similar to wild type (Figure 2A). It is possible to visualize simultaneously the GFP signal marking chromosome V (30 kb away from its centromere) and the presence of Scc1myc18p marked by Cy3. In both wild-type and *pds1*Δ *cdc26*Δ double mutant cells, Scc1p vanishes from chromosomes by the time that sister centromeres disjoin, whereas it never dissociates in *cdc26*Δ mutants that fail to separate sisters (Figure 2B). These data suggest that the APC's sole task in promoting dissociation of Scc1p from chromosomes during anaphase is to mediate destruction of Pds1p.

Pds1p Forms a Stable Complex with a 180 kDa Protein Encoded by the *ESP1* Gene

We failed to detect an association between the bulk of Pds1 protein and chromatin using the same chromosome spreading technique used to demonstrate Scc1p's association. The vast majority of Pds1p is washed away to an extent similar to nonchromosomal nuclear proteins like the exportin Cse1p (Tanaka et al., 1997). To investigate how Pds1p might block sister chromatid separation without binding to chromatin, we analyzed whether Pds1p associates with other proteins. Protein extracts were prepared from *PDS1myc18* cells grown in the presence of ^{35}S methionine and subjected to immunoprecipitation with an antibody to the myc-epitope (Figure 3A). A protein of 180 kDa was detected in immunoprecipitates from *PDS1myc18* strains but not from strains expressing non-tagged Pds1p or other myc-tagged proteins such as Cse1p or the APC subunit Apc2p. The Pds1p-p180 complex was purified by a large-scale immunoprecipitation from unfractionated whole-cell extracts, which yielded sufficient material to detect p180 on gels stained with silver. After in-gel digestion with trypsin, the recovered peptide mixture was analyzed by nano-electrospray tandem mass spectrometry (Mann and Wilm, 1995; Wilm and Mann, 1996). Sequences derived from four different peptides unambigously identified p180 as the product of the *ESP1* gene (Figures 3B and 3C). We estimate that the molar ratio of Pds1myc18p to Esp1p



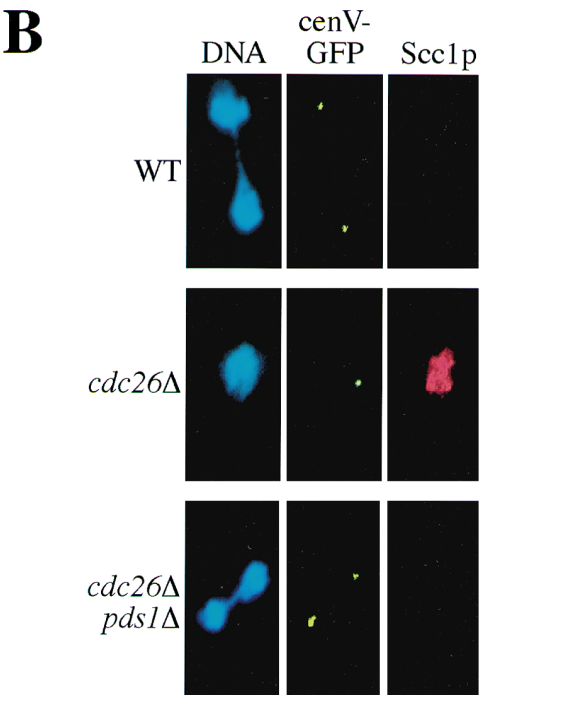


Figure 2. Deletion of the *PDS1* Gene Allows Sister Chromatid Separation and Dissociation of Scc1p from the Chromatin in the Absence of APC Function

(A) Dissociation of Scc1p from the chromatin in *cdc26*Δ *pds1*Δ cells. Small G1 cells of wild-type (K7056), *cdc26*Δ (K7042), and *cdc26*Δ *pds1*Δ (K7026) strains containing *SCC1myc18* and cenV-GFP were incubated at 37°C. Scc1myc18p associated with chromatin was detected by indirect immunofluorescence on chromosome spreads. (B) Chromosome spreads of cells taken at 135 min. DNA stained with DAPI. The centromeric region of chromosome V visualized by GFP (cenV-GFP). Scc1myc18p associated with chromatin detected by indirect immunofluorescence after chromosome spreading. Note that the delay in sister separation caused by loss of Pds1p is partly alleviated by the myc tag on Scc1p, which possibly reduces Scc1p's ability to maintain sister chromatid cohesion.

was 1 to 0.4 in immunoprecipitates prepared from ^{35}S-labeled cells, which suggests that a sizeable fraction of soluble Pds1p is bound to Esp1p. We confirmed the association between Esp1p and Pds1p using a strain expressing Esp1myc18p and Pds1HA6p. Pds1HA6p was detected by Western blotting in anti-myc immunoprecipitates prepared using myc-specific antibody, both from wild-type and *cdc26*Δ mutant cells arrested at 37°C (Figure 3D).

Esp1p Is Required for Sister Chromatid Separation

esp1 mutants were first identified because they accumulate extra spindle pole bodies (Baum et al., 1988). When G1 cells of an *esp1-1* strain are incubated at the restrictive temperature, they duplicate DNA and form mitotic spindles; they then fail to segregate chromosomes but nevertheless proceed with cyclin destruction, cytokinesis, and genome reduplication (McGrew et al., 1992; Surana et al., 1993). To investigate whether Esp1p is needed for sister separation, we compared the kinetics of sister separation in *esp1-1 cdc15-2* double mutants with *cdc15-2* single mutants (i.e., "wild type"), as unbudded G1 cells isolated by elutriation were incubated at 37°C. This showed that *esp1-1* mutant cells separate centromere proximal sequences very inefficiently (Figures 1 and 4A). *esp1-1* mutant cells were equally defective in separating sequences 30 kb from the right hand telomere of chromosome V (data not shown). *esp1-1* mutants' failure to separate sisters is clearly more severe than that of *pds1*Δ mutants. This phenotype was confirmed using time-lapse video microscopy of live cells, which showed that paired sister chromatids in *esp1-1* mutants were pulled from one to the other side of the nucleus at the time when disjunction should have occured (data not shown). The lack of sister separation in *esp1-1* mutant cells is not due to activation of the Mad/Bub mitotic spindle surveillance mechanism (checkpoint) because neither *mad2*Δ *esp1-1* nor *bub2*Δ *esp1-1* double mutants separated sisters any more efficiently than *esp1-1* single mutants (data not shown).

