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Regulation of Human Separase by Securin Binding and Autocleavage

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Summary

Background: Sister chromatid separation is initiated by separase, a protease that cleaves cohesin and thereby dissolves sister chromatid cohesion. Separase is activated by the degradation of its inhibitor securin and by the removal of inhibitory phosphates. In human cells, separase activation also coincides with the cleavage of separase, but it is not known if this reaction activates separase, which protease cleaves separase, and how separase cleavage is regulated.

Results: Inhibition of separase expression in human cells by RNA interference causes the formation of polyploid cells with large lobed nuclei. In mitosis, many of these cells contain abnormal chromosome plates with unseparated sister chromatids. Inhibitor binding experiments in vitro reveal that securin prevents the access of substrate analogs to the active site of separase. Upon securin degradation, the active site of full-length separase becomes accessible, allowing rapid autocatalytic cleavage of separase at one of three sites. The resulting N- and C-terminal fragments remain associated and can be reinhibited by securin. A noncleavable separase mutant retains its ability to cleave cohesin in vitro.

Conclusions: Our results suggest that separase is required for sister chromatid separation during mitosis in human cells. Our data further indicate that securin inhibits separase by blocking the access of substrates to the active site of separase. Securin proteolysis allows autocatalytic processing of separase into a cleaved form, but separase cleavage is not essential for separase activation.

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Introduction

Cohesion between sister chromatids is required to allow the biorientation of chromosomes on the mitotic spindle and is therefore essential for equal segregation of sister chromatids in anaphase. Cohesion is mediated by a chromosomal protein complex called cohesin [1]. In budding yeast, cleavage of the cohesin subunit Scc1/Mcd1 is required and sufficient to dissolve cohesion [2, 3]. Cohesin cleavage is mediated by Esp1/separase, a member of the CD clan of cysteine proteases that also comprises caspases [3]. In metaphase, separase is activated by the anaphase-promoting complex (APC), which mediates the ubiquitin-dependent degradation of the separase inhibitor Pds1/securin [4, 5].

Separase orthologs are also required for anaphase in *S. pombe*, *A. nidulans*, *C. elegans*, and *Drosophila* [6–9]. It was initially less clear whether separase is also required for anaphase in vertebrates, because the large bulk of vertebrate cohesin is removed from mitotic chromosomes in prophase by a mechanism that does not depend on the APC and cohesin cleavage [10–13]. However, small amounts of cohesin remain on chromosomes until metaphase, and the cleavage of these complexes is required for the initiation of anaphase [13, 14].

As in budding yeast, separase is also bound to a regulatory protein through most of the cell cycle in other eukaryotes, and it is called Cut2 in *S. pombe*, Pimples in *Drosophila*, and PTTG in vertebrates [15–17]. Although these proteins are not similar in their sequences, their APC-mediated degradation is required for anaphase in all cases and for activation of human separase in vitro [14]. Like budding yeast Pds1, these proteins therefore function as separase inhibitors, but it remains unknown by which mechanism they inactivate separase. All of these proteins are now called securins. *Drosophila* separase is also bound to a second protein called Threerows, which may correspond to the N-terminal domain of separase in other species [8, 17].

Genetic and biochemical evidence suggests that securins do not only inhibit separase but that their presence is also required for proper separase activation. Like separase mutants, Cut2 mutants in *S. pombe* and Pimples mutants in *Drosophila* are unable to initiate anaphase [15, 18], and human cells lacking securin contain only low amounts of separase, whose specific activity is reduced [19].

Although securin genes are essential for viability in fission yeast and *Drosophila*, budding yeast cells can survive without securin at low temperatures and can initiate anaphase with fairly normal timing, indicating that mechanisms other than securin degradation must regulate separase activation [20, 21]. Phosphorylation of Scc1/Mcd1 by the Polo-kinase Cdc5 increases cohesin cleavage by separase and may therefore be one such mechanism [22]. Also, mice and human cultured cells lacking securin are viable [19, 23, 24], suggesting that also mammalian separase is regulated by additional mechanisms. In *Xenopus* egg extracts, high cyclin-

dependent kinase 1 activity inhibits sister chromatid separation through inhibitory phosphorylation of separase [25]. This modification alone is sufficient to inhibit separase in the absence of securin binding and possibly explains why mammalian cells lacking securin are viable.

In human cells, separase activation at the onset of anaphase does not only coincide with securin destruction and cohesin cleavage but also with the cleavage of separase itself [14]. Separase cleavage occurs at at least two closely spaced sites and separates the conserved C-terminal catalytic domain of separase from the N-terminal portion. The correlation between securin destruction and cohesin and separase cleavage implies that these events may be coregulated, but it is not known how separase cleavage is controlled, which protease cleaves separase, and if separase cleavage is required for its activation or inactivation.

Here, we show that securin degradation activates separase to undergo autocatalytic cleavage that can occur at one of three sites. The resulting separase fragments remain associated with each other. Nevertheless, separase cleavage is not essential for separase activation in vitro. We also provide evidence that securin inhibits separase by blocking access of substrates to the active site of separase and that separase is required for sister chromatid separation in human cells in vivo.

Results

Inhibition of Separase Expression in Human Cells by RNA Interference

To obtain insight into the in vivo function of human separase, we transiently transfected HeLa cells with small interfering RNAs (siRNAs) derived from the human separase cDNA. Separase levels were strongly reduced 18 hr after transfection with either one of two different separase siRNAs, called 1/2 and 3/4, whereas mock transfections or transfection with a reverse siRNA (5/6) had only little effect on separase levels (Figure 1A). Two days following transfection, securin levels also decreased (data not shown), but other proteins such as tubulin were not affected (Figure 1A). In dishes containing transfected cells increasing numbers of floating cells were seen, DAPI-stained fixed material revealed many cells with apoptotic morphology, and immunoblotting experiments showed cleavage of the caspase substrate poly-ADP ribose polymerase. However, similar apoptotic effects were observed in cells transfected with reverse siRNA (Figure 1A and data not shown), and these effects suggest that apoptosis may be caused by the RNA interference protocol and not by loss of separase expression.

