Chapter 4
Structure and Function of the Separase-Securin Complex

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Abstract Separase is a large cysteine protease in eukaryotes and has crucial roles in many cellular processes, especially chromosome segregation during mitosis and meiosis, apoptosis, DNA damage repair, centrosome disengagement and duplication, spindle stabilization and elongation. It dissolves the cohesion between sister chromatids by cleaving one of the subunits of the cohesin ring for chromosome segregation. The activity of separase is tightly controlled at many levels, through direct binding of inhibitory proteins as well as posttranslational modification. Dysregulation of separase activity is linked to cancer and genome instability, making it a target for drug discovery. One of the best-known inhibitors of separase is securin, which has been identified in yeast, plants, and animals. Securin forms a tight complex with separase and potently inhibits its catalytic activity. Recent structures of the separase-securin complex have revealed the molecular mechanism for the inhibitory activity of securin. A segment of securin is bound in the active site of separase, thereby blocking substrate binding. Securin itself is not cleaved by separase as its binding mode is not compatible with catalysis. Securin also has extensive interactions with separase outside the active site, consistent with its function as a chaperone to stabilize this enzyme.

Keywords Sister chromatids · Cohesin · Mitosis · Meiosis · DNA damage repair · Cancer

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Introduction

Precise segregation of chromosomes is a prerequisite of faithful cell division and hence it is a tightly regulated process. During DNA synthesis in the interphase, sister chromatids are paired to each other through cohesion by the ring-shaped cohesin complex. Discovered in the late 1990s, the cohesin ring is primarily composed of three subunits: Smc1, Smc3 and a bridging subunit Scc1 between them, and it is also involved in DNA loop extrusion (Davidson et al. 2019; Kim et al. 2019). Two mechanisms are mainly responsible for removing cohesins from chromosomes during cell division. The first is through the prophase pathway, by which the majority of cohesins located on chromosome arms are removed during prophase, a process involving the Wapl protein, polo-like kinase Plk1, and Aurora B kinase (Sumara et al. 2002; Kueng et al. 2006; Gimenez-Abian et al. 2004; Hauf et al. 2005; Gandhi et al. 2006). However, cohesins close to the centromere region are protected from the prophase pathway and are removed instead during the transition from metaphase to anaphase by the protease separase, thus this pathway is known as the separase pathway. Separase cleaves the Scc1 subunit at two specific sites and opens the cohesin ring to resolve chromosome cohesion in a spatial and timely manner.

Separase was first discovered by yeast genetics in the late 1980s and early 1990s, and its loss of function was related to spindle pole body and polyploid nuclei abnormalities (Uzawa et al. 1990; Baum et al. 1988; May et al. 1992). Separase was further revealed to be the protease that targets the cohesin subunit Scc1/Mcd1 for chromosome resolution (Buonomo et al. 2000; Uhlmann et al. 2000; Waizenegger et al. 2000). Multiple other functional roles have been discovered since then, including apoptosis regulation (Hellmuth and Stemmann 2020), DNA damage repair (Nagao et al. 2004; McAleenan et al. 2013; Hellmuth et al. 2018) and centrosome disengagement and duplication (Tsou et al. 2009; Schockel et al. 2011; Lee and Rhee 2012; Matsuo et al. 2012; Nakamura et al. 2009). Due to its crucial roles in important cellular processes, separase is tightly regulated at many levels, including the inhibition by two well-known inhibitors securin and CDK1-cyclin B1 (Ciosk et al. 1998; Zou et al. 1999; Stemmann et al. 2001; Gorr et al. 2005; Karasu et al. 2019; Li et al. 2019; Holland et al. 2007) and a recently discovered inhibitor shugoshin-MAD2 complex (Hellmuth et al. 2020). Dysregulation of separase leads to aneuploidy and chromosome instability, which has been linked to diseases including cancers (Zhang et al. 2008; Mukherjee et al. 2014a). The molecular mechanisms for the activity and regulation of separase have been unraveled recently by several structural studies (Viadiu et al. 2005; Luo and Tong 2017; Lin et al. 2016; Boland et al. 2017). In this chapter, we discuss the molecular characterization, functional roles, regulation and the structure of separase and its complex with securin.

