

Secured cutting: controlling separase at the metaphase to anaphase transition

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The final irreversible step in the duplication and distribution of genomes to daughter cells takes place at the metaphase to anaphase transition. At this point aligned sister chromatid pairs split and separate. During metaphase, cohesion between sister chromatids is maintained by the chromosomal multi-subunit cohesin complex. Here, I review recent findings as to how anaphase is initiated by proteolytic cleavage of the Scc1 subunit of cohesin. Scc1 is cleaved by a site-specific protease that is conserved in all eukaryotes, and is now called 'separase'. As a result of this cleavage, the cohesin complex is destroyed, allowing the spindle to pull sister chromatids into opposite halves of the cell. Because of the final and irreversible nature of Scc1 cleavage, this reaction is tightly controlled. Several independent mechanisms seem to impose regulation on Scc1 cleavage, acting on both the activity of separase and the susceptibility of the substrate.

Introduction

The DNA that comprises eukaryotic genomes is packaged into chromosomes. These must be accurately replicated to produce exact copies during S phase, and then correctly distributed during mitosis. Errors in distribution lead to cells with supernumerary or missing chromosomes. The resulting aneuploidy is associated with many cancers, and is also a leading cause of human birth defects.

It is crucial that the products of DNA replication, the sister chromatids, remain linked by sister chromatid cohesion. This prevents them from drifting apart after their synthesis. Sister chromatids remain linked throughout G₂ to facilitate DNA repair by homologous recombination (reviewed in van Heemst and Heyting, 2000). Sister cohesion is then fundamental to the alignment of chromosomes on the metaphase spindle, as it counteracts the pulling of microtubules toward the spindle poles (reviewed in Nasmyth *et al.*, 2000).

At the start of anaphase, cohesion is abruptly abolished by a tightly regulated proteolytic cascade that activates a caspase-related protease, known as separase. Separase cleaves one of the subunits of the cohesin complex, the Scc1 subunit, which is required earlier, during metaphase, for holding the sister chromatids together. Now that the molecular trigger of chromosome separation has been identified, we can question how cohesion is established, and how it is subsequently terminated. This review focuses on the regulation of separase activity, and on how cells ensure that cohesin cleavage occurs at the right time and place, making possible the complete and accurate distribution of their chromosomes.

Cleaving apart chromosomes

Recent years have seen great advances in the understanding, at a molecular level, of how chromatid cohesion is achieved (reviewed in Hirano, 2000; Koshland and Guacci, 2000; Nasmyth *et al.*, 2000). Genetic and biochemical approaches together have made possible the identification of the chromosomal cohesin complex, which seems to be the central mediator of chromosome cohesion. The cohesin complex consists of the four subunits, Smc1, Smc3, Scc1 and Scc3 (Figure 1), which are required to maintain cohesion between sister chromatids, from their synthesis in S phase until the end of metaphase. However, the actual molecular nature of the bridges that connect sister chromatids, and the role that the cohesin complex plays in such bridges, is still unknown. The mechanism by which cohesion between sister chromatids is established during DNA replication is also unclear. It is likely to be a process that requires a number of specialized accessory proteins (Hirano, 2000; Koshland and Guacci, 2000; Nasmyth *et al.*, 2000; Wang *et al.*, 2000). It is known that cohesin can bind to naked DNA, likely through the Smc subunits (Akhmedov *et al.*, 1998; Losada *et al.*, 2000). Figure 1 depicts the simplest model for the function of cohesin, in which bivalent DNA binding ties the sister chromatids