Loss of Separase Expression Induces Polyploidy in Human Cells

Fluorescence microscopy revealed that, 3 days following transfection with separase siRNAs, most cells were in interphase and contained nuclei that were highly abnormal in size and shape (Figures 1C and 1F), whereas control transfected cells showed normal nuclear morpholgy (Figure 1B). The nuclei were 1–3 times larger

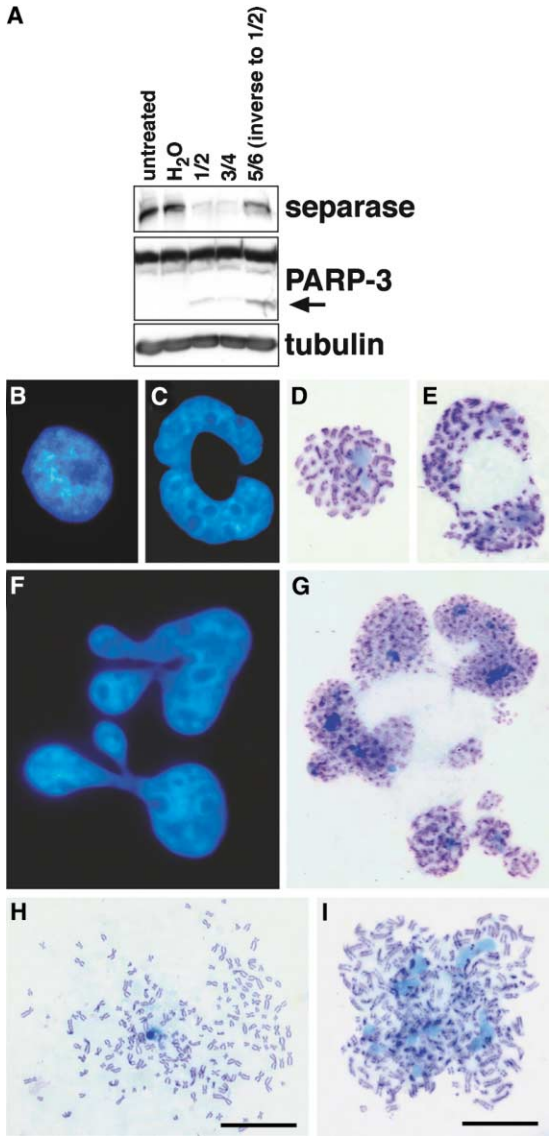


Figure 1. Inhibition of Separase Expression Causes Polyploidization in Human Cells

(A) HeLa cells were transfected with either one of two different separase siRNAs (1/2, 3/4), or with the inverse siRNA of 1/2 (5/6), or with control mixture (H_2O), or were left untreated. A total of 18 hr after transfection, total cell lysates were analyzed by immunoblotting with separase, PARP-3, and tubulin antibodies.

(B–I) (B, C, F) Three days after transfection, control cells show normal nuclear morphology, as revealed by DAPI staining (B), whereas separase RNAi cells contain larger nuclei that are either (C) rosette-shaped or (F) lobed. (D, E, G, I) Calyculin A-induced PCC followed by chromosome spreading and Giemsa staining reveals higher chromosome numbers in the morphologically abnormal nuclei of (E, G, and I) separase RNAi cells than in the nuclei of (D) control cells. The nucleus in (E) is near-tetraploid, and the nuclei in (G) and (I) are near-octoploid. Cells were analyzed 84 hr after transfection.

(H) Near-octoploid chromosome numbers are also seen in chromosome spreads from separase siRNA cells treated for 3 hr with nocodazole.

Images in (B)–(G) are at similar magnification (the scale bar represents 20 μM). The scale bar in (H) represents 30 μM.

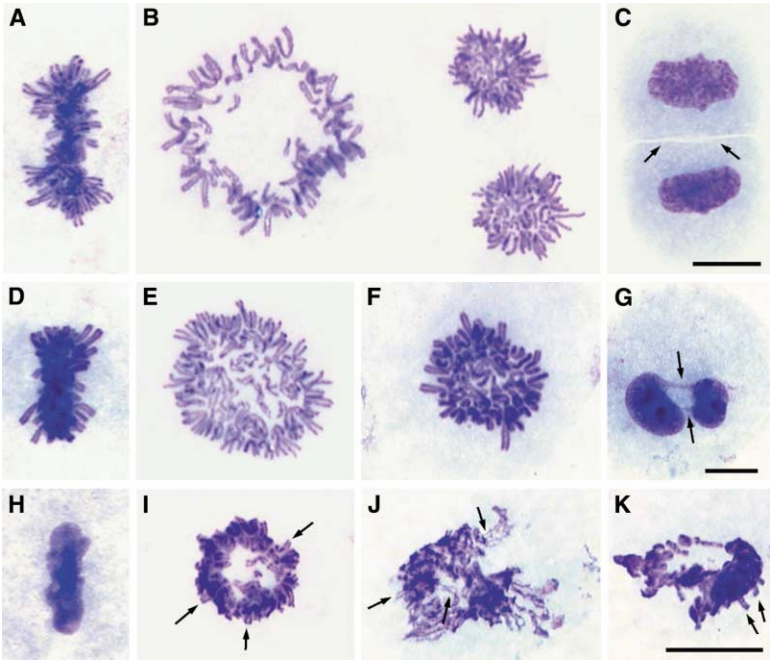


Figure 2. Inhibition of Separase Expression Causes Abnormal Mitotic Chromosome Figures

Chromosome spreads from mitotic HeLa cells transfected with separase siRNAs were analyzed by Giemsa staining. Chromosomes were isolated either (A, C–H, J, and K) 12 hr or (B and I) 24 hr after transfection. The scale bar represents 20 μM.

(A, B, C) Normal chromosome figures showing a (A) side view and a ([B], left figure) polar view of a metaphase plate, ([B], right figure) segregating chromatids in anaphase, and (C) cells that have just completed cytokinesis. The arrows in (C) indicate the cytokinesis furrow.

(D–K) Abnormal chromosome figures showing (D–F) smaller metaphase plates, (H and I) chromosomes in a metaphase-like plate that appear to decondense, and (J and K) irregularly shaped chromosome plates in which chromosomes appear to decondense. Sister chromatids have not separated in these abnormal chromosome figures (arrows in [I–K]).

(G) Decondensed chromatin from an interphase cell in which two nuclei are connected by chromatin bridges (indicated by arrows).

than normal, and they were usually lobed or adopted a rosette-like shape. To analyze if these abnormalities coincided with an increase in ploidy, we analyzed chromosomes in mitotic cells. Three days after transfection, only very few mitotic cells could be observed, but some mitotic cells could be recovered by the addition of nocodazole for 3 hr, followed by mitotic shake off. Chromosome spreads of these cells showed a wide range of chromosome numbers from near diploid to near octoploid (Figure 1H), and this is consistent with the possibility that the increased nuclear size is caused by polyploidization. To test directly if cells with abnormally shaped nuclei contained increased chromosome numbers, we treated cells 3 days after siRNA transfection with calyculin A, a phosphatase inhibitor that induces premature chromosome condensation (PCC) of interphase nuclei in a wide variety of cell lines [26]. When cells were fixed 2 hr after the induction of PCC and spread onto slides, chromosomes could be visualized before nuclear envelopes had broken down. Under these conditions, large lobed nuclei could clearly be seen to contain abnormally high chromosome numbers, confirming that these nuclei are polyploid (Figures 1E and 1G). The same specimens also contained chromosomes from cells whose nuclear envelope had already broken down. Also, these figures contained near tetraploid to near octoploid chromosome numbers (Figure 1I).

