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The Dual Mechanism of Separase Regulation by Securin

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Summary

Background: Sister chromatid separation and segregation at anaphase onset are triggered by cleavage of the chromosomal cohesin complex by the protease separase. Separase is regulated by its binding partner securin in two ways: securin is required to support separase activity in anaphase; and, at the same time, securin must be destroyed via ubiquitylation before separase becomes active. The molecular mechanisms underlying this dual regulation of separase by securin are unknown.
Results: We show that, in budding yeast, securin supports separase localization. Separase enters the nucleus independently of securin, but securin is required and sufficient to cause accumulation of separase in the nucleus, where its known cleavage targets reside. Securin also ensures that separase gains full proteolytic activity in anaphase. We also show that securin, while present, directly inhibits the proteolytic activity of separase. Securin prevents the binding of separase to its substrates. It also hinders the separase N terminus from interacting with and possibly inducing an activating conformational change at the protease active site 150 kDa downstream at the protein's C terminus.
Conclusions: Securin inhibits the proteolytic activity of separase in a 2-fold manner. While inhibiting separase, securin is able to promote nuclear accumulation of separase and help separase to become fully activated after securin's own destruction at anaphase onset.

Introduction

Replicated sister chromatids in the G2 phase of the cell cycle are held together pairwise by the chromosomal cohesin complex. In metaphase, cohesin provides the cohesion between sister chromatids to withstand the pulling force of the mitotic spindle. Sister chromatid separation at anaphase onset is triggered when the Scc1 subunit of cohesin is cleaved by the protease separase to destroy the cohesin complex (reviewed in [1, 2]). For the faithful execution of both the alignment of sister chromatid pairs on the metaphase spindle and their segregation into daughter cells in anaphase, the status of cohesin and the activity of separase must be tightly controlled. The nature and possible regulation of the cohesiveness of cohesin is still poorly understood. It is clear, however, that cohesin is the central mediator of sister chromatid cohesion in metaphase and that cleavage of its Scc1 subunit by separase destroys its cohesiveness in order to initiate sister chromatid separation in anaphase in, probably, all eukaryotes [3–7].

Separases are large proteins of 150–230 kDa in different species (an exception, *Drosophila*, in which the separase gene seems to have split in two, is described in the Discussion). Separases have been initially identified as Esp1 in budding yeast and Cut1 in fission yeast [8, 9]. A C-terminal region, spanning ca. 50 kDa, is conserved in all species and has been called "separase domain". The second half of this separase domain harbors the conserved cysteine and histidine residues of the protease active site and has been predicted to adopt the fold of CD clan proteases [4, 10]. The large regions N-terminal of the separase domain do not show obvious conservation between species. The contribution of these extended N termini to separase function has remained unclear. In fission yeast, N-terminal sequences are required for the function of separase and have been implicated in its nuclear localization, and more central sequences have been implicated in the possible cytoplasmic retention of the protein [11]. Also in fission yeast, as well as in budding yeast, N-terminal regions are thought to be the sites of interaction with separase's binding partner, securin [12–14].

The initial characterization of securin, Pds1 in budding yeast, uncovered two complementary roles: securin, although not essential, is required for efficient chromosome segregation in anaphase; and, at the same time, securin is needed to prevent anaphase in response to spindle and DNA damage [12, 13, 15–18]. It became clear that securin is a separase inhibitor that has to be degraded via ubiquitylation by the anaphase-promoting complex (APC) [19–21]. While present in cells, securin is bound to separase [13, 14], and, in crude in vitro systems, this prevents separase from cleaving Scc1 [3, 22]. Whether securin is itself sufficient to inhibit separase, and by which mechanism securin prevents separase from attacking Scc1, is unknown. In particular, it is unclear how binding of securin to the separase N terminus could influence the activity of the protease domain that is located far downstream at the protein's C terminus. We describe here that protease activity requires the separase N terminus that binds to the proteolytic site at the protein's C terminus. Securin disrupts this interaction, suggesting a mechanism for how securin inhibits separase activity.

The inhibition of separase by securin could suggest that, in cells lacking securin, separase is overly active, but this is not the case. Indeed, budding yeast and human cells lacking securin show compromised separase function [13, 23], and, in fission yeast and *Drosoph-*

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ila, securin is essential for sister chromatid separation and therefore apparently for separase activity [12, 24]. The supportive role of securin for separase activity has been explained in two ways. Securin might ensure that separase adopts its proper fold required for proteolytic activity [23]. Securin has also been implicated in the subcellular localization of separase [11, 14]. Here, we show that securin in budding yeast is both sufficient to drive the nuclear accumulation of separase and enable full catalytic activity of separase after its own destruction in anaphase.

Results

Securin Promotes Separase Nuclear Accumulation Independent of the Cell Cycle

A possible reason why cells lacking securin show reduced separase function in budding yeast might be the incorrect localization of separase in the absence of securin. This has recently been studied in budding yeast strains overexpressing separase [14]. To address this under more natural conditions, we observed separase Esp1 by virtue of myc epitopes that were added to the genomic copy of the *ESP1* gene (Figure 1A). In G1 cells, when securin was absent, about 50% of the cells showed a weak separase accumulation in the nucleus. At the G1/S transition, separase was enriched in the nucleus of over 80% of cells, and the nuclear accumulation further increased during the G2 and M period. All cells in early anaphase, when securin abruptly disappeared, showed a strong nuclear concentration of separase (Figure 1A). In marked contrast, cells deleted of securin showed a seeming exclusion of separase from the nucleus at all cell cycle stages (Figure 1A). This shows that securin is required for accumulation of separase in the nucleus. Although securin disappeared in anaphase and was absent in wild-type G1 cells, separase was still concentrated in the nuclei of some of these cells. In contrast, separase seemed to be excluded from all nuclei of cells deleted of securin. The reason for this is unclear; it could be because separase leaves the nucleus rather slowly after securin has been destroyed. Alternatively, separase that was bound to securin in the previous metaphase might be in a different functional state compared to separase that has never seen securin.