Pds1p Destruction Occurs Normally in *esp1* Mutants

One explanation for the lack of sister separation in *esp1-1* mutants is that they fail to destroy Pds1p. We therefore used indirect immunofluorescence to compare the kinetics of disappearance of a myc-tagged Pds1 protein as wild-type and *esp1-1* G1 cells isolated by centrifugal elutriation are incubated at 37°C. Pds1p disappeared with similar kinetics in wild-type and *esp1-1* mutant cells (Figure 4B). The mutant cells transiently accumulate with unseparated sister chromatids

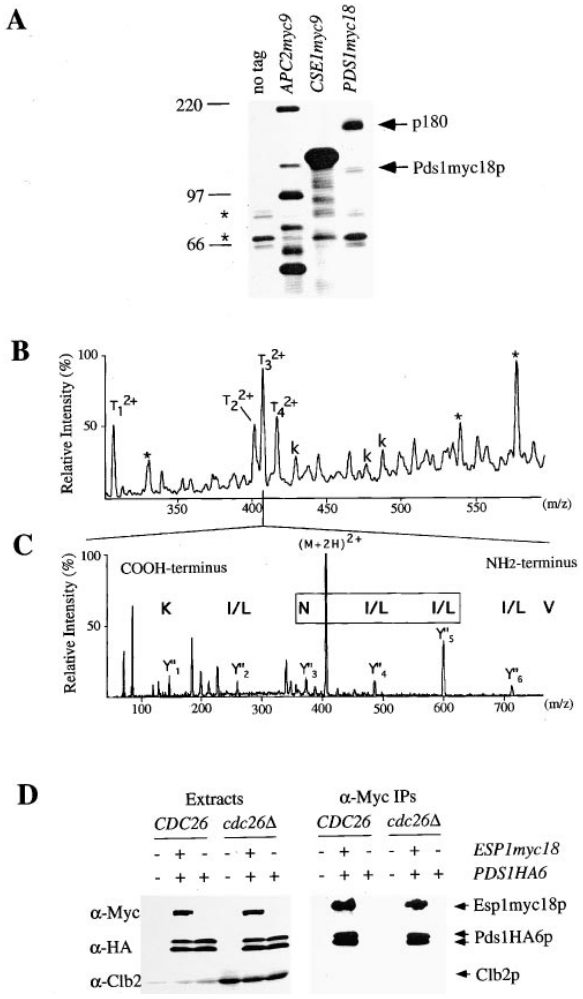


Figure 3. Pds1p Forms a Complex with Esp1p

(A) Coimmunoprecipitation of a 180 kDa protein with Pds1p. Control cells (no tag) and cells containing myc-tagged versions of *APC2*, *CSE1*, and *PDS1* were grown in medium containing ^{35}S methionine and cysteine. Protein extracts were immunoprecipitated with an antibody specific for the myc epitope. Bound proteins were separated on SDS-polyacrylamide gels and detected by fluorography. Proteins whose precipitation does not depend on the myc epitope are marked by asterisks. To identify p180 by nano-electrospay tandem mass spectrometric sequencing, immunoprecipitates prepared from unlabeled *PDS1myc18 Δpep4* cells were separated on a SDS-polyacrylamide gel followed by silver staining.

(B) Part of the mass spectrum of the peptide mixture extracted after in-gel digestion of the 180 kDa band with trypsin. The spectrum was acquired in parent ion scan mode, which detects peptide ions present in very low amounts by scanning for a fragment ion specific for peptides but not for the background (Wilm et al., 1996a). Ions are detected that yield, upon collisional fragmentation, daughter ions with a mass to charge ratio (m/z) of 86, the immonium ions of leucine and isoleucine. Tandem mass spectra were aquired upon fragmentation of selected peaks. Peaks were identified as trypsin autolysis products (*), peptides from human keratins (k, common impurities observed at low protein levels), and peptides originating from Esp1p (T).

(C) Tandem mass spectrum of the doubly charged ion T_3^{2+} at m/z 407.0. Collisional fragmentation of tryptic peptides produces mainly ions containing the COOH-terminal part of the peptide (Y" ions). A peptide sequence tag (Mann and Wilm, 1994) was assembled from the mass differences between adjacent Y" ions (boxed) and used for database searches. A single match, the tryptic Esp1p peptide VILLNIK, was retrieved and confirmed by the complete Y" ion series. Three other Esp1 peptides were identified: T_1, LPLIR; T_4, ELLESLK (in a mixture with the trypsin autolysis product SISISVAR which has the same nominal mass); and T_2, SLTDLPR. Peptide T_2 was identified by an error tolerant search (Mann and Wilm, 1994) because it is not preceeded by a trypsin cleavage site (K or R) in the Esp1p sequence.