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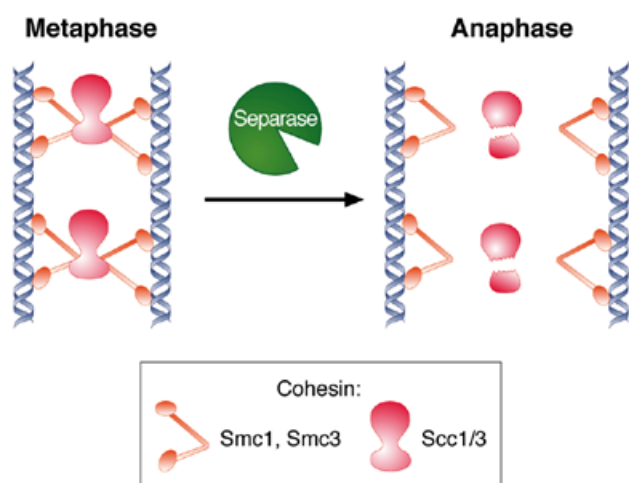


Fig. 1. A model for how bivalent DNA binding of the cohesin complex (consisting of Smc1, Smc3, Scc1 and Scc3) might hold sister chromatids together during metaphase. At anaphase, separase recognizes and cleaves two distinct sites in the Scc1 subunit. This destroys the interactions within the cohesin complex, leading to dissociation of the Scc1 and Scc3 subunits from chromosomes. Cohesion is lost, and the pulling force of the mitotic spindle segregates the sister chromatids towards opposite poles.

together. This model, although hypothetical, could explain how the proteolytic cleavage of the Scc1 subunit at the onset of anaphase dismantles the cohesin complex, and thereby frees the chromatids to move polewards (Uhlmann *et al.*, 1999).

Separase, the protease that cleaves cohesin

Separase, the protease responsible for cleaving cohesin at anaphase onset has now been identified (Uhlmann *et al.*, 2000). It is, in fact, a protein that had been implicated in the regulation of anaphase much earlier. Separase homologues probably exist in all eukaryotes, and mutations in the separases in *Schizosaccharomyces pombe* (Cut1), *Saccharomyces cerevisiae* (Esp1) and *Aspergillus nidulans* (BimB) have been characterized (Uzawa *et al.*, 1990; May *et al.*, 1992; McGrew *et al.*, 1992). All prevent chromosome segregation at anaphase without halting the continuation of the cell cycle. This leads to cells with re-replicated chromosomes and excess spindle pole bodies, explaining the original phenotypic description of the *Extra Spindle Poles* mutant *esp1* (Baum *et al.*, 1988).

The primary defect in *esp1* mutant cells only became apparent following the discovery of cohesin. During anaphase, two of cohesin's subunits, Scc1 and Scc3, suddenly disappear from the chromosomes of wild-type cells (Figure 1) (Michaelis *et al.*, 1997; Toth *et al.*, 1999). In *esp1* mutant cells, however, these subunits fail to dissociate from chromosomes, and sister chromatids remain paired even after they should have separated (Ciosk *et al.*, 1998). This observation led to the hypothesis that separases are cohesin removal factors. Meanwhile, the separases in a number of other organisms had been identified by the genome sequencing projects. Separases are generally large proteins of close to 200 kDa, and only a C-terminal domain seems to be conserved amongst them. This conserved 'separase domain'

contains the signature motif for cysteine proteases of the CD-clan, a superfamily of proteases that also includes the caspases. Indeed, separases purified from both budding yeast and human cells possess proteolytic activity against Scc1. In addition, a specific Scc1-derived peptide inhibitor binds the active site of the yeast separase, thereby confirming its identity (Uhlmann *et al.*, 2000; Waizenegger *et al.*, 2000).

Triggering anaphase

Is cleavage of cohesin sufficient to trigger anaphase? If true, any protease capable of cleaving cohesin should be able to do so. This has been tested in budding yeast. One of the two separase cleavage sites in Scc1 was replaced by the specific recognition sequence of a plant virus protease. Cleavage of Scc1 in yeast metaphase by this protease, under conditions in which the separase was kept inactive, resulted in a strikingly efficient anaphase in terms of sister chromatid separation and segregation. This shows that yeast cells in metaphase are prepared for anaphase, just waiting for cohesin cleavage as the final trigger (Uhlmann *et al.*, 2000). However, the anaphase spindle produced after cohesin cleavage by the ectopic protease seemed faint and brittle, suggesting that separase might have an additional role in stabilizing the elongating spindle (see below).