Inhibition of Separase Expression Blocks Sister Chromatid Separation in Mitosis

To test if separase is required for sister chromatid separation in human cells, we isolated mitotic separase RNAi cells by shake off without prior nocodazole treatment and analyzed the morphology of metaphase and anaphase chromosomes in spread preparations. For this experiment, we used cells 12–24 hr after siRNA transfection because it was difficult to recover mitotic cells at later stages when many cells were dying. At this early stage, separase levels were greatly reduced but were still detectable (Figure 1A). Some cells might therefore still have contained sufficient amounts of separase for normal mitotic progression. Consistent with this possibility, we observed some normal metaphase, ana-telophase, and early G1 figures in these preparations (Figures 2A–2C). However, 62% of all metaphase-like configurations showed highly abnormal morphologies, whereas only 8% of all metaphases from mock-transfected cells showed abnormalities. Many figures were seen that resembled metaphase plates in side views (Figures 2D and 2H) or polar views (Figures 2E, 2F, and 2I), but the chromosomes in these plates were arranged closer to each other than normal (see Figure 2B for comparison). These chromosome plates often had a more disk-like shape and not the ring shape with a central opening that is rather typical for the arrangement of chromosomes in normal human metaphase plates. We never observed sister chromatid separation in the abnormal chromosome plates. Instead, we observed figures in which chromosomes had apparently begun to decondense without separating sister chromatids (Figure 2I), and more irregular mitotic figures apparently decondensed without separating sister chromatids (Figures 2J and 2K). These observations are consistent with the possibility that cells lacking sufficient amounts of separase fail to separate sister chromatids properly but nevertheless exit from mitosis and therefore decondense unseparated chromosomes.

Inhibition of Separase by Peptide Inhibitors

To address if separase cleavage is required for its activation, we first used peptide inhibitors that can covalently bind to the active site cysteine in separase. Many proteases are regulated by controlling the access of substrates to their active sites. We therefore reasoned that

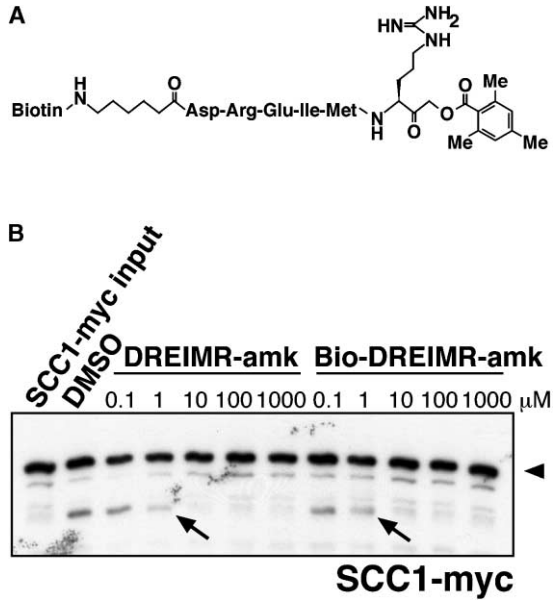


Figure 3. A Peptide Inhibitor of Human Separase

(A) The structure of the acyloxymethyl ketone derivative of the human SCC1 cleavage site peptide, synthesized as a separase inhibitor.

(B) Inhibition of human separase by the inhibitor shown in (A), Bio-DREIMR-amk, and a similar derivative lacking the N-terminal biotin moiety (DREIMR-amk). Activated separase from HeLa cells was incubated with the indicated amounts of the inhibitors, washed, and analyzed for its ability to cleave in vitro-translated full-length human SCC1-myc (arrowhead). The generation of a C-terminal SCC1 cleavage product was detected by immunoblotting with myc antibodies (arrows).

the ability of peptide inhibitors to bind to either full-length or cleaved separase could indicate which of these proteins represents an active enzyme.

We synthesized inhibitors based on the hexapeptide DREIMR, which represents amino acid residues P6– P1 in the first cleavage site of human SCC1 [13]. The N terminus of the peptide was either biotinylated or was left unmodified, whereas the C terminus was modified to an acyloxymethyl ketone (amk; Figure 3A). Similar inhibitors based on a cleavage site found in yeast Scc1/Mcd1 have previously been shown to bind to the active site cysteine in yeast separase [3]. To analyze the effects of these inhibitors on separase activity, we immunoprecipitated separase from human HeLa cells arrested in mitosis, activated separase by securin degradation in mitotic *Xenopus* egg extracts, and incubated the reisolated separase with in vitro-translated human SCC1 either in the presence or absence of the peptide inhibitors. Both the biotinylated and the nonbiotinylated peptides inhibited SCC1 cleavage with an IC_{50} between 1 and 10 μM (Figure 3B), whereas an unrelated fluoromethyl ketone inhibitor of caspase V, Z-VK-X-(Biotin)-D-FMK, had no effect (data not shown). Human separase could also be inhibited by amk and chloromethyl ketone derivatives of the yeast Scc1/Mcd1 cleavage site (Figure S1) at concentrations similar to the ones required to inhibit yeast separase [3]. However, for recognition of physiological protein substrates, more than this hexapeptide seems to be required, as human separase is not able to cleave yeast Scc1/Mcd1 and yeast separase cannot cleave human SCC1 (data not shown; F. Uhlmann, personal communication).