(D) Coimmunoprecipitation of Pds1p with Esp1p. Wild-type and *cdc26Δ* strains containing *PDS1HA6* and *ESP1myc18* were grown at 25°C and then shifted to 36°C for 3 hr. Protein extracts were subjected to immunoprecipitations with an antibody to the myc epitope. Protein extracts and immunoprecipitates were analyzed by immunoblotting with anti-myc, anti-HA, and anti-Clb2p antibodies. +, epitope-tagged allele integrated at the genomic *PDS1* or *ESP1* locus; −, wild-type allele.

within nuclei that lack Pds1p. Subsequently, they proceed with the inactivation of cyclin B/Cdk1 kinases and reembark on a new cell cycle, during which Pds1p reaccumulates. *esp1-1* mutants are therefore not defective in the APC-mediated proteolysis of either Clb2p (Surana et al., 1993) or Pds1p.

esp1-1 pds1Δ Double Mutants Are Inviable

apc mutants fail to separate sister chromatids because they do not degrade Pds1p, whereas *esp1-1* mutants fail to separate sisters despite having destroyed Pds1 protein. This suggests that the lack of sister chromatid separation in *esp1-1* mutants should not be suppressed by deletion of *PDS1*. While trying to test this, we found that *pds1Δ esp1-1* double mutants were inviable, even when spores were germinated at 19°C (data not shown). This suggests that deletion of *PDS1* exacerbates the *esp1-1* defect.

Sister Chromatid Cohesion in *esp1-1* Mutants Depends on Scc1p

To test whether the persistence of sister chromatid cohesion in *esp1-1* mutants depends on Scc1p, we compared the kinetics of sister separation in *esp1-1* single mutants with that in *esp1-1 scc1-73* double mutants. Loss of Scc1 function permitted efficient separation of sister chromatids in *esp1-1* mutants (Figure 5A). To investigate whether Scc1p dissociates from chromosomes in *esp1-1* mutants, we repeated the experiment with an Scc1myc18p strain and followed the association between Scc1myc18p and chromatin using chromosome spreads as unbudded G1 *esp1-1* mutant cells progressed through the cell cycle at 37°C. Scc1p associated with chromatin in late G1 but failed to dissociate following destruction of Pds1p (Figure 5B). Cells accumulated with DNA masses containing a single GFP dot (i.e., paired sisters), and chromatin stained strongly with Cy3 (the marker for Scc1myc18p) (Figure 5C). Similar results were obtained using a version of Scc1p tagged with six HA epitopes (data not shown). We conclude that Esp1p is required for both sister separation and dissociation of Scc1p from chromatin.

Esp1p Localizes to Spindles and Their Poles at the Onset of Anaphase

We modified the endogenous *ESP1* gene to encode a functional fusion protein carrying 18 myc epitopes at its C terminus. Esp1myc18p was distributed throughout

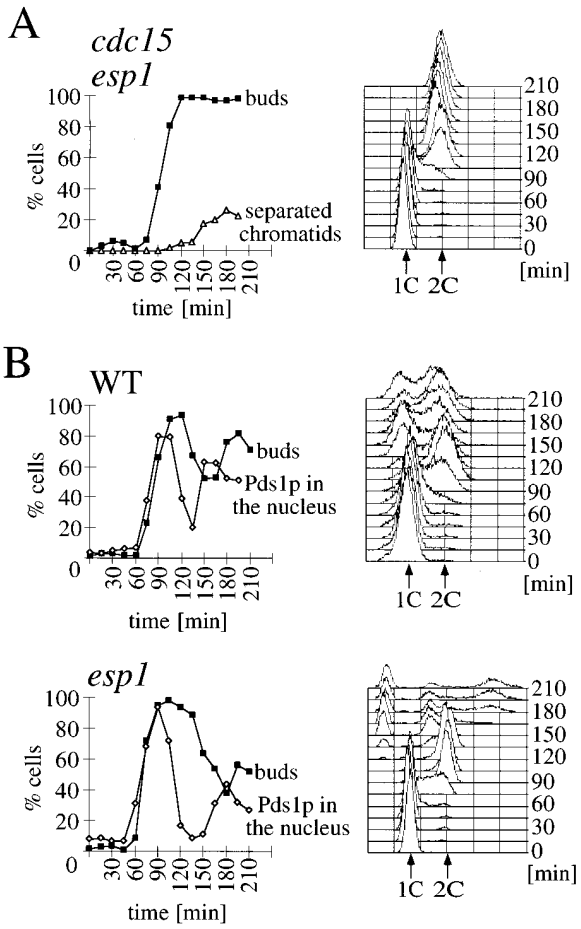


Figure 4. *ESP1* Is Required for Sister Chromatid Separation but Not for Degradation of Pds1p

(A) Lack of sister chromatid separation in *esp1-1* mutant cells. Small G1 cells of an *esp1-1 cdc15-2* strain containing cenV-GFP (K7183) were incubated at 37°C. The control experiment with an *ESP1 cdc15-2* strain is shown in Figure 1.

(B) Degradation of Pds1p does not depend on Esp1 function. Small G1 cells of wild-type (K6803) and *esp1-1* (K6999) strains containing *PDS1myc18* and cenV-GFP were incubated at 37°C. Pds1myc18 protein was detected by indirect immunofluorescence. Only cells completely lacking nuclear Pds1myc18p staining were scored as negative.

the cell during G1, S, G2, and early M phases but was concentrated within dividing nuclei (data not shown). Esp1myc18p's abundance did not vary much during the cell cycle (data not shown). Most nuclear spreads lacked any Esp1myc18p-specific staining, but some contained two large Esp1p-specific dots at opposite ends of the DNA mass. Such nuclei frequently also contained smaller Esp1p-specific dots situated between these two major foci. We never detected any association between Esp1p and bulk chromatin. Most if not all cells with Esp1p dots had two green GFP dots (data not shown), implying that sister kinetochores had separated. The Esp1p-specific dots were seen in *cdc5-1* mutants, which separate sister chromatids but fail to complete anaphase B (Shirayama et al., 1998), but not in *cdc26*Δ mutants or in cells arrested with nocodazole (data not shown). The Esp1p-specific dots at opposite ends of the DNA mass colocalized with tubulin at spindle poles, whereas the Esp1p dots situated midway between these poles were always closely associated with tubulin of the mitotic spindle, at least in those spreads where the latter was visible (Figure 6A). These data suggest that Esp1p associates with mitotic spindles and their poles at the onset of anaphase. Surprisingly, the Esp1p dots associated with spindles, and their poles could not be detected in *pds1*Δ mutants at any temperature, which was not due to lower amounts of Esp1p (data not shown).