In fission yeast, cleavage of the Scc1 homologue Rad21 also takes place at anaphase onset and is essential for chromatid segregation (Tomonaga *et al.*, 2000). In human cells the situation is more complex. Most cohesin actually dissociates from chromosomes as they compact during condensation in prophase, without any sign of cleavage (Losada *et al.*, 1998, 2000; Darwiche *et al.*, 1999; Sumara *et al.*, 2000). A fraction of cohesin, however, remains on human and *Drosophila* chromosomes during metaphase, and it is this fraction that seems to be cleaved by separase to initiate anaphase (Waizenegger *et al.*, 2000; Warren *et al.*, 2000).

Cohesin cleavage is essential not only during mitosis, but also meiosis. During meiosis, it is required for homologue segregation in the first division and probably also for sister centromere separation in the second (Buonomo *et al.*, 2000). Cohesin cleavage is therefore a universal mechanism for triggering anaphase. It is an irreversible step; once executed, chromosome segregation cannot be reversed. This suggests that cohesin cleavage must be tightly controlled, by mechanisms responding to cell cycle progression and also to surveillance mechanisms that might seek to halt cells from progressing into anaphase if, for example, unrepaired DNA damage persists in the genome.

Securins: cellular separase inhibitors

The best known regulators of separases are the securins. First discovered in both budding and fission yeast (Funabiki *et al.*, 1996b; Yamamoto *et al.*, 1996b), securins have since also been characterized in metazoans (Zou *et al.*, 1999; Leismann *et al.*, 2000; reviewed in Yanagida, 2000). They are functionally conserved proteins, although there is little conservation of their primary amino acid sequence. Securins bind to and inhibit separase for most of the cell cycle (Ciosk *et al.*, 1998; Uhlmann *et al.*, 1999), but are degraded at the onset of anaphase, thus releasing separase. Their degradation is triggered via ubiquitylation by the anaphase promoting complex/cyclosome (APC/C)