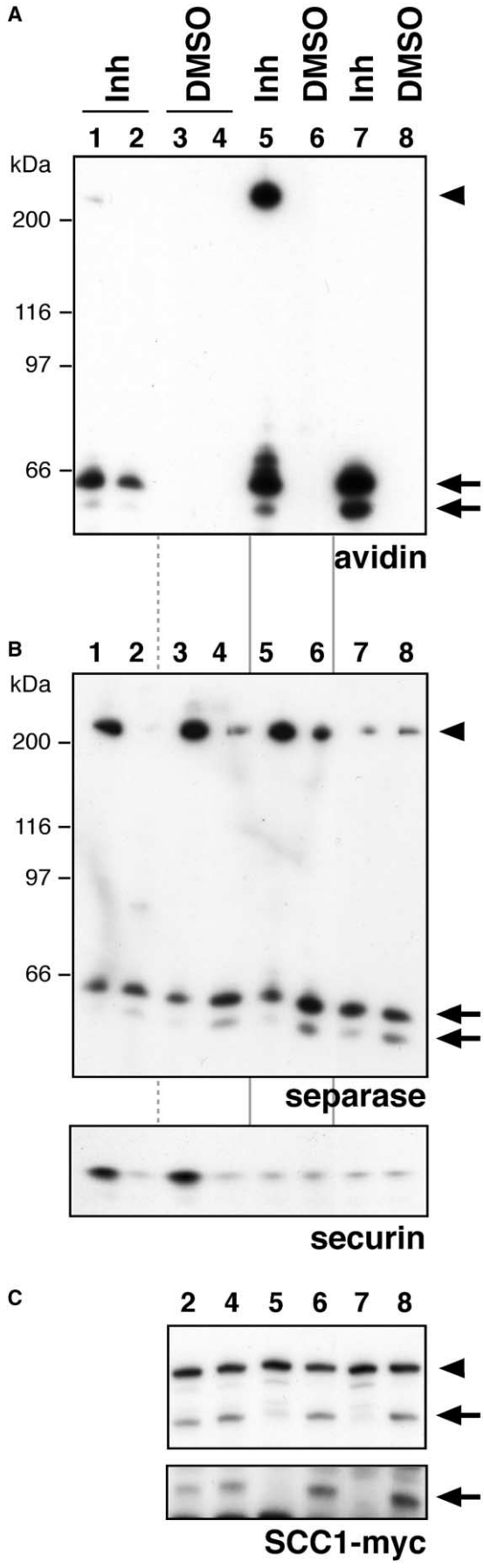
Analysis of Separase Activity by Peptide Inhibitors

To understand when separase's active site is accessible, we added the biotinylated amk inhibitors either prior, during, or after activation of separase-securin complexes in mitotic *Xenopus* extracts. Subsequently, we analyzed inhibitor binding to separase by avidin blotting and separase activity in SCC1 cleavage assays. When biotinylated inhibitor was added to separase-securin complexes before mitotic activation, the cleaved forms of separase that are already present in these immunoprecipitates were clearly detected by avidin blotting, whereas full-length separase was only labeled very weakly (Figure 4A, lane 1). This result implies that the active site of full-length separase is not accessible in separase-securin complexes. When the inhibitor was washed away from these complexes and securin was degraded in mitotic extracts, active separase was recovered, further indicating that the inhibitor had not bound to full-length separase in separase-securin complexes (Figure 4C, lane 2).

When inhibitor was present during mitotic activation of separase-securin complexes in mitotic *Xenopus* extracts, a very different result was obtained. In this case, both full-length and cleaved forms of separase were strongly labeled with inhibitor (Figure 4A, lane 5), and separase remained inactive (Figure 4C, lane 5) despite the fact that securin had been destroyed (Figure 4B, bottom panel, lane 5). These observations imply that the active site of separase can also be accessible to inhibitor, but only once securin is destroyed.

Yet another result was obtained when inhibitor was added to separase after it had been activated by securin degradation in mitotic extracts. In this case, most separase had been cleaved and only the resulting separase fragments were labeled with inhibitor, whereas residual amounts of full-length separase were not (Figures 4A and 4B, lanes 7). The inhibitor treatment abolished SCC1 cleavage under these conditions (Figure 4C, lane 7) and thus confirmed the data shown in Figure 3B.

Together, these observations show that the active site cysteine of C-terminal separase cleavage fragments is accessible to inhibitors under all conditions, and these data are consistent with the possibility that cleaved separase is an active protease. In contrast, full-length separase can only be bound by inhibitor if the inhibitor is present while securin is degraded. These results suggest that securin proteolysis may allow access of inhibitors to the active site of separase. Once bound, the inhibitor might prevent autocatalytic processing of separase into cleaved forms, which would otherwise occur rapidly following securin proteolysis. According to this hypothesis, the residual full-length separase molecules that are present after separase activation in mitotic extracts (Figure 4B, upper panel, lane 7) might not bind inhibitor because they would represent inactive separase that is still bound to securin. Consistent with this interpretation, some securin can still be detected in the same fraction (Figure 4B, lower panel, lane 7).



Securin Binding Prevents the Access of Peptides to the Active Site of Separase

We next analyzed if rebinding of securin to activated separase prevents the binding of peptide inhibitors. Activated separase bound an amount of recombinant purified securin that was similar to the amount detected in inactive separase immunoprecipitates, despite the fact that activated separase contained predominantly cleaved separase (Figure 5A, compare lanes 1 and 4). This implies that securin can also bind to cleaved separase. The rebinding of securin to separase correlated with the complete loss of separase activity in SCC1 cleavage assays and indicated that securin inhibits separase by direct binding (Figure 5B). A destruction box mutant of securin that can not be ubiquitinated by the APC had the same effect, whereas bovine serum albumin (BSA) used as a control did not inhibit separase (Figure 5B). During the preparation of this manuscript, Stemmann et al. [25] showed similarly that recombinant securin can inhibit purified separase.

We next analyzed if peptide inhibitors can still bind to activated separase once securin has been rebound (Figure 5C). We observed that no peptide labeling was obtained if separase was preincubated with securin, whereas preincubation with a recombinant purified cyclin fragment had no effect. Conversely, the binding of inhibitor to separase did not detectably reduce the binding of securin to separase, indicating that securin can bind to separase by contacting residues outside the active site of separase.

The N- and C-Terminal Cleavage Products of Separase Remain Bound to Each Other

To analyze if the separase cleavage products remain associated with each other, we transiently coexpressed

Figure 4. Securin Proteolysis Allows Binding of Peptide Inhibitors to Separase

(A–C) The yeast separase inhibitor Bio-SVEQGR-amk (Inh) or its solvent DMSO was added to separase from HeLa cells either before (lanes 1 and 3), during (lanes 5 and 6), or after (lanes 7 and 8) activation in mitotic *Xenopus* extracts. Unbound inhibitor was washed away, and the separase immunoprecipitates were analyzed by (A and B) SDS-PAGE and blotting and in (C) activity assays. Separase to which the inhibitor or DMSO had been added before activation (lanes 1 and 3) was subsequently also incubated in mitotic *Xenopus* extracts (lanes 2 and 4) and was analyzed as above. The inhibitor was used at a concentration of 85 μM when added before or after separase activation. In *Xenopus* extracts, 750 μM inhibitor was used. At lower concentrations, separase could not be labeled, possibly because these extracts contain 50 mg/ml protein. (A) The binding of inhibitor to separase was analyzed by SDS-PAGE and by detecting the inhibitor's biotin moiety in avidin blots. The position of full-length separase and its C-terminal cleavage products is marked by an arrowhead and arrows, respectively. (B) Separase (top panel) and securin (bottom panel) were analyzed by SDS-PAGE and immunoblotting. The separase antibodies used are directed against the C terminus of separase. The arrowhead and arrows indicate full-length and cleaved separase, respectively, as in (A). (C) Separase activity was analyzed in SCC1-myc cleavage assays as in Figure 3B. The top panel shows full-length SCC1-myc (arrowhead) and its larger C-terminal cleavage product (arrow). The bottom panel shows the smaller C-terminal cleavage of SCC1-myc (arrow). Similar results were obtained with the human peptide inhibitor Bio-DREIMR-amk (data not shown).