Esp1p Promotes Loss of Sister Chromatid Cohesion in the Absence of Mitotic Spindles

The phenotype of *esp1-1* mutants is at least superficially similar to that of *ndc10-1* mutants, which are known to be defective in a centromere binding complex (Doheny et al., 1993). *ndc10-1* mutants also cytokinese and rereplicate their genomes without segregating chromosomes (Goh and Kilmartin, 1993). Furthermore, Esp1p's association with spindles and their poles during anaphase raises the possibility that it might promote sister separation by modulating mitotic spindle activity. To address whether sister separation induced by Esp1p depended on functional kinetochores, we isolated by centrifugal elutriation G1 cells of *ndc10-1* mutants and incubated them at 37°C. In contrast to *esp1-1* mutant cells, sister chromatid separation occcured efficiently in *ndc10-1* mutants, though it was slightly delayed, due possibly to the lack of kinetochore activity (Figure 6B). Sister separation in *ndc10-1* mutant cells even occured in the presence of nocodazole (data not shown) as occurs in *mad* or *bub* mutants (Straight et al., 1996), suggesting that *ndc10-1* mutants are defective in the surveillance mechanism that detects spindle damage and blocks anaphase onset (Murray, 1995). Crucially, loss of cohesion in *ndc10-1* mutants was dependent on Esp1p, because sister chromatids failed to separate in *esp1-1 ndc10-1* double mutants (Figure 6B). Esp1p clearly promotes loss of sister chromatid cohesion by a mechanism that requires neither functional kinetochores nor mitotic spindles.

Overexpression of Esp1p Permits Sister Separation in the Presence of Pds1p

The strict dependence of sister separation on both Esp1p activity and Pds1p destruction suggests that Esp1p might be a sister-separating protein whose activity is blocked by its association with Pds1p. Do cells contain sufficient Pds1p for such a role? Comparisons of the abundance of Pds1myc18p and Esp1myc18p by Western blotting in both cycling cells and *cdc26*Δ mutants arrested at 37°C (data not shown) suggest that, from late G1 till its destruction shortly before anaphase, Pds1p (which is a nuclear protein) is as abundant as Esp1p (which is present both in the nucleus and cytoplasm). These data suggest that there is an excess of Pds1p over Esp1p within nuclei.

To test whether overproduction of Esp1p could titrate out Pds1p and permit cells to separate sisters without Pds1p destruction, we elevated the Esp1p level by integrating three copies of the *GAL1-10* promoter-*ESP1* construct (*GAL-ESP1*) into ts *cdc20-3* mutant cells.

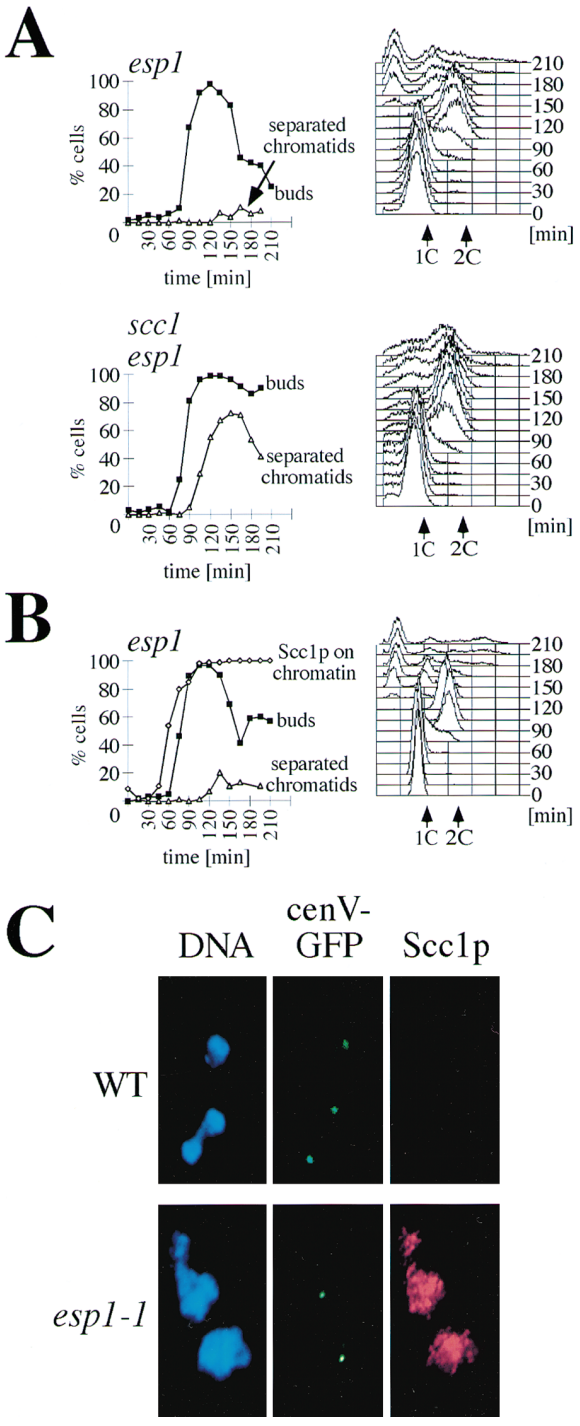


Figure 5. Scc1p Is Required to Maintain Sister Chromatid Cohesion in *esp1-1* Mutants

(A) Sister chromatids separate in *esp1-1 scc1-73* double mutants. Small G1 cells of *esp1-1* (K6842) and *esp1-1 scc1-73* (K6859) strains containing cenV-GFP were incubated at 37°C. Control experiments showing sister separation in wild-type and *scc1-73* strains have already been published (Michaelis et al., 1997).