Domain Organization of the Cysteine Protease Separase

Separase is an endopeptidase that belongs to the CD clan of cysteine proteases, related to the well-known caspases in the same clan. It's substrate contains an arginine at the P_1 position before the scissile bond and several additional residues are conserved for recognition (Lin et al. 2016). The size of separase varies substantially, ranging from 140 to 250 kD in eukaryotes. Sequence analysis shows that the C-terminal region where the catalytic domain is located (Fig. 4.1) has the highest conservation across species. Sequence diversity is observed in the N-terminal region and the unstructured segment in the middle, which contributes to the size variations among separases. Atypical separases have also been found in several species. For example, *Drosophila* separase consists of two proteins, THR and SSE, which correspond to the N- and C-terminal regions of single-chain separase (Jager et al. 2001).

The N-terminal region is mainly composed of helices and was predicted to be ARM or HEAT repeats (Fig. 4.1) (Viadiu et al. 2005). For example, human separase was reported to contain 26 ARM repeats and is responsible for securin binding. This region was also predicted to have a super-helical structure like TPR repeats (Winter et al. 2015). Structural studies of separase and its complex with securin from yeast (*S. cerevisiae*) and *C. elegans*, however, show that it is a mixture of both (Luo and Tong 2017; Boland et al. 2017).

The C-terminal region was originally predicted to have two consecutive caspase domains. The structural studies reveal that this region is composed of two domains, a substrate-binding domain (SD) or pseudo protease domain (PPD), followed by the catalytic domain (CD) or the active protease domain (APD) (Luo and Tong 2017; Lin et al. 2016). Only the CD/APD has activity and structural similarity to caspases.

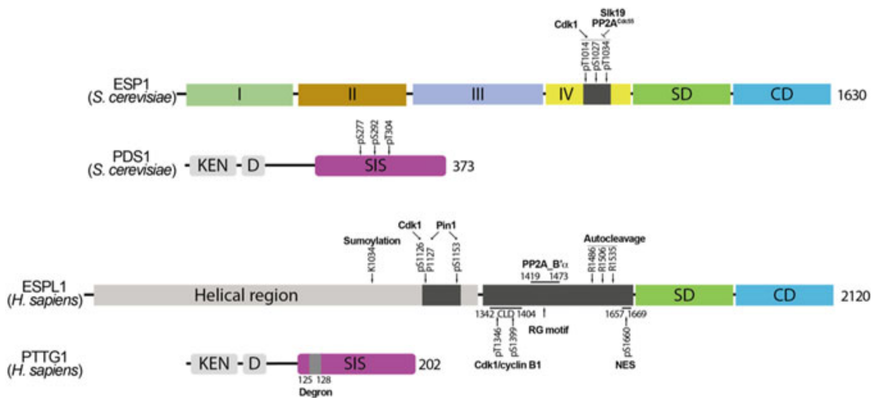

Fig. 4.1 Domain organization of budding yeast and human separase and securin. Domains are depicted as color-coded boxes and labeled. The regulatory segments are shown in dark gray. Modification sites, motifs, and interacting factors are also labeled. The N-terminal helical region of human separase without high-resolution structural information is colored gray

The CD contains a catalytic dyad of a histidine and a cysteine, and the substrate recognition is ensured by a few conserved residues in this domain.