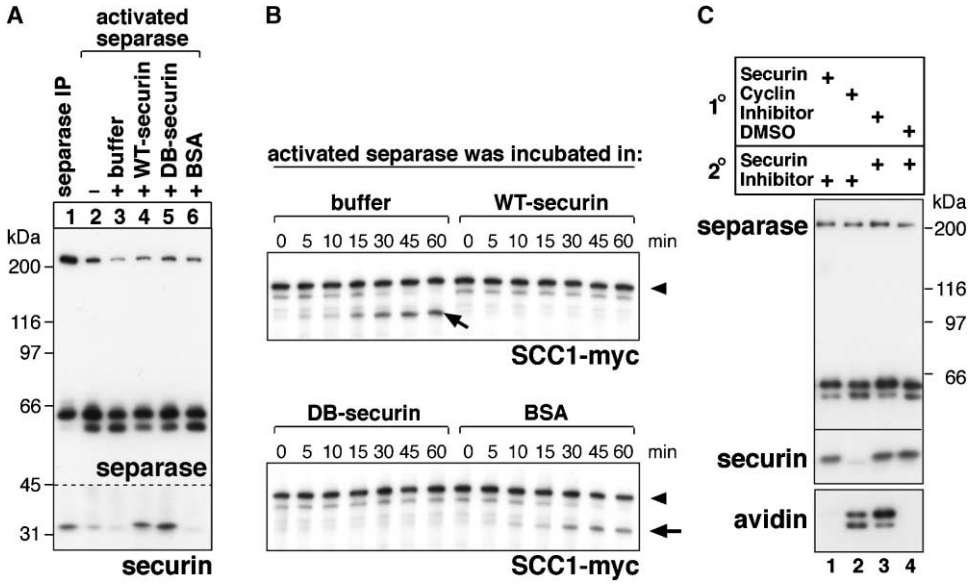


Figure 5. Securin Prevents Binding of Peptide Inhibitors to Separase

(A) Securin can rebind to activated separase. Separase immunoprecipitates (IP) from HeLa cells (lane 1) were incubated in mitotic *Xenopus* extracts (lane 2). After reisolation, separase was incubated in the presence of buffer (lane 3), wild-type securin (WT, lane 4), a destruction box mutant of securin (DB, lane 5), or BSA (lane 6) and was subsequently washed again. Aliquots were analyzed by immunoblotting with securin (bottom panel) and C-terminal separase antibodies (top panel).

(B) Securin binding inhibits activated separase. Separase samples prepared as in (A) were analyzed in SCC1-myc cleavage assays as in Figure 3B. Full-length and cleaved SCC1-myc are marked by an arrowhead and an arrow, respectively.

(C) Securin prevents binding of peptide inhibitors to separase. Activated separase was incubated with securin (lane 1) or as a control with a fragment of cyclin B (lane 2). Unbound proteins were washed away, and separase was incubated with 90 μM Bio-DREIMR-amk, washed again, and analyzed by blotting with avidin or with securin and separase antibodies. Alternatively, active separase was first incubated either with peptide inhibitor (lane 3) or DMSO (lane 4) and was subsequently incubated with securin.

N-terminally FLAG-tagged separase with securin in HeLa cells. FLAG immunoprecipitates were incubated in mitotic *Xenopus* extracts to allow securin destruction, and the resulting separase cleavage fragments were analyzed by immunoblotting with FLAG antibodies and with antibodies to the C terminus of separase. Both N- and C-terminal fragments could clearly be detected in the immunoprecipitates, indicating that they remain bound to each other after cleavage (Figure 6). Although separase is cleaved at at least two sites, only a single 170-kDa band could be detected in the FLAG immunoblots. However, it is possible that the 170-kDa band contains two N-terminal fragments of slightly different lengths that were not resolved by SDS-PAGE. Transfection of separase without securin confirmed the earlier observation [19] that securin is required for the accumulation of separase (Figure 6).

Separase Cleavage Is Not Essential for SCC1 Cleavage

To address whether separase cleavage is required for its activation, we first mapped the cleavage sites in separase. We generated a series of N-terminal deletion mutants of separase and compared their electrophoretic mobilities to those of the C-terminal separase cleavage fragments. This identified the sequences EILR^{1506} and ELLR^{1535} as putative cleavage sites in which cleavage would occur after the arginine residues (Figures 7A and S2). These sites were also independently identified by M. Kirschner and colleagues (cited in [1]). We mutated these sites by exchanging the position of the glutamate and the arginine residue with each other, because swapping these residues in the SCC1 cleavage sites had abolished cleavage completely [13]. To analyze the cleavability of the mutants, we used an N-terminally FLAG-tagged version of separase that contains a TEV protease recognition site between the tag and the first amino acid residue of separase. We coexpressed this form of separase together with securin in HeLa cells, isolated it by FLAG immunoprecipitation, activated it in mitotic *Xenopus* extracts, and eluted it from the antibody beads by incubation with TEV protease. We then analyzed different separase mutants for the presence of C-terminal cleavage products by immunoblotting and for their ability to cleave SCC1. We also analyzed the FLAG immunoprecipitates by immunoblotting with securin antibodies, which revealed that securin bound to all mutants of separase equally well and that securin was effectively destroyed in mitotic *Xenopus* extracts in all cases (Figure S3).