(B) Esp1p is required for the dissociation of Scc1p from chromatin. Small G1 cells of an *esp1-1 SCC1myc18* cenV-GFP strain (K7030) were incubated at 37°C. Scc1myc18p associated with chromatin was detected by indirect immunofluorescence on chromosome spreads.

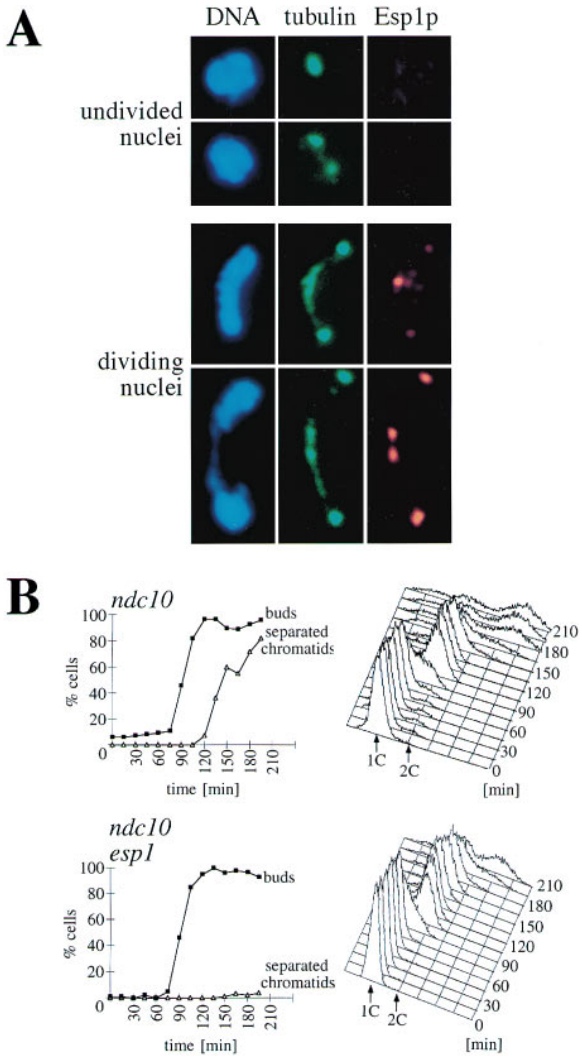


Figure 6. Esp1p Associates with Spindles and Their Poles during Anaphase but Promotes Sister Separation in the Absence of Functional Kinetochores

(A) Chromosome spreads of the *ESP1myc18* cells (K7025) at different stages of the cell cycle. DNA was stained with DAPI. Spindle pole bodies, spindle structures, and Esp1myc18p were detected by indirect immunofluorescence. Two top images, nuclei before and after SPB duplication. Two bottom images, anaphase nuclei with detectable spindles spanning the spindle poles.

(B) Small G1 cells of *ndc10-1* (K6841) and *esp1-1 ndc10-1* (K6884) strains containing cenV-GFP were incubated at 37°C. Cytokinesis takes place in neither strain, so there was no need to use a *cdc15* mutation.

These mutants are defective in the ubiquitination of Pds1p by the APC and separate sister chromatids only if *PDS1* is deleted (Shirayama et al., 1998). Unbudded G1 cells were isolated by elutriation from a culture grown in raffinose and incubated at 37°C in the presence of

(C) Chromosome spreads of cells taken at 135 min. DNA stained with DAPI. The centromeric region of chromosome V visualized by GFP (cenV-GFP). Scc1myc18p associated with chromatin detected by indirect immunofluorescence.

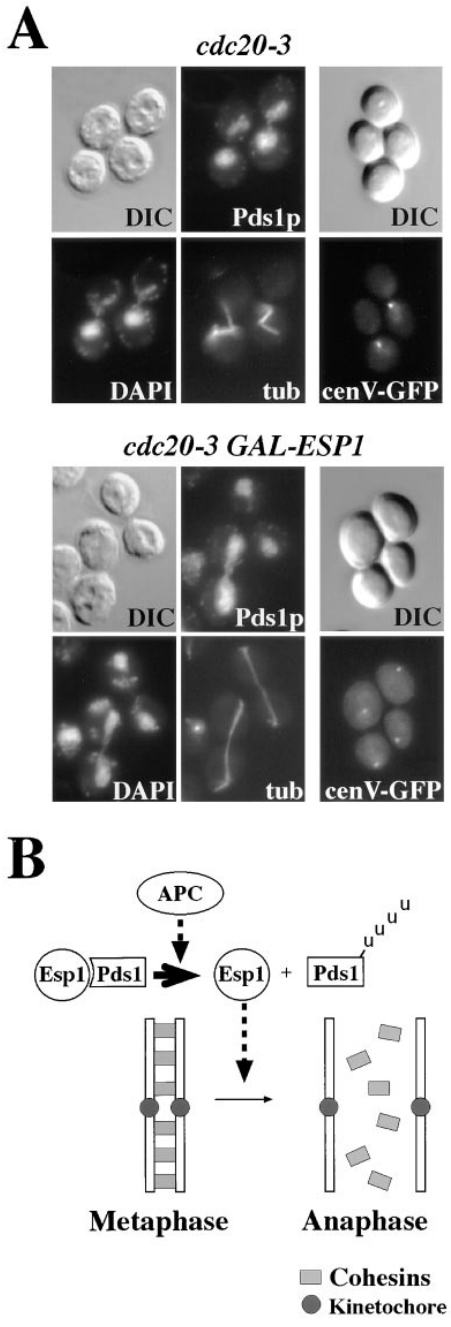


Figure 7. Elevating Esp1p Levels Triggers Anaphase in the Presence of Pds1p as Predicted by the Model