The cohesin subunit Scc1 is cleaved on time in budding yeast cells lacking securin [25], suggesting that a certain level of separase, sufficient to cleave Scc1, can reach the nucleus even in the absence of securin. To address this directly, we observed separase on chromatin spreads in which cytoplasmic components of the cell are washed away [26, 27]. Separase was seen associated with metaphase chromatin in wild-type cells, and to a lesser but still significant extent, it was seen on spreads from metaphase cells lacking securin (Figure 1B). Separase was no longer chromatin-associated in anaphase. Instead, and as reported previously [13, 14], separase was visible at spindle poles and, in wild-type cells, also at the anaphase spindle. The association of a low level of separase with chromatin in the absence of securin indicates that separase can indeed enter the nucleus independently of securin.

Securin binds and inactivates separase before cells enter S phase, but separase gradually accumulates in nuclei throughout G2, reaching maximum levels only in mitosis. This could mean that cell cycle-dependent events other than the presence of securin contribute to nuclear accumulation of separase. However, separase nuclear accumulation was unchanged when DNA replication was blocked using the replication inhibitor hydroxyurea or when all of the mitotic cyclins, Clb1–4, were inactivated [28] (data not shown). Alternatively, the presence of securin might be sufficient to cause nuclear accumulation of separase, but it might be a relatively slow process or require an excess of securin over separase. In this case, even G1 cells should accumulate nuclear separase if securin is ectopically expressed. To test this, we arrested cells lacking securin stably in G1 by pheromone treatment and then induced expression of securin from the galactose-inducible *GAL1* promoter (Figure 1C) [20]. As soon as securin appeared in the nucleus, separase had redistributed and was also concentrated in the nucleus, reminiscent of cells in mitosis. (Figure 1C). This demonstrates that expression of securin in G1 is sufficient to cause nuclear accumulation of separase. Because levels of securin after expression from the *GAL1* promoter were about 10-fold higher than endogenous levels in metaphase, an excess of securin might be sufficient to promote fast nuclear separase accumulation. Together, this suggests that separase can enter the nucleus independently of securin, but that the presence of securin is required and sufficient to cause nuclear concentration of separase.

One possible mechanism to explain this observation is that separase is in an equilibrium of import and export from the nucleus. Securin, once present in the nucleus, could act to prevent nuclear export of separase. To test this hypothesis, we searched the amino acid sequence of separase and found a putative bipartite nuclear localization sequence (NLS) RKAQNLALSLLKKKNK at amino acids 798–813 as well as several possible nuclear export sequences, $Lx_{1-3}Lx_{2-3}LxL$, for the major nuclear export receptor exportin Crm1/Xpo1. However, mutation of the putative NLS did not interfere with nuclear localization or the functionality of separase, and interference with nuclear export by the *xpo1-1* mutation [29] did not promote nuclear accumulation of separase (data not shown). We conclude that securin causes nuclear concentration of separase by a mechanism different from that which prevents exportin-mediated nuclear export.

Securin Supports Full Proteolytic Activity of Separase

In addition to its role in separase localization, securin might act as a chaperone, possibly facilitating proper folding of the large separase polypeptide. This has recently been suggested for separase isolated from human cells lacking securin [23]. To address this directly in budding yeast, we measured the proteolytic activity of separase in wild-type and securin-deleted cells that were both arrested in G1 by α-factor treatment (Figure 1D). At this stage, securin is absent from both cell types, as it is degraded in anaphase and does not reaccumulate before the next S phase. In contrast to securin-