A mutant in which ELLR^{1535} was changed to RLLE^{1535} was still cleaved at the first site, but cleavage at the second was almost completely abolished, confirming that separase is normally cleaved at ELLR^{1535} (Figure 7B, lane 6). In contrast, a mutant in which EILR^{1506} was changed to RILE^{1506} was still cleavable at two sites (Figure 7B, lane 3). The shorter C-terminal fragment appeared unchanged in this mutant, but the longer one migrated more slowly than its wild-type counterpart,

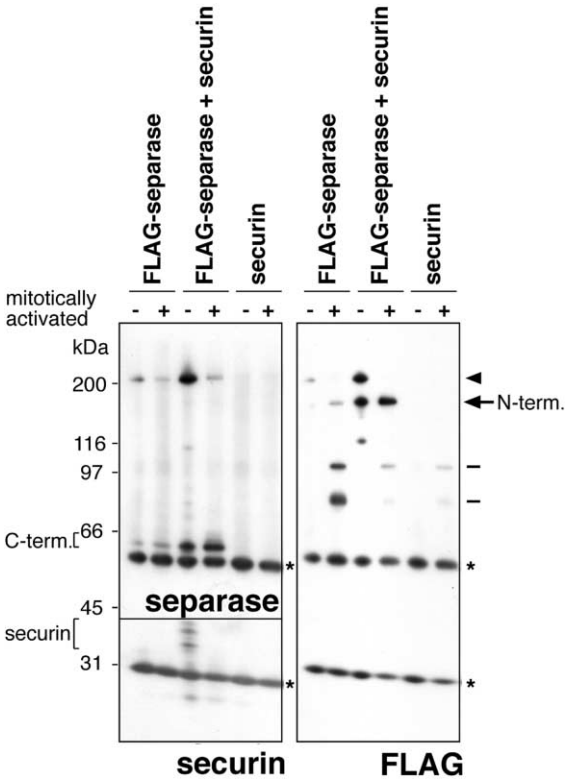


Figure 6. N- and C-Terminal Separase Fragments Remain Associated with Each Other

Extracts of HeLa cells expressing N-terminally FLAG-tagged separase, myc-tagged securin, or both were used for immunoprecipitation with anti-FLAG antibodies. The immunoprecipitates were analyzed by immunoblotting with FLAG, securin, and C-terminal separase antibodies before (−) and after (+) incubation in mitotic *Xenopus* extracts. Full-length separase is marked by an arrowhead, an N-terminal cleavage product is marked by an arrow, and IgG chains are marked by stars. Two proteins that bind nonspecifically to the antibody beads in *Xenopus* extracts and are recognized in FLAG immunoblot reactions are indicated by bars.

and this suggests that cleavage had now occurred after a residue other than 1506. Inspection of sequences upstream of EILR^{1506} identified another putative cleavage site, EIMR^{1486}. Mutation of this site together with mutation of the other two sites abolished separase cleavage almost completely (Figure 7B, lane 5). These observations suggest that separase is normally cleaved at ELLR^{1506} and ELLR^{1535}, but that mutation of the first of these sites results in cleavage at the more upstream site, EIMR^{1486}. Long immunoblot exposures revealed that some fragments could still be detected in EIMR^{1486}-EILR^{1506}-ELLR^{1535} triple mutants (Figure 7A, lane 5), and the presence of these fragments implies that some cleavage of the triple mutant may still occur, albeit at very low levels. We furthermore observed one additional band that was migrating more slowly than the fragment generated by cleavage after the very first site, EIMR^{1486} (Figure 7B, marked by a dot in lane 4). This band disappeared following treatment of separase samples with λ-protein phosphatase, indicating that it might represent a phospho-isoform and not a distinct cleavage product (data not shown).

We also generated mutants in which the putative active site cysteine^{2029} was mutated to alanine, and a mutant in which serine^{1126} was changed to alanine. Stemmann and colleagues [25] had shown that phosphorylation of serine^{1126} inhibits separase. We found that separase^{S1126A} was cleaved to the same degree as wild-type separase and also showed similar SCC1 cleavage activity (see below), suggesting that separase was not subject to inhibitory phosphorylation under our isolation and assay conditions. In contrast, separase^{C2029A} did not yield any cleavage products and was unable to cleave SCC1, and this confirmed that cysteine^{2029} is critical for protease activity (Figures 7B and 7C).

When we tested the different separase mutants in SCC1 cleavage assays, we did not observe obvious differences in their activity, with the exception of separase^{C2029A}, which was inactive (Figure 7C). Because limiting amounts of the substrate SCC1 were used in this assay, we performed a dose-response experiment with the same amount of SCC1 and varying amounts of either wild-type separase or the EIMR^{1486}-EILR^{1506}-ELLR^{1535} triple mutant. Also under these conditions, in which separase activity was limiting, no differences between the activities of wild-type and mutant separase could be detected (Figure 7D). These results suggest that SCC1 cleavage by separase does not depend on the cleavage of separase.

Separase Cleavage Occurs Autocatalytically

During the course of our experiments, we made a number of observations that suggest that separase cleavage occurs autocatalytically. First, we noticed that inhibition of separase by peptide derivatives inhibited separase cleavage (Figures 4A and 4B, lanes 5, and S1). Second, rebinding of securin to activated separase also correlated with a slight decrease in the abundance of cleaved separase (Figure 5C, upper panel, compare lanes 1 and 2). Third, all three cleavage sites that we identified in separase contain the consensus ExxR, which is also found in the cleavage sites of known separase substrates (Figure 7A). Finally, we observed that in vitro-translated separase can be cleaved by active separase isolated from HeLa cells (Figure S4). Together, these observations indicate that separase cleaves itself once it has been activated by securin destruction. Recently, Stemmann et al. also reported autocatalytic cleavage of separase [25].

Discussion

Genetic and biochemical observations in different organisms indicate that sister chromatid separation is initiated by the protease separase, which dissolves cohesion between sister chromatids by cleaving cohesin. Our work further supports this model by showing that inhibition of separase expression in human cells causes phenotypes in interphase and mitosis that are consistent with an essential role of separase in anaphase. We observed that HeLa cells lacking physiological amounts of separase become polyploid and form unusually large and lobed nuclei, suggesting that they were able to rereplicate their DNA but failed to undergo proper mito-