(A) Small G1 cells isolated by elutriation of *cdc20-3* (K7108) or *cdc20-3 GAL-ESP1x3* (K7445) strains containing *PDS1myc18* and cenV-GFP were incubated at 37°C in the presence of galactose. Timing of budding and DNA replication were similar in both strains. All pictures show cells at 165 min. Panels on the left show cells fixed with formaldehyde and processed for indirect immunofluorescence. Panels on the right show cells fixed with ethanol and processed for GFP fluorescence. Pds1myc18p and spindles were detected with antibodies to the myc epitope and tubulin (tub), respectively. Sequences close to cenV were visualized with GFP (cenV-GFP). In wild-type cells sister separation is always preceeded by Pds1p destruction (Michaelis et al., 1997). Overexpression of Esp1p allowed at least 20% of *cdc20-3* mutant cells to completely segregate their genomes in the presence of Pds1p.

(B) From the end of S phase until the onset of anaphase, sister chromatids are held together by cohesins like Scc1p. During this time, Esp1p is inhibited by its association with Pds1p. Proteolysis of Pds1p, which is mediated by the APC, allows Esp1p to trigger sister separation, possibly by causing dissociation of Scc1p from chromosomes.

galactose. *cdc20-3 ESP1* cells did not segregate their genomes, whereas at least 20% of the *cdc20-3 GAL-ESP1* cells did so within 3 hr. Crucially, the nuclei of *GAL-ESP1* cells that separated sister chromatids in the absence of Cdc20 activity contained high levels of Pds1p (Figure 7A). These cells also contained a fully elongated mitotic spindle stretching between segregated chromosomes (Figure 7A). The presence of a single GFP dot associated with cen V (cenV-GFP) at each end of these "anaphase" cells (Figure 7A) shows that *GAL-ESP1* caused proper disjunction of sister chromatids in the presence of Pds1p. *GAL-ESP1* also permitted sister separation in cells whose Pds1p proteolysis was prevented (at 37°C) by a deletion of *CDC26* (data not shown). We conclude that the dependence of sister separation on APC activity can be bypassed not only by deleting *PDS1* but also by elevating Esp1p levels.

Discussion

The APC is a multisubunit ubiquitin protein ligase necessary for mitotic cyclin proteolysis. The APC is also necessary for sister chromatid separation, even though cyclins themselves need not be destroyed. Sister chromatid separation is therefore thought to depend on ubiquitination by the APC of proteins other than cyclins. One suggestion has been that cohesins holding sister chromatids together might be the key APC targets. Scc1p in yeast is indeed destroyed in an APC-dependent manner, but the timing of this event suggests that it might be a consequence rather than a cause of its dissociation from chromosomes. Pds1p is another candidate; it is degraded shortly before the onset of anaphase and nondegradable versions block sister separation (Cohen-Fix et al., 1996). We have now extended these findings by comparing the kinetics of sister separation in *pds1*Δ, *apc*, and *pds1*Δ *apc* double mutants. Our finding that inactivation of the APC fails in any way to retard sister separation in mutants lacking Pds1p suggests that the APC might promote sister separation by mediating destruction of Pds1p and no other protein. How then does Pds1p block sister separation? In *apc* mutants, which fail to destroy Pds1p, the cohesin Scc1p remains associated with sister chromatids. We show here that deletion of *PDS1* permits its dissociation with almost wild-type kinetics. The implication is that in *apc* mutants, the persistence of Pds1p alone blocks the dissociation of Scc1p from sister chromatids. One possibility is that Pds1p interacts directly with cohesins and prevents their dissociation from chromosomes. If so, one might expect to find Pds1p bound tightly to chromosomes before the onset of anaphase. We have been unable, however, to demonstrate any association between Pds1p and chromosomes using the same spreading technique used successfully for cohesins. We found

instead that Pds1p is tightly associated with a 180 kDa protein, which was identified by mass spectrometric sequencing as the product of the *ESP1* gene.

We show here that *ESP1*, like the APC, is essential for sister separation and for dissocation of Scc1p from chromosomes. The resemblance between the phenotypes of *esp1* and *apc* mutants ends here. In *apc* mutants Scc1p remains on chromosomes because Pds1p is not destroyed, whereas in *esp1* mutants Scc1p remains on chromosomes despite Pds1p proteolysis occuring normally. Indeed, all cell cycle events except sister separation continue unabated in *esp1-1* mutants, which even undergo cytokinesis and rereplicate their genomes (McGrew et al., 1992). This phenotype contrasts with the metaphase arrest of *apc* mutants and the telophase arrest of *apc pds1*Δ double mutants. These observations suggest that Esp1p has a very specific role in promoting the loss of sister chromatid cohesion and that it is not directly required for any other anaphase process. Because Pds1p inhibits sister separation whereas Esp1p promotes it, we suggest that Pds1p's association with Esp1p inhibits Esp1p's ability to trigger sister chromatid separation (Figure 7B). According to this hypothesis, the APC promotes sister separation only indirectly by allowing the release of Esp1p, a specialized sister-separating protein, from its guardian Pds1p. As predicted by our hypothesis, overproduction of Esp1p permits sister separation in *cdc20* or *cdc26* mutant cells that cannot destroy Pds1p.