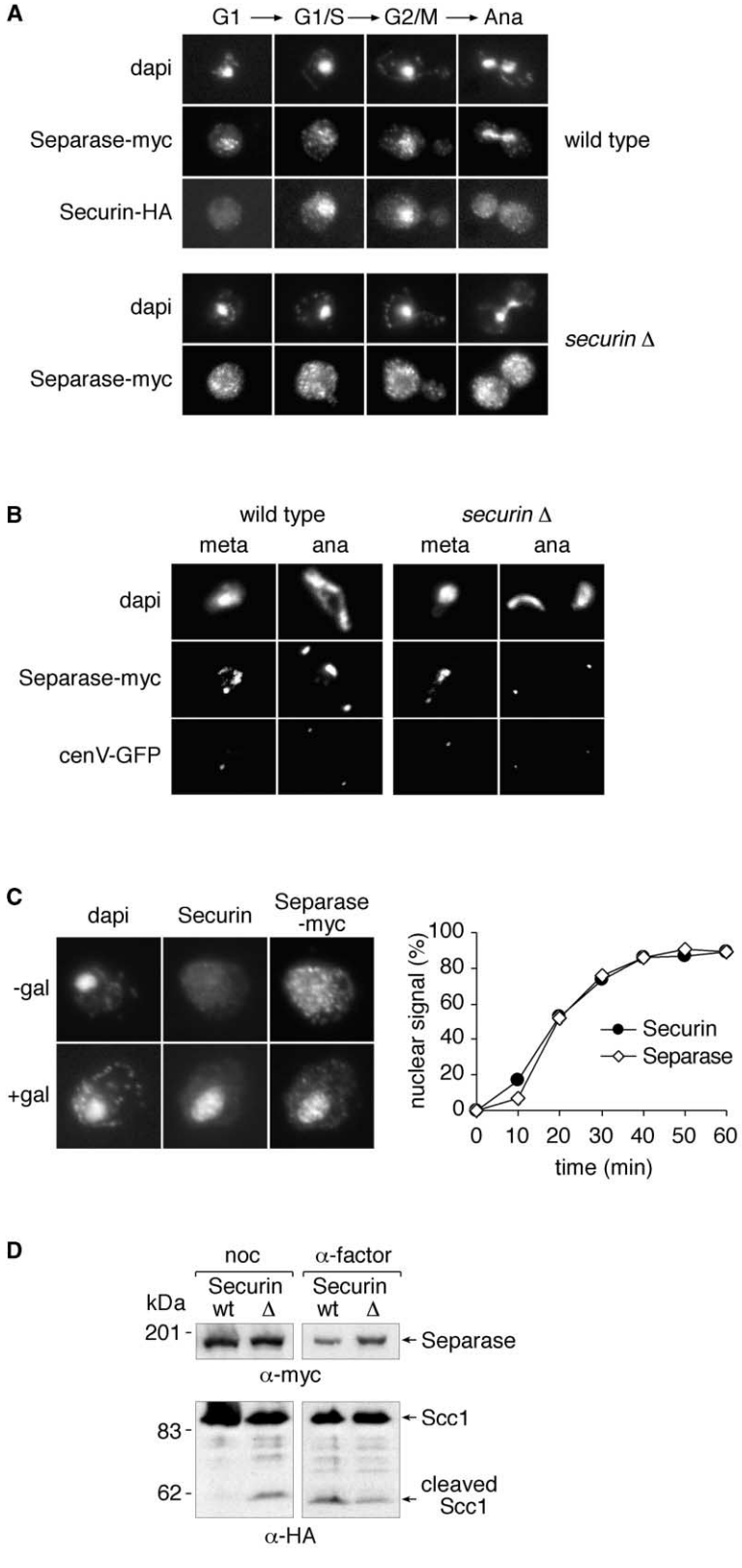

Figure 1. Securin Is Required and Sufficient for Separase Nuclear Accumulation and Enables Separase to Gain Full Activity

(A) Wild-type cells, Y489 (*MATa, ESP1-myc18, POS1-HA6*), and cells deleted for securin, Y291 (*MATa, pds1Δ, ESP1-myc18, TetOs::URA3, TetR-GFP*), were arrested in G1 by mating pheromone α-factor and were released into a synchronous cycle. Cells were fixed and processed for in situ immunofluorescense against the myc epitope on separase using monoclonal antibody 9E10 and against the HA epitope on securin using monoclonal antibody 16B12 (Babco). DNA was visualized by staining with 4′,6-diamidino-2-phenylindole (dapi). Cells at characteristic cell cycle stages are shown.

(B) Chromosome spreads of wild-type cells, Y292 (*MATa, ESP1-myc18, TetOs::URA3, TetR-GFP*), and cells deleted for securin, Y291, in metaphase and anaphase from a similar experiment. The cell cycle stage of individual chromosome spreads was confirmed by visualizing sister chromatids close to centromere V by GFP [41].

(C) Cells deleted for securin, Y576 (*MATa, pds1Δ, ESP1-myc18, GAL1-PDS1mdb*), were arrested in G1 using α-factor, and expression of securin from the *GAL1* promoter was induced by the addition of 2% galactose to the culture. Complete arrest in G1 before and throughout the experiment was confirmed by FACS analysis. Securin was detected using antibody sc-9076 (Santa Cruz Biotechnology). Examples of cells before and 30 min after galactose addition are shown.

(D) Separase activity in wild-type, Y189 (*MATa, ESP1-myc18*), and securin-deleted, Y543 (*MATa, pds1Δ, ESP1-myc18*), cells was measured. Cells were arrested in metaphase (noc) or G1 (α-factor), extracts were prepared, and Scc1 cleavage activity was measured using chromatin from metaphase cells as a substrate [25].

deleted cells, separase in wild-type cells was previously bound to securin. We prepared extracts from both cells that contained comparable levels of separase (Figure 1D). We then measured the protease activity of separase in both extracts by incubating them with saturating levels of Scc1 on a metaphase chromatin preparation. Sep-

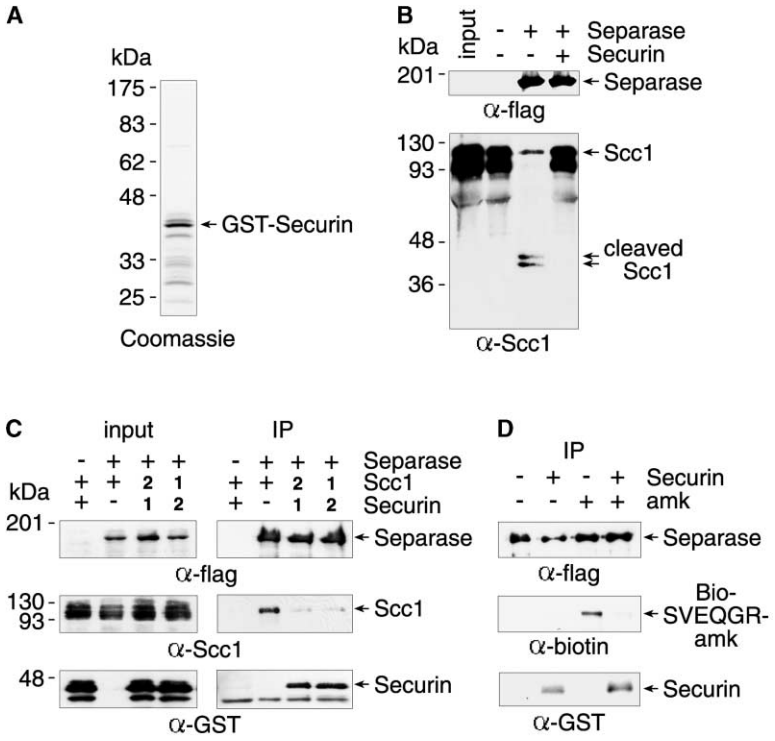

Figure 2. Securin Inhibits Separase Proteolytic Activity, Substrate Binding, and Reaction with a Cleavage Site Peptide

(A) Preparation of the securin fragment (amino acids 256–359) used for the experiments. The fragment, fused to GST, was purified on glutathione sepharose. Numerous breakdown products of the fragment are present in the preparation.