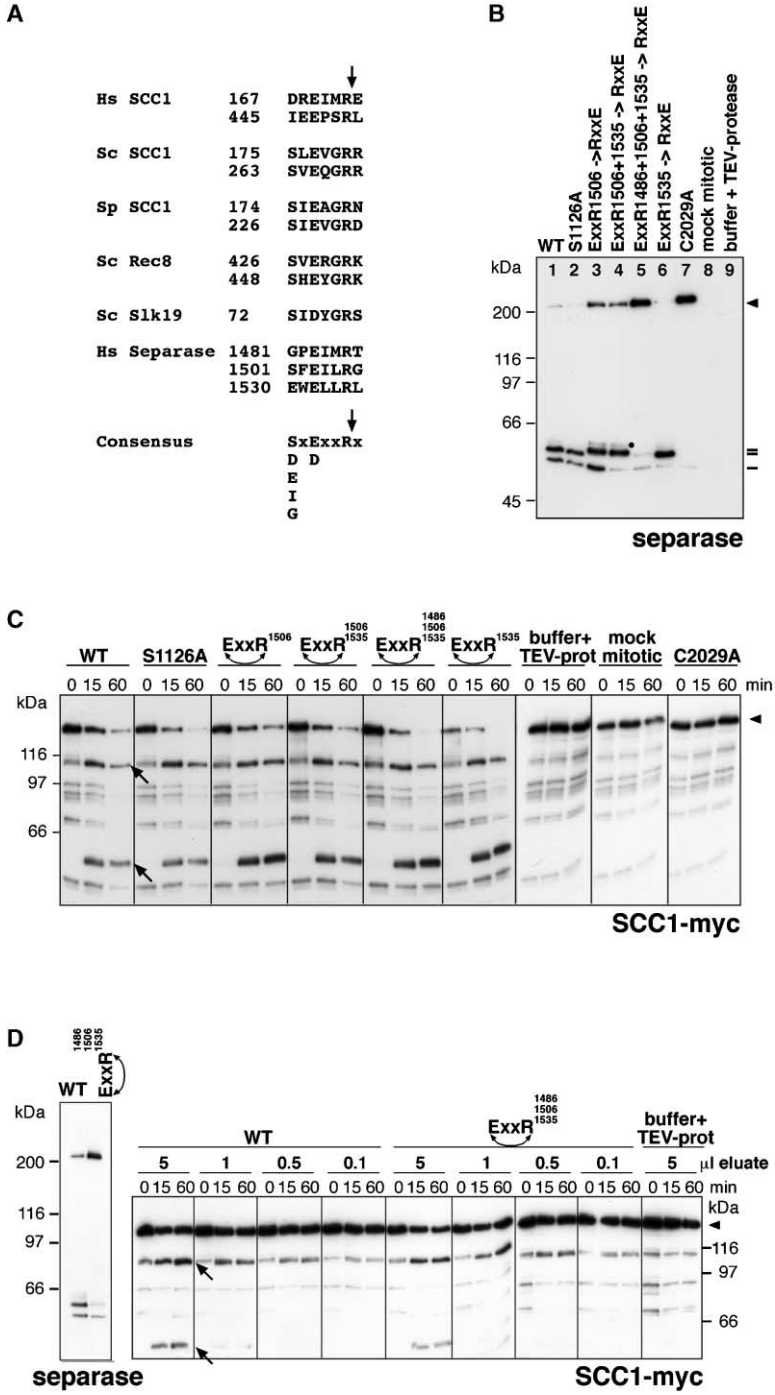


Figure 7. Noncleavable Separase Can Cleave SCC1

(A) Sequence alignment of cleavage sites in human separase and known separase substrates in humans (Hs), budding yeast (Sc), and fission yeast (Sp). The arrow indicates the arginine residue after which cleavage occurs. "x" represents any amino acid residue.

(B) Isolation of recombinant soluble forms of wild-type (WT) and mutated separase. Extracts of HeLa cells coexpressing securin and different forms of FLAG-TEV-separase were used for immunoprecipitation with anti-FLAG antibodies. After incubation in mitotic *Xenopus* extracts, separase was eluted with TEV protease and was analyzed by immunoblotting with antibodies to its C terminus. Full-length separase is marked by an arrowhead, three different C-terminal separase cleavage fragments are marked by bars, and a phosphorylated isoform of a C-terminal fragment is marked by a dot.

(C) The activities of wild-type and mutated forms of separase isolated as in (B) were analyzed in SCC1-myc cleavage assays as in Figure 3B. Full-length SCC1-myc is marked by an arrowhead, and C-terminal cleavage fragments are marked by arrows.

(D) Titration of wild-type and noncleavable separase in SCC1-myc cleavage assays. Wild-type and noncleavable separase were isolated as in (B) and were analyzed by immunoblotting with C-terminal separase antibodies (left panel). Different amounts of the separase preparations were used in SCC1-myc cleavage assays (right panel).

sis and cytokinesis. Consistent with this interpretation, polyploid cells have also been observed in yeast and *Drosophila* cells containing mutant alleles of separase [6, 8, 27, 28] and in human cells expressing noncleavable cohesin mutants [13].

We further observed that chromosome spreads from separase knockdown cells contained abnormal metaphase-like figures. Chromosomes were often arranged in groups that were smaller than normal metaphase plates. We presently do not know the molecular cause of this phenotype, but it is conceivable that sister chromatid cohesion could not be resolved in these chromosomes. As a consequence, these chromosomes may have been grouped together more closely because they were subject to increased spindle pulling forces in anaphase. Alternatively, it is possible that loss of separase expression had effects on spindle function, although, so far, we have been unable to detect obvious spindle defects by immunofluorescence microscopy (data not shown). Importantly, we observed numerous chromosome figures in which unseparated sister chromatids had begun to decondense, suggesting that they were

derived from cells that were exiting mitosis without having separated their sister chromatids.

We performed our RNA interference experiments in HeLa cells, which are unable to arrest in G1 in the presence of a tetraploid or polyploid DNA content and therefore continue to rereplicate their DNA. If loss of separase expression caused a defect in sister chromatid separation, one would expect to observe chromosomes containing four chromatids (diplochromosomes) in these cells. Such chromosomes have been observed in separase-defective *Drosophila* mutants [8, 18], whereas separase mutants in other organisms have not been analyzed for the presence of diplochromosomes yet. To our surprise, we have so far been unable to observe diplochromosomes in separase knockdown cells. Low but significant numbers of diplochromosomes have been found in HeLa cells expressing noncleavable cohesin, but, unexpectedly, these showed only some cohesion along chromosome arms and not at centromeres, where sister chromatids are normally tightly held together in metaphase [13]. One possible explanation for these conflicting observations is that separase is required for the timely resolution of sister chromatid cohesion at the onset of anaphase but that sisters can nevertheless be separated from each other by an unknown mechanism in the subsequent interphase, perhaps during DNA replication. In this respect, it would be interesting to analyze if diplochromosomes in *Drosophila* separase mutants contain fully replicated DNA, or if centromeres were not replicated in this case.

Expression of noncleavable cohesin often blocks sister chromatid separation without inhibiting the initiation of cytokinesis, resulting in a "cut" phenotype [13]. Surprisingly, we did not observe any cells with this phenotype after inhibition of separase expression. We presently do not know if this difference is due to technical aspects; for example, differences in the strength of phenotypes obtained by separase RNAi and by expression of noncleavable cohesin, or if the difference is due to additional functions of separase. For example, the difference in phenotype could be explained by the existence of additional separase substrates whose cleavage is required for the initiation of cytokinesis.

