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Separase Is Recruited to Mitotic Chromosomes to Dissolve Sister Chromatid Cohesion in a DNA-Dependent Manner

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SUMMARY

Sister chromatid separation is triggered by the separase-catalyzed cleavage of cohesin. This process is temporally controlled by cell-cycle-dependent factors, but its biochemical mechanism and spatial regulation remain poorly understood. We report that cohesin cleavage by human separase requires DNA in a sequence-nonspecific manner. Separase binds to DNA in vitro, but its proteolytic activity, measured by its autocleavage, is not stimulated by DNA. Instead, biochemical characterizations suggest that DNA mediates cohesin cleavage by bridging the interaction between separase and cohesin. In human cells, a fraction of separase localizes to the mitotic chromosome. The importance of the chromosomal DNA in cohesin cleavage is further demonstrated by the observation that the cleavage of the chromosome-associated cohesins is sensitive to nuclease treatment. Our observations explain why chromosome-associated cohesins are specifically cleaved by separase and the soluble cohesins are left intact in anaphase.

INTRODUCTION

Stable cohesion between sister chromatids before anaphase and their timely separation during anaphase are critical for chromosome inheritance. Sister chromatid cohesion is mediated by the cohesin complex, consisting of four core subunits: an α-kleisin subunit (SCC1/MCD1/RAD21), SCC3 (known as SA1 or SA2 in vertebrates), SMC1, and SMC3 (Huang et al., 2005a; Nasmyth, 2005). Although the cohesin complex is conserved from yeast to humans, the regulation of sister chromatid cohesion is more complex in metazoans. In vertebrates, sister chromatid cohesion is released in two steps via two distinct mechanisms. The first step occurs in prophase, which involves phosphorylation of SA1/2 (Hauf et al., 2005) and dissociates most cohesins from chromosome arms but not the heterochromatin or centromeric regions. The second step occurs in anaphase, when separase cleaves SCC1 and initiates the final separation of sister chromatids (Hauf et al., 2001; Uhlmann et al., 2000).

In vertebrates, separase cleaves SCC1 exclusively in anaphase. This cleavage does not occur during the rest of the cell cycle because separase is inhibited by two independent and sometimes redundant mechanisms: binding by securin (Zou et al., 1999) and phosphorylation of serine 1126 (S1126) of separase (Stemmann et al., 2001). Moreover, the bulk of separase is excluded from the nucleus in interphase, avoiding the possibility of direct contact with cohesin (Sun et al., 2006). However, additional regulation is strongly suggested by the lack of expected phenotype in cells and animals missing securin and/or S1126 phosphorylation (Huang et al., 2005b; Jallepalli et al., 2001; Mei et al., 2001; Pfleghaar et al., 2005; Wang et al., 2001).

In addition to temporal regulation, cohesin cleavage is regulated spatially after separase activation. In budding yeast, cohesins are mostly chromosome bound and nearly completely cleaved in anaphase. Nonetheless, chromosome-associated cohesins are a preferred substrate for separase. Polo-like kinase (Plk1) phosphorylates the chromosome-associated cohesin on the SCC1 subunit, which stimulates cohesin cleavage 2- to 3-fold over that of the unphosphorylated soluble cohesins (Alexandru et al., 2001; Hornig and Uhlmann, 2004). In vertebrates, only a small fraction of cohesin, which is thought to be the chromosome-associated pool, is cleaved in anaphase (Waizenegger et al., 2000). The stimulation of cohesin cleavage by Plk1 is moderate (Hauf et al., 2005). Furthermore, it is unclear whether the chromosome-associated cohesins are phosphorylated by Plk1. In fact, phosphorylation of SA2 by Plk1 causes the prophase removal of cohesin from chromosomes (Hauf et al., 2005; Waizenegger et al., 2000). The remaining chromosome-associated cohesins are protected from Plk1 by Shugoshin and PP2A (Kitajima et al., 2005, 2006; McGuinness et al., 2005; Tang et al., 2006). Therefore, the mechanism that limits cohesin cleavage to the chromosome-associated pool in vertebrate cells remains unclear.

In order to better understand the spatiotemporal regulation of cohesin cleavage by separase in vertebrates, we searched for