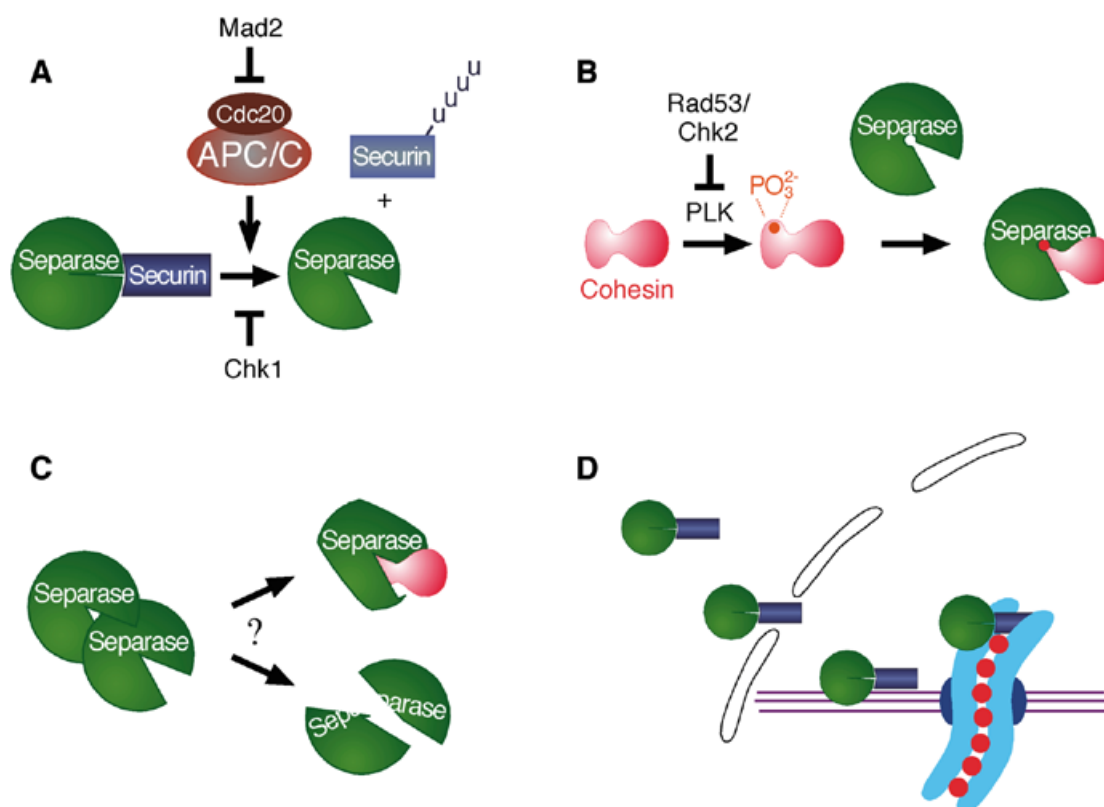


Fig. 2. Mechanisms of separase regulation at a variety of levels. **(A)** From late G₁ until metaphase, securin binds to and inhibits separase. At anaphase, securin is targeted for destruction via ubiquitylation by the APC/C. The APC/C is activated by the regulatory subunit Cdc20/Fizzy. As long as chromosomes are not properly aligned on the mitotic spindle, Mad2 prevents activation of the APC/C. After DNA damage, Chk1 kinase phosphorylates securin and prevents its destruction. **(B)** Phosphorylation of the Scc1 subunit of cohesin by polo-like kinase (PLK) is required for its efficient cleavage. Phosphorylation might be prevented by Rad53/Chk2 after DNA damage. **(C)** Human separase itself undergoes cleavage at anaphase. The consequences of this cleavage on separase activity have not yet been determined, but may include activation of the protein, or its inactivation after cohesin cleavage. **(D)** Localization of separase to the vicinity of its targets might be helped by securin.

(Figure 2A) (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996b). Although securins are potent separase inhibitors, in budding yeast, securin is not essential for cell cycle regulation of Scc1 cleavage, indicating that other control mechanisms exist (see below).

Securins are not simply inhibitors of separase. In fission yeast and *Drosophila*, the absence of securin does not lead to a prematurely active separase as one might predict, but rather paradoxically to an apparent lack of separase activity. This suggests a dual role for securins: the priming of separase activity via binding, and the unleashing of separase activity after securin is degraded by the APC/C (Funabiki *et al.*, 1996b; Stratmann and Lehner, 1996). Even in budding yeast, where securin is not essential, separase function is impaired in its absence. This results, at least at elevated temperature, in anaphase delay (Ciosk *et al.*, 1998). How securin acts as a primer for the separase is unclear. It may serve to correctly localize the protease (Jensen *et al.*, 2001) or, alternatively, may be necessary for the separase to acquire its active conformation.

When the human securin was identified it was found to be the product of the *pituitary tumor-transforming gene*, which is over-expressed in certain tumours and exhibits transforming activity

in NIH 3T3 cells (Zou *et al.*, 1999). Chromosome missegregation is thought to be a cause of the genetic instabilities found associated with many cancers (Lengauer *et al.*, 1997), and overexpressed securin might lead to incomplete chromosome segregation due to inhibition of separase activity during anaphase.

Securin regulation by the anaphase promoting complex

The APC/C appears to be the mastermind of the metaphase to anaphase transition. It controls the degradation of numerous proteins in addition to securin at this stage (reviewed in Zachariae and Nasmyth, 1999). Whereas in yeast, the APC/C exerts its effect on sister chromatid separation solely by targeting securin (Yamamoto *et al.*, 1996a; Ciosk *et al.*, 1998) (Figure 2A), *Xenopus* meiotic divisions also require the APC/C-dependent degradation of a chromosomal spindle motor to allow chromosome movement (Funabiki and Murray, 2000). The APC/C is activated at anaphase by the Cdc20/Fizzy protein, whose expression, in turn, is cell cycle regulated (see Zachariae and Nasmyth, 1999). Cell cycle-dependent phosphorylation of APC/C

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subunits is also required for the activation of the APC/C complex (Kotani *et al.*, 1999; Shteinberg *et al.*, 1999).