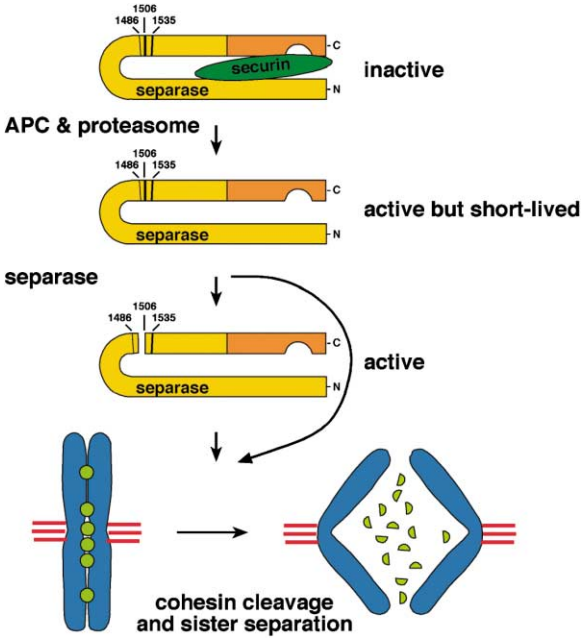


Figure 8. A Model Illustrating How Securin Destruction May Activate Separase

The model proposes that securin inhibits separase by shielding its active site. Securin destruction mediated by the APC and the proteasome would therefore allow access of substrates to separase's active site and would result in autocatalytic cleavage of separase into fragments that remain bound to each other. Both full-length and cleaved separase may be able to cleave cohesin and to initiate sister chromatid separation. It is not yet known to which parts of human separase securin binds, but data in budding yeast suggest that securin can bind to both the evolutionary conserved C terminus of separase (shown in orange) and to nonconserved N-terminal regions [29]. In *Drosophila*, the securin Pimples binds to the separase C terminus and to Three-rows, which may correspond to the N terminus of separase in other organisms [8, 35].

How Does Securin Destruction Activate Separase?

Several observations have shown that securin proteolysis is essential for separase activation, but it has so far been unknown how securin inhibits separase and how securin destruction relieves this inhibition. Our biochemical experiments with peptide inhibitors suggest that securin inhibits separase by preventing the access of substrates to the active site of separase (Figure 8). During preparation of this manuscript, Hornig et al. also reported that securin prevents the binding of substrates to separase in budding yeast [29]. We do not yet know if securin shields separase's active site directly or if it induces a conformational change in separase that blocks substrate access indirectly. If the latter possibility were true, the securin-induced conformational change had to be reversible because we observed that securin is able to reinhibit already-activated separase. We further note that caspases, which are distantly related to separase, can be inhibited by the insertion of inhibitor of apoptosis (IAP) proteins into the active site of caspases [30, 31]. It is therefore possible that securin is a noncleavable pseudosubstrate that inhibits separase by directly binding to its active site.

This interpretation does not imply, however, that securin does only bind to the active site and not to other regions of separase. In fission yeast, the securin Cut2 has been shown to bind to an N-terminal part of separase [32], and, in budding yeast, Pds1 interacts with both the N- and C-terminal parts of separase [29]. Likewise, *Drosophila* Pimples has been shown to bind to both Three-rows and separase, and this binding is consistent with the possibility that Three-rows corresponds to an N-terminal portion of separase in other organisms [8]. Furthermore, we observed that securin can still bind to separase, whose catalytic site had been occupied by a peptide inhibitor, also suggesting that securin can interact with separase regions outside the active site.

Our finding that peptide inhibitors can bind to the active site of full-length separase only if the inhibitor is present during securin degradation implies that securin proteolysis uncovers the active site of separase that then rapidly cleaves itself. This cleavage reaction nor-

mally occurs at either one of two sites. However, if the first of these is mutated, a third site further upstream can also be used, as if separase "searches" for a cleavable site nearby if the usual site is changed in its sequence. This implies that both the amino acid sequence around the cleavage site and its position within the protein are important determinants of autocatalytic cleavage.

Why Is Human Separase Cleaved?

Active preparations of separase contain predominantly cleaved separase. Only these, and not the residual amounts of full-length separase that are also present, can be labeled with peptide inhibitors, indicating that cleaved separase must be an active protease, whereas full-length separase in these preparations may still be bound and inhibited by securin. This notion is also supported by the observation that separase cleavage coincides with cohesin cleavage in vivo, which is in line with the model that securin degradation initiates separase autocleavage into a processed form that then catalyzes cohesin cleavage.

Do these findings imply that separase cleavage is required to generate a form of the protease that is able to cleave cohesin? Although an attractive hypothesis, our present data suggest that this is not the case. Separase mutants whose autocleavage sites have been mutated are as active in cleaving SCC1 in vitro as is wild-type separase, despite the fact that only little residual autocatalytic processing of the mutated separase could be detected. It remains possible that the conditions of our in vitro SCC1 cleavage assay are so different from cohesin cleavage in vivo, that differences in the activities of wild-type and noncleavable separase were simply not revealed. We consider it more likely, however, that separase cleavage may serve some other unknown function in vivo.

Rao et al. [33] showed recently that Scc1/Mcd1 cleavage products in budding yeast are rapidly degraded by the N-end rule pathway. This pathway recognizes proteins depending on the identity of their N-terminal residue and targets proteins with certain "destabilizing" N termini for degradation via ubiquitination [34]. Interestingly, autocleavage of separase at the most C-terminal site, ELLR^{1535}, generates a C-terminal fragment whose N-terminal leucine residue is predicted to be highly destabilizing. Although the stability of separase cleavage products has not been measured yet, we note that the steady-state level of this C-terminal fragment is lower than the level of the longer fragment [14]. It is therefore possible that separase autocleavage generates a cleaved form that can be recognized by the N-end rule and is therefore rapidly degraded. The longer separase fragment generated by cleavage after EILR^{1506} carries the "stabilizing" amino acid residue glycine at its N terminus, but it is possible that this fragment could also be converted into an unstable form by further autocleavage at ELLR^{1535}. Separase autocleavage could thereby prepare the enzyme for its own destruction, which could be an important prerequisite for the establishment of new cohesion in the subsequent cell cycle. This hypothesis will require further testing by an in vivo analysis of noncleavable separase mutants.

Conclusions

Our separase siRNA experiments suggest that separase function is required for sister chromatid separation during mitosis in human cells. Our data are consistent with the idea that securin prevents access of substrates to the active site of separase. Upon release of its inhibitor securin, separase cleaves itself at one of three potential sites into fragments that remain noncovalently associated with each other. As separase cleavage is not essential for its activation in vitro, it is conceivable that separase cleavage prepares the enzyme for its subsequent inactivation.

Supplementary Material

Supplementary Material including the Experimental Procedures and additional data about separase cleavage and cloning of full-length separase is available at http://images.cellpress.com/supmat/supmatin.htm.

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