(B) Inhibition of separase cleavage of Scc1. For the cleavage assay, separase was purified on chitin beads, and recombinant Scc1 was added as described [4]. This reaction was performed with or without the addition of the securin fragment.

(C) Separase, which was catalytically inactive due to the active site point mutation H1505A, was purified from strain Y364 (*MATa, pep4Δ, GAL1-flag-ESP1(H1505A)-CBD*) on chitin beads, and the binding of recombinant Scc1 was analyzed. Securin was added either before (1) or after (2) the incubation with Scc1.

(D) Separase was purified from strain Y334 (*MATa, GAL1-flag-ESP1-CBD*), and the binding of the peptide inhibitor Bio-SVEQGR-amk (amk) was measured with or without the addition of securin.

arase in wild-type cell extracts was at least 2-fold more active in cleaving Scc1 compared to separase from securin-deleted cells. This suggests that the initial binding to securin helps separase to acquire activity after securin is destroyed. The 2-fold difference in separase activity is likely an underestimate. Western blotting revealed residual low levels of securin in the wild-type cell extract that may have partly inhibited separase and that might stem from minor impurities of non-G1 cells in our preparation.

Securin Is a Protease Inhibitor of Separase

We then addressed the mechanism by which securin, while bound to separase, inhibits it from cleaving Scc1. Securin could directly inhibit the protease activity of separase, or other proteins might cooperate with securin in rendering or maintaining separase inactive. We have previously established the use of virtually pure separase and Scc1 for an in vitro assay to measure separase proteolytic activity [4]. We now added purified securin to this reaction to see whether it is sufficient to inhibit separase. The C-terminal half of securin is expected to bind to separase [12], so we expressed in bacteria and purified a fragment spanning a C-terminal domain (amino acids 256–359) of securin Pds1 fused to GST (Figure 2A). When we added this recombinant securin fragment to the separase assay, Scc1 cleavage was efficiently inhibited (Figure 2B). In a control reaction, GST alone did not inhibit separase (data not shown). This demonstrates that securin is indeed a protease inhibitor for separase.

Securin is thought to bind to the N terminus of separase [12, 14] while the protease active site is situated close to its C terminus [4]. Securin might therefore cause an allosteric change within separase that inactivates the protease active site, or it might prevent substrates from binding to separase. To test the latter, we measured the binding of recombinant Scc1 to separase that was immobilized via a chitin binding domain tag on chitin beads. We used a separase variant carrying an active site point mutation (H1531A, [4]) to prevent Scc1 from being cleaved after binding. Figure 2C shows that Scc1 binding to separase was readily detected in this assay. When the inactivating securin fragment was incubated with the separase beads before adding Scc1, securin bound to separase, and the binding of Scc1 was prevented. When separase was incubated with Scc1 first to allow binding, and then securin was added, Scc1 was displaced by securin from separase (Figure 2C). This indicates that securin binding to separase prevents the interaction of separase with its substrate, and that the interaction of securin with separase is likely to be stronger, or more stable than, the interaction with Scc1.

We then analyzed the effect of securin on the binding to separase of the protease inhibitor Bio-SVEQGR-amk [4]. This inhibitor is a short, biotinylated peptide spanning an Scc1-derived cleavage site motif linked to a reactive group that covalently binds to and inhibits the separase active site cysteine residue. If securin prevents Scc1 binding to separase by occupying a substrate binding site at the separase N terminus, the small peptide inhibitor might still gain access to the active site at the C terminus. Separase was purified again on chitin beads and was incubated with or without securin, and Bio-SVEQGR-amk was added (Figure 2D). The inhibitor bound to separase, as detected by Western blotting against its biotin moiety, but binding was prevented by preincubation of separase with securin (Figure 2D). This indicates that securin does not only hinder access of