APC/C activation is also the entry point for the Mad2-dependent checkpoint pathway that monitors the bipolar attachment of chromosomes to the mitotic spindle (Figure 2A; reviewed in Clarke and Gimenez-Abian, 2000). Unattached kinetochores send a signal via Mad2 that keeps the APC/C inactive, potentially through the binding of Mad2 to the APC/C activator Cdc20/Fizzy. Indeed, the budding yeast securin, Pds1, had initially been identified as a protein required to prevent sister chromatid separation when the Mad2-dependent checkpoint pathway is activated (Yamamoto *et al.*, 1996a). In yeast, this pathway for regulating securin destruction only becomes essential once actual damage to spindle kinetochore attachment occurs. In contrast, in higher eukaryotes it acts during each cell cycle to ensure timely sister chromatid separation (Basu *et al.*, 1999; Kitagawa and Rose, 1999; Dobles *et al.*, 2000).

Controlling the onset of anaphase based on the state of chromosome attachment to the mitotic spindle seems inherently to be of greatest importance. But it is not the only control. If DNA is damaged, anaphase onset is delayed to allow repair before sister sequences are segregated from each other. In budding yeast, DNA damage elicits a response pathway which uses two routes that act together to prevent anaphase (Cohen-Fix and Koshland, 1997; Gardner *et al.*, 1999; Sanchez *et al.*, 1999). One route again acts via securin; the other is understood more poorly (see below). Securin is stabilized in response to DNA damage mainly through the action of the Chk1 kinase, and Chk1 can phosphorylate the budding yeast securin Pds1 (Sanchez *et al.*, 1999). Although not addressed experimentally, it is tempting to speculate that phosphorylation may protect securin from ubiquitylation by the APC/C. In higher eukaryotic cells, the majority of sister DNA sequences become separated during chromosome condensation in prophase. Accordingly, the DNA damage response mainly downregulates Cdk activity, which blocks cells from entering prophase (see Clarke and Gimenez-Abian, 2000). Human securin has been reported to interact with the Ku70 subunit of DNA-dependent protein kinase (Romero *et al.*, 2001), and whether securin is also a target of the DNA damage response pathway in higher eukaryotes remains to be determined.

Preparing the cleavage target

As described above, securin is essential for the prevention of anaphase onset in response to spindle damage or DNA lesions. During undisturbed cell cycle progression, however, the budding yeast securin is entirely dispensable. Cleavage of cohesin still occurs in a regulated fashion, its kinetics unchanged (Alexandru *et al.*, 2001). Is there a second regulator besides securin that can inhibit premature activation of separase? Probably not, since the overall separase activity in cells lacking securin no longer undergoes major changes during the cell cycle. Instead, regulation occurs at the level of the separase cleavage target. Scc1 is a phosphoprotein whose phosphorylation is crucial for its cleavage by separase (Figure 2B) (Uhlmann *et al.*, 2000). The polo-like kinase, Cdc5 in budding yeast, is responsible for phosphorylation of Scc1 during metaphase. Preventing Scc1 phosphorylation decreases the rate of Scc1 cleavage *in vivo*. This effect is especially pronounced in the

absence of securin, possibly due to the impairment of separase activity. Of several sites phosphorylated in Scc1 by Cdc5 kinase, two phosphorylated serines lie adjacent to the separase cleavage sites, and the affinity of separase for the cleavage sites is dramatically increased by phosphorylation of these residues (Alexandru *et al.*, 2001).