How might Esp1p promote loss of sister chromatid cohesion upon its release from Pds1p? We know that Esp1p must cause a global change to chromosomes, because it is needed for the dissociation of Scc1p from a large number of loci throughout the genome. We also know that Esp1p causes loss of cohesion even in the absence of any connection between centromeres and microtubules, because sister separation occurs in an Esp1p-dependent manner in *ndc10-1* mutants, even in the presence of nocodazole. The splitting of acentric chromosome fragments obtained by laser microsurgery at the same time as intact chromosomes is also consistent with such a notion (Liang et al., 1993).

We find Esp1p distributed throughout the cell from late G1 until metaphase. It is neither concentrated in nor excluded from the nucleus. Pds1p in contrast is concentrated within the nucleus during this period (Cohen-Fix et al., 1996). At the metaphase to anaphase transition, Esp1p concentrates within nuclei, and a fraction of it associates with spindles and their poles. The latter depends on the APC and presumably occurs as a consequence of Pds1p proteolysis. More surprisingly, it also depends on the prior presence of Pds1p. We saw little or no association of Esp1p with spindles and their poles in *pds1*Δ mutants, even when they separate sisters on time when grown at their permissive temperature, which casts doubt as to whether Esp1p's assocation with spindles and their poles is relevant to its role in promoting sister chromatid separation.

Only a fraction of Esp1 molecules associate with spindles and their poles during anaphase, and the free Esp1 molecules that are not associated with spindles might be those actually responsible for loss of sister cohesion. Esp1p might, for instance, interact transiently with cohesins and facilitate their dissocation from chromosomes. Alternatively, it might destroy cohesion by an indirect mechanism, by generating a global change within nuclei that is more directly responsible for weakening sister chromatid cohesion. A candidate would be the concentration of Ca^{2+}, which appears to change at the metaphase to anaphase transition (Groigno and Whitaker, 1998).

If loss of sister chromatid cohesion were solely regulated by APC-mediated removal of Pds1p from Esp1p/Pds1p complexes, then one would expect sister separation to occur prematurely in *pds1*Δ mutants, which could be a lethal event. However, this is not the case. In cells growing at low temperatures (e.g., 25°C), *PDS1* is neither necessary for proliferation (Yamamoto et al., 1996a) nor for preventing premature separation of sister chromatids (G. Alexandru, personal communication). The implication is that the putative inactivation of Esp1p by the binding of Pds1p cannot be the only mechanism for regulating sister chromatid separation. In addition to its control by an unstable inhibitor, Esp1p may be subject to other controls, such as phosphorylation by mitotic kinases, for example Cdks, which ensure that Esp1p is only active once cells have aligned sister chromatids on the mitotic spindle. The separation of sister chromatids is one of the most important transitions in the cell cycle, and it comes as no surprise that this event is regulated by more than one mechanism. Destruction of Pds1p might merely "unlock the door," which must nevertheless still be "opened."

The lethality of *pds1*Δ mutants at 37°C is not due to premature separation of their sister chromatids (Figure 1). Instead, there are four reasons for believing that *pds1*Δ lethality might be due to a reduction in Esp1p activity. First, Esp1p's association with spindles and their poles is greatly reduced by a deletion of *PDS1*. Second, *pds1*Δ *esp1-1* double mutants are inviable. Third, the lethality of *pds1*Δ mutants at high temperatures is suppressed by multicopy plasmids containing the *ESP1* gene or by *GAL-ESP1* (data not shown; B. Byers, personal communication). Fourth, *pds1*Δ mutants separate sister chromatids inefficiently at 37°C. This suggests that Esp1p's guardian, Pds1p, regulates Esp1p's activity both negatively and positively. For example, Esp1p might only be fully active when released (by the APC) from its complex with Pds1p.

In addition to controls that operate in cycling cells, sister separation is blocked by surveillance mechanisms (checkpoints) that detect damage to DNA or to spindles. A key question is whether such checkpoints block sister separation solely by blocking the proteolysis of Pds1p or whether they also act on components such as Esp1p. *pds1*Δ mutants are at least partially defective in blocking sister chromatid separation in the presence of damaged DNA or microtubules, suggesting that at least some surveillance mechanisms work by regulating APC-mediated proteolysis (Yamamoto et al., 1996b).

Esp1p is homologous to Cut1p from *Schizosaccharomyces pombe* (Uzawa et al., 1990) and to BimB in *Aspergillus nidulans* (May et al., 1992), both of which are also required for nuclear division but not for reentry into the next cell cycle. The homology between these proteins is largely confined to their C-terminal 300 amino acids,

which are similarly conserved in potential homologs from humans and *Caenorhabditis elegans*. *cut1* mutants manage to separate chromatids at centromeric regions but do not segregate sister chromatids fully from each other (Funabiki et al., 1993). We suggest that these *cut1* alleles are "leaky" and that Cut1p is not merely needed for the proper segregation of chromatids but is required like Esp1p to initiate sister chromatid separation. Thus, the Esp1/Cut1 class of sister-separating proteins might be called "separins."