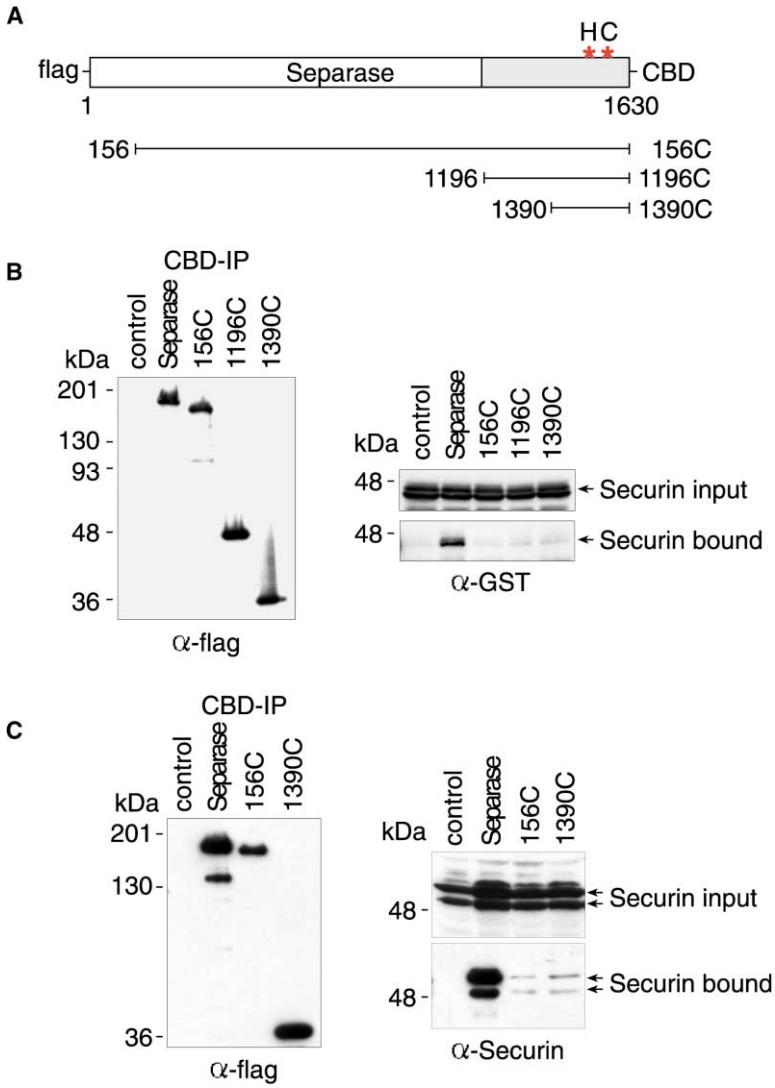

Figure 3. Securin Interactions with Separase

(A) A scheme of the separase N-terminal deletion mutants used in the experiments. The separase domain that shows conservation between all species is shaded. The histidine (H) and cysteine (C) residues that are predicted to form the catalytic dyad are marked with asterisks. The scheme is drawn to scale. The strains that expressed these deletion mutants were Y373 (*MATα, GAL1-flag-ESP1 (156C)-CBD*), Y367 (*MATa, GAL1-flag-ESP1 (1196C)-CBD*), and Y336 (*MATa, GAL1-flag-ESP1(1390C)-CBD*).

(B) Separase and the deletion mutants were purified on chitin beads, and binding of the securin fragment (see Figure 2) was analyzed.

(C) Separase and the deletion mutants together with full-length securin were coover-expressed in yeast, and their association was analyzed after binding separase to chitin beads. Securin was detected using antibody sc-9076.

the substrate protein Scc1, but also of a reactive cleavage site peptide. Peptide binding could be blocked if securin binds tightly into the separase active site groove. However, when the protease active site was occupied by Bio-SVEQGR-amk, this did not perturb the subsequent interaction of securin with separase (data not shown). Indeed, securin makes only a weak contact with the separase C terminus (see below). Therefore, securin might prevent binding of the cleavage site peptide to separase by an alternative mechanism, maybe by causing an allosteric change that distorts the active site.

Securin Interacts with Separase N and C Termini

Because the protease active site is located at the separase C terminus, we wanted to see which parts of separase directly interact with securin. We constructed N-terminal deletion mutants of separase that were overexpressed in yeast and purified on chitin beads as above (Figure 3A). One of the constructs lacked only the first 155 amino acids from the N terminus (156C), another construct comprised the conserved separase domain (1196C), and a third construct only included the C-terminal half of the separase domain that is predicted to fold into the protease domain (1390C). When the inhibiting GST-securin fragment was added, it bound efficiently to full-length separase but bound significantly less well to any of the deletion mutants (Figure 3B). Thus, the very N terminus of separase contributes to the inhibitory interaction with securin. Because we used only a C-terminal part of securin in this binding assay, we could not exclude the possibility of additional contacts between securin and separase. We therefore coexpressed full-length securin and the different separase constructs in yeast and isolated complexes by binding separase to chitin beads (Figure 3C). Again, only full-length separase interacted efficiently with securin. But a weaker interaction of securin with separase was evident after the N terminus was deleted. The efficiency of this interaction was similar when separase lacked only 155 N-terminal amino acids or when only the short C-terminal protease domain was expressed (Figure 3C). This shows that, while the separase N terminus is important for securin binding, securin also makes contact with the C-terminal protease domain in separase.

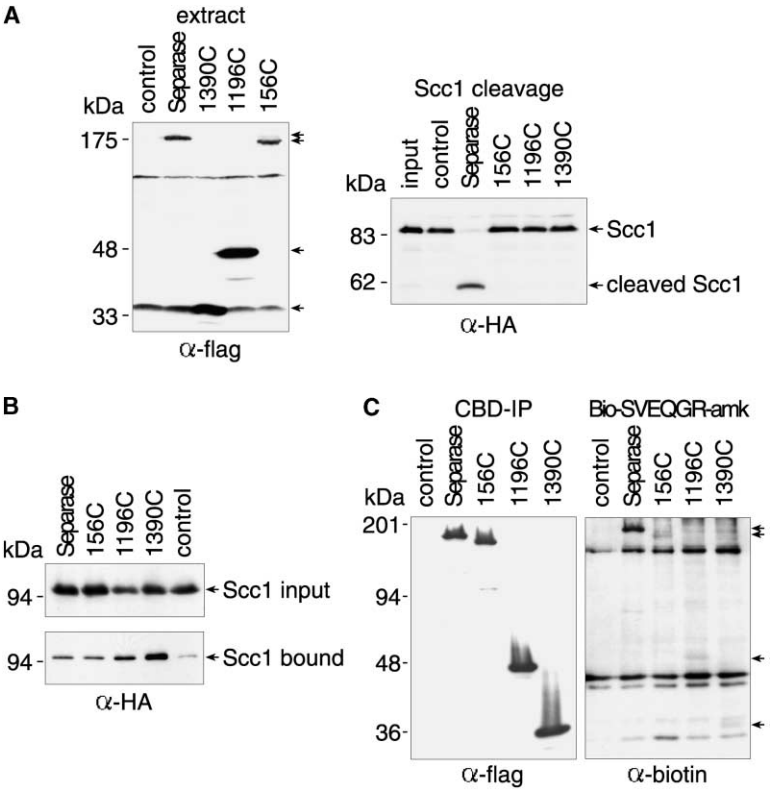

Figure 4. The Separase N Terminus Is Required for Catalytic Activity

(A) Separase and N-terminal deletion mutants were overexpressed in yeast, and separase activity in the extracts was measured using yeast chromatin as the substrate as described [3]. The arrows in the left panel indicate the migration of the individual separase fragments.