The effect that phosphorylation has on the cleavability of Scc1 certainly contributes to the cell cycle regulation of anaphase onset. Could changes in the phosphorylation status of Scc1 also be used to prevent anaphase onset after DNA damage? The securin-independent branch of the DNA damage response pathway acts through the Rad53 kinase in budding yeast, and requires the downregulation of Cdc5 (Sanchez *et al.*, 1999). Amongst other effects, diminished Cdc5 activity could block cohesin from becoming a substrate for cleavage by the separase. After prolonged arrest following DNA damage, cells adapt and overcome the metaphase block. This adaptation in turn depends on active Cdc5 (Toczyski *et al.*, 1997). Perhaps phosphorylation of Scc1 is particularly important in allowing anaphase to occur even under conditions where part of the damage remains unrepaired.

What cleaves the separase?

While total levels of budding or fission yeast separase do not undergo obvious changes during the cell cycle (Funabiki *et al.*, 1996a; Ciosk *et al.*, 1998), the abundance of human separase fluctuates. In human cells, separase levels are high during metaphase and decline during anaphase. Furthermore, in anaphase, intact separase itself seems to be processed into distinct cleavage fragments, reminiscent of the processing of cohesin (Figure 2C) (Waizenegger *et al.*, 2000). What are the consequences of separase cleavage for its own proteolytic activity? The answer is open. It could be just as for the related caspases: cleavage of an inactive proform of separase may be necessary to produce the actual protease. If separase is a self-cleaving protease, a positive feedback loop may exist, and this could cause the sudden activation of many molecules after an initial triggering event. Such a system could provide the decisiveness required for anaphase onset. Alternatively, cleavage may inactivate separase, rapidly terminating its activity after anaphase. This might be necessary to allow rebinding of cohesin to chromosomes even during telophase (Darwiche *et al.*, 1999; Losada *et al.*, 2000; Waizenegger *et al.*, 2000). In budding yeast, separase remains active throughout the G₁ phase (Uhlmann *et al.*, 1999), presumably until it is inactivated by the resynthesis of securin shortly before the next S phase.

Positioning the separase

In order to cleave cohesin at anaphase onset, not only must separase be active and Scc1 phosphorylated, but also separase must locate Scc1 on the chromosomes. Might additional regulation take place at the level of the localization of separase (Figure 2D)? In budding yeast, separase is found distributed throughout the cell, although an accumulation in dividing nuclei has been reported (Ciosk *et al.*, 1998; Jensen *et al.*, 2001). Surprisingly, separase is found on the mitotic spindle during anaphase. Spindle binding of separase has been studied in detail in fission yeast (Funabiki *et al.*, 1996a; Kumada *et al.*, 1998). Here,

separase localizes to the spindle from metaphase until mid-anaphase. The spindle localization requires active securin, implicating securin in the localization of separase. Does the interaction of separase with the mitotic spindle indicate a role for separase in regulating microtubule dynamics during anaphase? This would be consistent with the observed spindle defect after anaphase triggered by ectopic cohesin cleavage in yeast (Uhlmann *et al.*, 2000). Localization of separase to chromosomes has so far not been demonstrated, although cohesin, its best characterized target to date, resides there. This illustrates the problem associated with drawing conclusions regarding function from mere localization data.

Conclusions

We now understand the molecular principle of how chromosome segregation is triggered at anaphase onset. A specific protease, the separase, cleaves a protein that is required to hold the chromosomes together. We are also beginning to understand how this reaction is regulated at a number of levels to ensure that chromosomes are not prematurely separated. It may be that additional controls become important under certain conditions. For example, calcium waves are thought to play a role in triggering anaphase, and separase has a potential calcium binding site (Kumada *et al.*, 1998), suggesting that separase activity might be regulated by calcium levels. In addition, different sized complexes of separase with securin have been detected in fission yeast cells, but their roles have not yet been explored (Funabiki *et al.*, 1996a). Finally, separase may do more than just cleave cohesin to ensure a smooth transition from metaphase into anaphase. It would not be surprising if there were more tasks for this formidable protease.

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