Cut1p binds to Cut2p, a protein with Pds1-like properties, and is associated with mitotic spindles (Uzawa et al., 1990; Funabiki et al., 1993, 1996a). Though Cut1p and Esp1p are clearly related proteins with similar functions, there is little obvious sequence similarity between Pds1p and Cut2p. Furthermore, *cut2*$^+$, unlike *PDS1*, is an essential gene and is necessary for chromosome segregation (Funabiki et al., 1996a). Despite these differences, we suspect that Cut1p and Cut2p in *S. pombe* perform similar functions to Esp1p and Pds1p in *S. cerevisiae*. We suggest that during S, G2, and early M phase, Esp1p's "guardian" Pds1p not only inhibits Esp1p activity but also prepares it for activation by as yet unkown cell cycle signals. This "helpful" role is important but not essential for *S. cerevisiae*, except at 37°C. We propose that Cut2p performs both roles for Cut1p, with the difference that Cut1p's prior association with Cut2p is not merely helpful but actually essential for Cut1p function. The lack of any striking homology between Pds1p and Cut2p may stem from the fact that Pds1p and Cut2p are primarily inhibitors of Esp1/Cut1-like proteins and do not themselves have a conserved role in the enzymology of loosening cohesion between sisters. Inhibitors of S phase Cdks, whose proteolysis has an important role in promoting S phase, are likewise not highly conserved. If we are correct in thinking that Cut1/Cut2 complexes are mechanistically equivalent to Esp1/Pds1 complexes, then the existence of animal homologs of Esp1- and Cut1-like proteins make it seem likely that similar complexes will also regulate the onset of anaphase in humans.

Experimental Procedures

Yeast Strains and Growth Conditions

All strains were derivatives of W303. YEP medium (Rose et al., 1990) was supplemented with either 2% raffinose (YEPraf) or 2% glucose (YEPD). To obtain synchronous cultures, cells were grown in YEPraf medium at 25°C (or at 19°C in case of *pds1*Δ strains), and small, unbudded G1 cells were isolated by centrifugal elutriation (Schwob and Nasmyth, 1993). These cells were then inoculated into YEPD medium to a density of 6×10^6 cells per ml, usually at 37°C. Chromosomes were visualized by a tet repressor-GFP fusion protein (tetR-GFP) binding to an array of tet operators (tetOs) integrated at either the *ura3* locus near the centromere of chromosome V (Michaelis et al., 1997, referred to as cenV-GFP) or at the *BMH1* locus 30 kbp from the right end of chromosome V (telV-GFP).

Strain Constructions

To tag the *ESP1* gene, a YIplac204 (Gietz and Sugino, 1988) based tagging plasmid was constructed, which contained a fragment spanning the C-terminal part of *ESP1* and a part of its 3′UTR, with an SpeI site created in front of the stop codon. Two SpeI cassettes containing 9 myc epitopes each (Michaelis et al., 1997) were cloned into the SpeI site. The resulting plasmid was linearized with ClaI and integrated into the yeast genome at the endogenous *ESP1* locus. The *ESP1myc18* strain (K7025) grew normally at 37°C, demonstrating that the epitope-tagged version is functional. To overproduce the *ESP1* gene, a YIplac204-based plasmid was constructed that contained *ESP1* coding sequence with 1 kb of its 3′UTR with a HindIII site created before the start codon, to which the *GAL1-10* promoter was attached. The resulting plasmid was linearized with BstXI and integrated into the yeast genome at the *trp1* locus. For further studies we chose a triple integrant of the *GAL-ESP1* construct. A *PDS1HA6::HISMX* allele was constructed by PCR-mediated epitope tagging of the genomic *PDS1* locus. *PDS1HA6* strains grew normally at 37°C.

Mass Spectrometric Identification of Esp1p

Immunoprecipitations from extracts of metabolically labeled cells were carried out as described (Zachariae et al., 1996b). For mass spectrometric analysis, the Pds1myc18p-p180 complex was purified by one-step immunoprecipitation from an unfractionated whole-cell extract prepared from *PDS1myc18* Δ*pep4* cells (1.0×10^{10}) using the procedure of Zachariae et al. (1998). The 180 kDa band was excised from a single one-dimensional gel stained with silver and digested in-gel with trypsin (Shevchenko et al., 1996). The recovered unseparated peptide mixture was analyzed by tandem mass spectrometric sequencing as described (Wilm et al., 1996b) using an API III triple quadrupole mass spectrometer (Perkin Elmer-Sciex, Ontario, Canada) equipped with the nano-electrospray ion source (Wilm and Mann, 1996a). The gel actually used to identify Esp1p is shown in Figure 1A of Zachariae et al. (1998). For immunoprecipitation-immunoblotting experiments (Figure 3D), 1.5×10^6 cells were broken in 0.4 ml buffer B70. Extracts (0.33 ml, 6 mg) were incubated with 0.1 ml protein A-Sepharose and then with 0.033 ml protein A-Sepharose carrying the anti-myc antibody 9E10. Preparation of extracts and immunoprecipitations were carried out essentially as described previously (Zachariae et al., 1998). Extracts and immunoprecipitates were separated on 0.8 mm, 7.5% SDS-polyacrylamide gels and analyzed by immunoblotting.

Other Techniques

A FACScan (Becton-Dickinson) was used for flow cytometric analysis of cellular DNA content as described (Epstein and Cross, 1992). Chromosome spreading and visualisation of yeast chromosomes using the tetR-GFP/tetO system were performed as described previously (Michaelis et al., 1997). Cells were prepared for indirect immunofluorescence according to Piatti et al. (1996). Myc-tagged proteins were detected with the 9E10 monoclonal antibody and a CY3-conjugated secondary antibody. Spindles were detected with a rabbit antiserum to yeast tubulin and a fluorescein isothiocyanate-conjugated secondary antibody. Pictures were taken with a Quantix charge-coupled device camera (Photometrics) mounted on a Zeiss Axioplan 2 microscope.

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