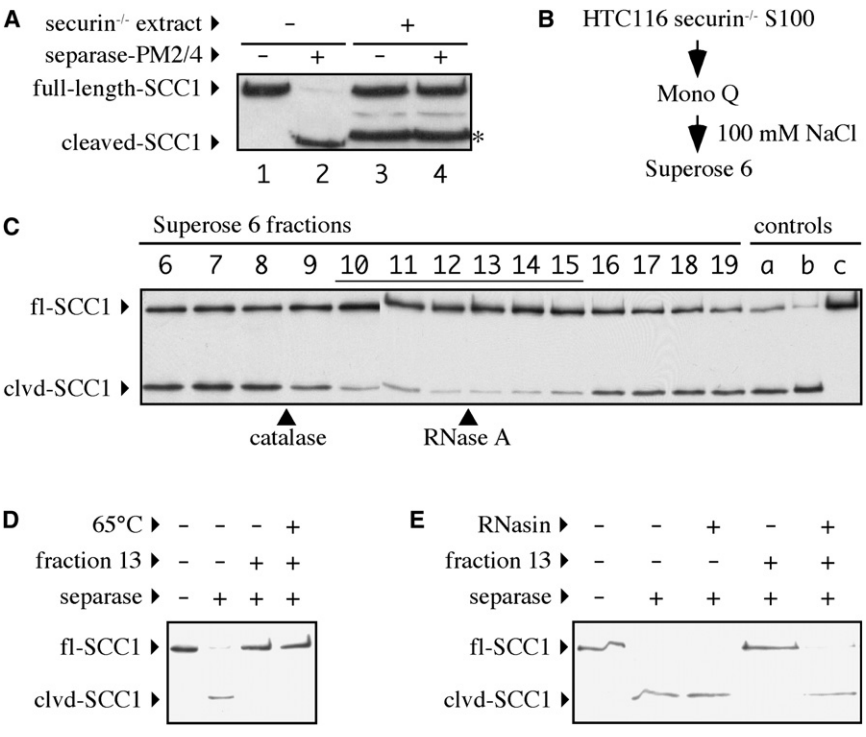

Figure 1. The In Vitro Cohesin Cleavage Assay Is Inhibited by Ribonuclease Activity

(A) The in vitro cohesin cleavage by separase-PM2/4 is sensitive to S100 prepared from *securin*$^{-/-}$ HCT116 cells. The cleavage of SCC1 was determined by the disappearance of the full-length SCC1 (fl-SCC1) and the appearance of a cleaved SCC1 fragment (clvd-SCC1). The clvd-SCC1 is a product of the cleavage of the N-terminal site on SCC1 (Hauf et al., 2005). In lanes 3 and 4, the bands marked by an asterisk represent a protein from the cell lysate that cross-reacted with anti-SCC1.

(B) Schematic diagram of the purification scheme. The S100 was prepared from *securin*$^{-/-}$ HCT116 cells. The activity was eluted at 100 mM NaCl from the mono Q column.

(C) The inhibitory activity in the Superose 6 column fractions. The fraction numbers are indicated above the blot. The active fractions are underlined. The controls include the cohesin cleavage reactions in the column buffer (a), in the cleavage buffer (b), and in the absence of separase (c). The protein standards are indicated below the blot.

(D) The inhibitory activity is resistant to heat. The peak fraction (#13) described in (C) was analyzed in a standard cohesin cleavage assay. Before adding into the cleavage reaction, 2 μl of fraction 13 was incubated at 65°C for 20 min.

(E) The inhibitory activity is sensitive to RNasin. Before adding into the cleavage reaction, 2 μl of fraction 13 was mixed with 10 units of RNasin.

a new biochemical activity that would regulate cohesin cleavage by separase in the absence of securin, the phosphorylation inhibitions, and the nuclear exclusion. This study led to the discovery that cohesin cleavage is dependent on the presence of DNA. The protease activity of separase per se does not exhibit this dependency, as evidenced by the ability of separase to cleave itself in the absence of DNA. Instead, our results indicate that DNA facilitates the cleavage reaction by bridging separase and cohesin. We propose that chromosomal DNA functions as this molecular bridge in vivo. In support of this model, we detected separase on mitotic chromosomes by immunofluorescent microscopy and cellular fractionation. We also demonstrated that chromosome-associated mitotic cohesin is no longer cleaved when chromosomal DNA is removed by nuclease digestion. Our findings help to explain why only chromosome-associated cohesins are cleaved by separase in anaphase.

RESULTS

Polynucleotide Is Required for SCC1 Cleavage by Separase In Vitro

The cleavage of cohesin SCC1 by separase was previously reconstituted in vitro using partially purified enzyme and substrate (Fan et al., 2006; Stemmann et al., 2001). In order to identify additional regulators of cohesin cleavage, we tested whether the crude extract prepared from the *securin*$^{-/-}$ HCT116 cells (Jallepalli et al., 2001) could inhibit cohesin cleavage in this assay. The separase S1126A mutant (separase-PM2/4), which is resistant to phosphorylation inhibition, was used as the enzyme. In this system, all known mechanisms of separase regulation were absent. Remarkably, as little as 2 μl of this extract completely blocked SCC1 cleavage (Figure 1A, lane 4). This result suggested that SCC1 cleavage by separase was inhibited by an unknown activity. We performed biochemical purifications to identify this activity (Figure 1B). The activity was eluted from a gel filtration column with an apparent molecular weight of about 15 kDa, which overlapped with RNase A (Figure 1C). Furthermore, the activity was resistant to heat (Figure 1D) and sensitive to RNasin (Figure 1E). This prompted us to investigate whether it was actually a ribonuclease. Indeed, both RNase A and RNase T1 inhibited SCC1 cleavage (Figure S1 available online). Finally, the inhibition was not caused by the hydrolyzed ribonucleotides because, at concentrations up to 100 μM, they did not affect the cleavage reaction (data not shown). Therefore, we concluded that a ribonuclease activity was responsible for the inhibition of cohesin cleavage.

The fact that the reaction was sensitive to various RNases suggested that an RNA component might be required for SCC1 cleavage in our assay. Further analyses revealed that the separase preparation was contaminated with a noticeable amount of RNA (Figure S2). These RNA molecules originated from the *Xenopus* egg extract, which was used to degrade securin, because they were not detected prior to the extract treatment. The *Xenopus* egg extract is known to contain a large amount of maternal mRNA. We directly analyzed whether RNA or other polynucleotide facilitates SCC1 cleavage. We pretreated the recombinant separase and the cohesin complex with RNase A to destroy the contaminating RNA and supplemented the reaction