(B) A catalytically inactive version of full-length separase and the deletion mutants together with Scc1 were cooverexpressed in yeast. Association of Scc1 with separase was measured after binding separase to chitin beads.

(C) Separase and deletion mutants were immobilized on chitin beads and were incubated with the cleavage site peptide inhibitor Bio-SVEQGR-amk. Binding of the inhibitor was analyzed by probing the Western blot against the biotin moiety of the inhibitor.

The Separase N Terminus Is Required for Protease Activity

What role does the N terminus play for the activity of separase and its inhibition by securin? We first analyzed the separase deletion mutants for protease activity against Scc1. After overexpression in yeast, only full-length separase was capable of cleaving Scc1, while removing the first 155 amino acids completely abolished its protease activity (Figure 4A). This shows that the separase N terminus plays an essential role in the proteolytic activity of the protein.

It might be that separase lacking the N terminus can no longer interact with its substrate Scc1. Alternatively, the N terminus might be required for the C terminus to adopt a proteolytically active conformation. To distinguish between these possibilities, we first analyzed the ability of the N-terminal separase deletion mutants to interact with Scc1 (Figure 4B). All of the deletion mutants interacted with Scc1. Indeed, the interaction was reproducibly enhanced when only the C-terminal protease domain of separase, 1390C, was expressed (Figure 4B). This demonstrates that the separase N terminus is not required for interaction with the substrate Scc1. N-terminal sequences might even be involved in loosening the protease substrate interaction to facilitate turnover of processed substrate.

We then analyzed the binding of the peptide inhibitor Bio-SVEQGR-amk to the separase deletion mutants, which all interacted with Scc1. If the protease domain is in an active state, we would expect the cleavage site peptide inhibitor to be able to bind to these fragments as well. But, while full-length separase efficiently bound the peptide inhibitor, binding was strongly reduced in any N-terminal deletion (Figure 4C). This shows that, while the N terminus is not required for overall interaction of separase with its substrate Scc1, it is required for the C-terminal protease active site to react with the peptide inhibitor. The separase N terminus might be required to stabilize an active conformation of the protease active site. Only the active conformation would allow access of the cleavage site peptide to the active site groove or its attack by the active site cysteine residue. To achieve this, the N terminus might have an allosteric effect on the active site. We cannot exclude that the N terminus might itself become part of the active site or might, for another reason, be required for an active conformation.

Separase N and C Termini Bind Each Other

The dependence of separase activity on its own N terminus predicts that separase N and C termini may interact. To test whether there might be a direct physical association, we cooverexpressed fragments from both ends of separase in yeast. The C-terminal fragments were purified via the attached chitin binding domain, and association with the N-terminal fragments was analyzed by Western blotting against the flag epitopes present on both fragments (Figures 5A and 5B). We first expressed the entire separase sequence in two parts: the C-terminal separase domain (1196C) together with the N terminus (N1195). These two fragments of separase associated with each other in stoichiometric amounts, indicating a stable interaction between them (Figure 5B, lane 3). The interaction was not diminished when only the shorter C-terminal protease domain, 1390C, was expressed (Figure 5B, lane 4), indicating that the contact

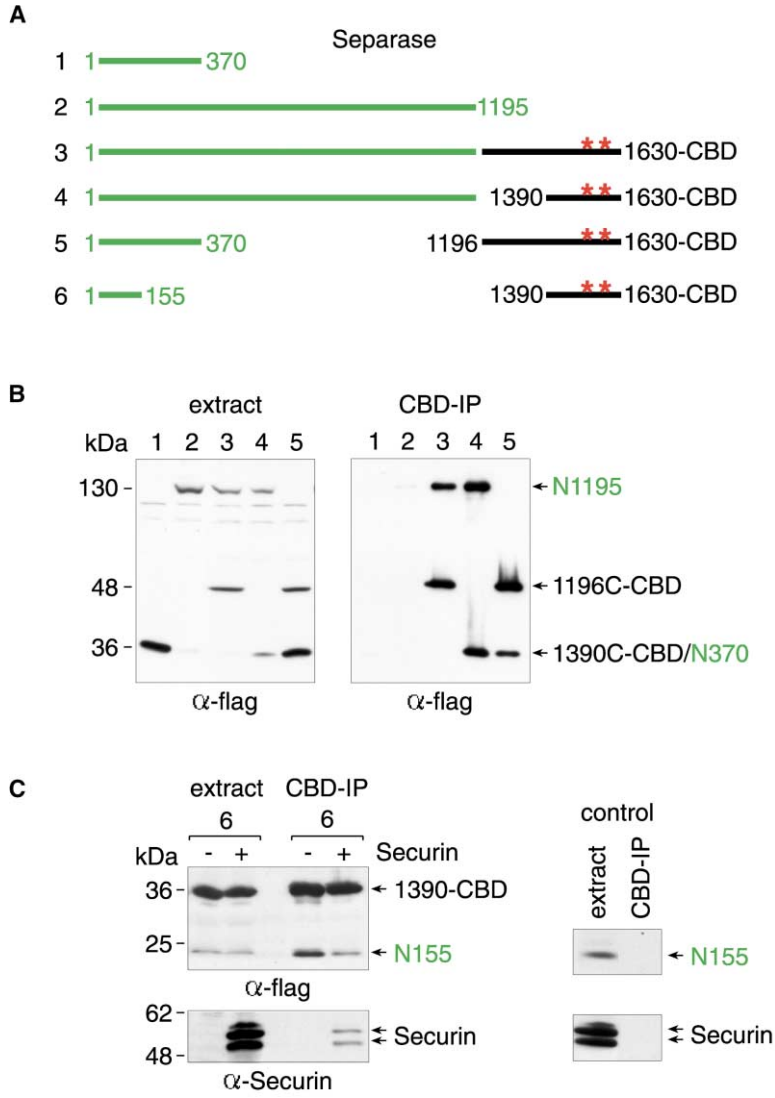

Figure 5. An Interaction between the Separase N and C Termini Is Disrupted by Securin

(A) A scheme of the separase fragments expressed in the experiment. The strains expressing these fragments were (1) Y538 (*MATα, GAL1-flag-ESP1(N370)*), (2) Y490 (*MATα, GAL1-flag-ESP1(N1195)*), (3) Y533 (*MATa/MATα, GAL1-flag-ESP1(N1195), GAL1-flag-ESP1 (1196C)-CBD*), (4) Y532 (*MATa/MATα, GAL1-flag-ESP1(N1195), GAL1-flag-ESP1(1390C)-CBD*), (5) Y541 (*MATa/MATα, GAL1-flag-ESP1(N370), GAL1-flag-ESP1(1196C)-CBD*), and (6) Y561 (*MATa/MATα, GAL1-flag-ESP1 (N155), GAL1-flag-ESP1(1390C)-CBD*).

(B) Extracts from these strains were prepared, and interactions between N- and C-terminal pairs were analyzed after binding of the C-terminal fragments to chitin beads. The numbers correspond to the combinations of fragments, as indicated in (A).

(C) Extracts were prepared and interactions were analyzed as in (B), but securin was cooverexpressed in addition to the separase fragments.

of the N terminus may occur at the protease domain. When a shorter N-terminal fragment comprising residues 1–370 was expressed, this N370 fragment also interacted with the C-terminal separase domain, although with somewhat reduced efficiency (Figure 5B, lane 5). Therefore, while N370 makes contact with the separase C terminus, more central parts of the protein may also be involved.

Securin Disrupts the Separase N/C-Terminal Interaction

We then addressed how the presence of securin would influence the interaction between separase N and C termini. We now expressed 155 N-terminal amino acids (N155) together with the C-terminal protease domain (Figures 5A and 5C). Expression of the N155 fragment from the *GAL1* promoter consistently yielded poor expression levels compared to other separase fragments. Nevertheless, we could clearly detect an interaction of this fragment with the C-terminal protease domain (Figure 5C). When securin was cooverexpressed in the same cells, the interaction between the N155 fragment and the protease domain was markedly reduced. Instead, we could detect securin bound to the protease domain. This indicates that securin is capable of displacing the separase N terminus from the C terminus. The low levels of N155 still bound to the separase domain in the presence of securin could mean that the displacement was not complete under our conditions. Alternatively, it might stem from a trimeric complex in which securin might bind separase N and C termini simultaneously. The recovery of a trimeric complex is expected to be poor because of the relatively weak interaction of securin with the separase C terminus. We conclude that securin interrupts interactions between the separase N and C terminus. Therefore, securin has the potential to disrupt contacts within separase that may be crucial for the activation of the protease active site.

Discussion

We present here studies that address the molecular nature of the dual regulation of separase by securin. An

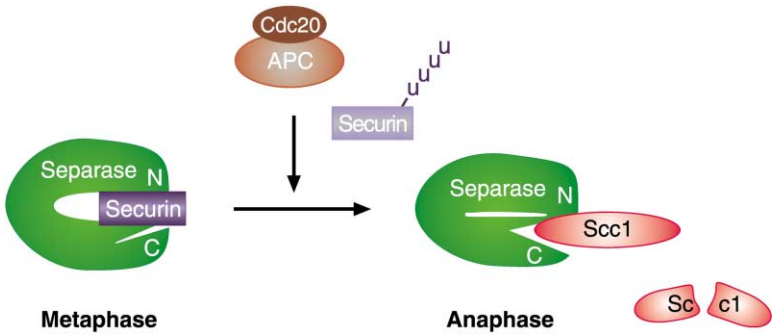

Figure 6. A Model of Separase and Its Inhibition by Securin

Securin binds to both separase's N and C terminus. Thereby, securin may separate these two parts of the protein, acting as a molecular wedge. After securin degradation via ubiquitylation by the anaphase-promoting complex (APC), the separase N terminus is free to interact with the protein's C terminus. Securin might promote the interaction by prepositioning the separase termini. The interaction within separase might induce an activating conformational change at the protease active site at the separase C terminus. Securin, while bound to separase, also prevents access of Scc1 to separase.

analysis of domains and their interactions in fission yeast separase and securin has been reported previously [12], and, more recently, interactions between the *Drosophila* equivalents have been studied [30]. But only since the discovery of separase as a site-specific protease that cleaves the cohesive bond between sister chromatids are we in a position to analyze separase regulation on a molecular level.

Securin forms a tight complex with separase, and we show here that this directly inhibits the protease activity of separase. The architecture of the complex between separase and securin shows unexpected features. Securin binds to both N- and C-terminal regions of separase, and, by doing so, securin disrupts, like a molecular wedge, interactions within separase (Figure 6). Thereby, securin might prevent the separase N terminus from inducing an activating conformational change at the protease active site required to recognize or attack a substrate cleavage site. At the same time, this model also offers a possible explanation for securin's proposed chaperone function that enables efficient activation of separase. By bridging the separase N and C termini, which are separated from each other by over 1500 amino acids, securin might bring them in juxtaposition, preparing them for interaction upon securin destruction. We currently do not know whether the interaction within separase is truly intramolecular, i.e., whether the N terminus of the polypeptide folds back onto its own C terminus. The interaction might likewise happen between two molecules of separase in an intermolecular fashion, thereby forming separase dimers. Dimer formation has been observed for proteases of the CD clan, and, in the case of caspase 9, it has been implicated in the activation of the enzyme [31].

The above observations also go some way in explaining why separases are such large proteins. Sequences very close to the N terminus of the protein are required for protease activity at the protein's C terminus (Figure 6). What about the sequences in between? When we coexpressed fragments from the N and C termini of separase, they efficiently formed complexes, but we were unable to reconstitute protease activity from these fragments (data not shown). This indicates that the intactness of the middle portion of separase is also important for the function of the protease. Consistent with this, when ten temperature-sensitive alleles of the fission yeast separase Cut1 were sequenced, eight were found to encode single amino acid changes in the middle of the protein [12]. This might mean that this region also contributes to the proteolytic activity of separase.

A seeming exception to the separase-securin architecture exists in *Drosophila*. Here, separase is much smaller, and the N terminus does not extend far beyond the conserved separase domain. However, it has recently been suggested that the THR protein in *Drosophila*, which is required for separase activity, might play the role of the separase N terminus. The pattern of interactions of THR with separase and the *Drosophila* securin PIM is reminiscent of the interactions of the budding yeast's separase N terminus with its catalytic C terminus and securin [30]. We therefore suggest that our results for the activation of separase's protease activity and its inhibition by securin might be applicable to *Drosophila* as well, supporting a model in which THR activates separase by an interaction that is prevented by PIM [30].

Will this model of separase inhibition and activation also be applicable to vertebrates? *Xenopus* and human securin must be degraded for separase activation [6, 21], and human securin inhibits human separase in vitro ([22]; I. Waizenegger and J.-M. Peters, personal communication). Securins are poorly conserved between species on the amino acid level, but all contain equivalent clusters of charged residues. While the primary amino acid sequence therefore differs between securins and separases in different species, their overall structure and organization begins to appear very similar. A distinct feature of human separase is that, after its activation by securin degradation, separase cleaves itself into two halves at a position upstream of the separase domain [6, 22]. Processed separase is still active to cleave cohesin. And, consistent with the idea that the N terminus might be required for proteolytic activity, it stays associated with the C terminus after cleavage (I. Waizenegger and J.-M. Peters, personal communication). We have not been able to detect any evidence for self-cleavage of separase in budding yeast (data not shown). Another level of regulation of human separase is its inhibition by Cdk(CDC2)-dependent phosphorylation. This inhibition is effective even after securin is degraded. The phosphorylation takes place in the center of separase, and it will be interesting to see whether it influences an interaction between the N and C termini or inhibits separase by an alternative mechanism. In budding yeast, there is no evidence for Cdk-dependent inhibition of separase.

Sister separation can proceed in the presence of high kinase activity, securin destruction is sufficient to promote anaphase onset, and separase appears to be no longer regulated in the absence of securin [13, 25, 32]. Finally, whether human securin is also involved in recruiting separase to its places of action while it is still keeping the protease inactive has yet to be addressed.

Conclusions

The regulation of separase activity is of critical importance for cells to ensure faithful segregation of their genetic material. Separase is not only the trigger of sister chromatid segregation, but it orchestrates multiple mitotic events, including mitotic spindle stability and mitotic exit [33, 34]. A key regulator of separase, securin, acts to concentrate separase in the nucleus where separase cleaves its known targets in anaphase. While recruiting separase into the nucleus and preparing it for efficient activation, securin inhibits the protease activity of separase. Thereby, an accumulation of inactive separase is built up that can be suddenly unleashed after securin is destroyed at anaphase onset. Securin uses a double strategy to inhibit separase. Not only does securin prevent access of substrates to separase, but securin also seems to inhibit separase by preventing it from activating itself.

Experimental Procedures

Yeast Strains

Epitope tagging of the endogenous separase *ESP1* and securin *PDS1* genes and gene disruption of the *PDS1* gene were performed using PCR-based techniques as described [35, 36]. The construction of strains expressing from the galactose-inducible *GAL1* promoter Scc1 fused to haemagglutinine epitopes, and separase fused to a flag epitope and chitin binding domain, were described previously [4, 37]. Deletion mutants of separase were cloned using conventional techniques under the control of the *GAL1* promoter into vectors of the YIplac series [38]. To the N-terminal deletion mutants, a flag epitope was added at the N terminus, and the chitin binding domain was added at the C terminus. To all C-terminal deletions, a flag epitope was added at the N terminus. Constructs were integrated into the genome, and expression levels were analyzed by Western blotting. Strains overexpressing securin (containing a mutation in its destruction box) from the *GAL1* promoter were obtained as described using plasmid pOC70 [20].

Protein Purification and Biochemical Assays

Amino acids 256–359 of securin were cloned into the bacterial expression vector pGEX-KG [39] by PCR using genomic DNA as the template. Overexpression and purification via glutathione sepharose followed standard protocols [40]. Purification of Scc1 after overexpression in insect cells, and of separase on chitin beads after overexpression in yeast, were as described, as was the assay of endogenous or overexpressed separase activity against Scc1 and the binding of the peptide inhibitor Bio-SVEQGR-amk [4, 25]. To analyze binding of the GST-securin fragment to separase, 1 μg GST-securin was incubated with 20 μl chitin beads, to which separase had been bound as above, in a reaction volume of 25 μl FBX buffer [4]. Incubation occurred for 10 min on ice with agitation, after which the beads were washed and the bound protein eluted in SDS-PAGE loading buffer. To analyze the binding of recombinant Scc1 to separase, 0.2 μg Scc1 was added to the separase-containing beads for 10 min at 25°C. For binding of Scc1 to separase deletion mutants, both proteins were cooverexpressed in the same strain. To detect interactions between N- and C-terminal fragments of separase, the fragments in question were cooverexpressed in a diploid strain obtained by crossing strains expressing the individual fragments.

Other Techniques

In situ immunofluorescense and chromosome spreading were performed as previously described [37].

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