Between the N- and C-terminal regions of separase is a natively unstructured segment with variable length, which contains motifs for binding other proteins and modification (Fig. 4.1). In higher eukaryotes and yeast, this region contains residues that are phosphorylated by Cdk1, the master regulatory kinase of mitosis (Stemmann et al. 2001; Lianga et al. 2018). A Cdc6-like domain (CLD) in this segment is also phosphorylated and crucial for mutual inhibition of the Cdk1-cyclin B1 complex (Boos et al. 2008; Gorr et al. 2005). In addition, several auto-cleavage sites are located in this segment (Fig. 4.1) (Waizenegger et al. 2002), and their cleavage is important for mitosis progression (Papi et al. 2005) and the interaction with PP2A (Holland et al. 2007). Phosphorylation-dependent isomerization mediated by the peptidyl-prolyl isomerase Pin1 on the Ser-Pro motifs of this segment is crucial for separase regulation (Hellmuth et al. 2015b). A nuclear export sequence (NES) resides in this segment (Hellmuth et al. 2018; Sun et al. 2006), phosphorylation of which is crucial for separase's role in DNA damage repair (Hellmuth et al. 2018; Sun et al. 2006). Methylation of a RG-motif within the regulatory segment and sumoylation of the Lys1034 residue is also important for its function in DNA damage response (Hellmuth et al. 2018). A cysteine motif (CxCxxC) was recently identified in this segment of metazoan separases (Melesse et al. 2018).

Diverse Functions of Separase

Although the substrates for separase identified so far are limited to only a few proteins, the functions of separase are very diverse. They can be roughly divided into two categories, depending on whether the function requires its catalytic activity. The cohesin subunits Scc1 and its meiosis-specific counterpart Rec8 are the well-known substrates targeted by separase for spatial and temporal chromosome segregation during mitosis and meiosis, respectively (Uhlmann et al. 1999; Buonomo et al. 2000). Besides chromosome segregation, the catalytic activity is also required for DNA damage repair at interphase in yeast (Nagao et al. 2004; McAleenan et al. 2013) and separase is recruited to double-strand breaks (DSBs) to support homology-directed repair (HDR) in mammals (Hellmuth et al. 2018).

The kinetochore and spindle-associated protein Slk19 is another substrate of separase, discovered soon after Scc1. Unlike the Scc1 cohesin subunit, which is subjected to destruction, the cleavage product of Slk19 stabilizes the anaphase spindle (Sullivan et al. 2001) and regulate pericentric cohesion and anaphase onset together with PP2A by counteracting phosphorylation by Cdk1 (Lianga et al. 2018). Separase is localized to spindle pole bodies and spindle midzone via interactions with its inhibitor securin and the protease activity is required for spindle elongation (Jensen et al. 2001; Baskerville et al. 2008).

Kendrin or pericentric protein, a giant coiled-coil centrosome protein crucial for centriole engagement, is specifically cleaved by separase during mitosis which

ensures a timely centriole disengagement at late mitosis to license centriole duplication during S phase (Matsuo et al. 2012; Lee and Rhee 2012; Schockel et al. 2011; Tsou et al. 2009; Nakamura et al. 2009). Separase is responsible for the cleavage of the N-tail of the CENP-A related protein CPAR-1 at the meiosis I anaphase in *C. elegans* and plays a role in the centromere activity (Monen et al. 2015).

Independent of its protease activity, separase takes part in other important cellular events. Separase contributes to mitotic exit, which requires three pathways including the Cdc14 early anaphase release (FEAR) pathway, Cdk inactivation and mitotic exit network (MEN). Separase together with Slk19 play roles in the FEAR pathway to promote the release of Cdc14, a critical Cdk counteracting phosphatase required for mitotic exit, from the nucleolus to enable mitotic exit (Sullivan and Uhlmann 2003; Stegmeier et al. 2002). Separase also directly binds to the Cdc55 subunit of PP2A to suppress its phosphatase activity and facilitate Net1 phosphorylation in the FEAR pathway to initiate mitotic exit (Queralt et al. 2006). On the other hand, separase is phosphorylated by Cdk1 and its catalytic activity is further controlled by forming a stable complex with Cdk1 via its regulatory subunit cyclin B1. Interestingly, this interaction also enables separase as a Cdk1 inhibitor to block its kinase activity to regulate mitotic exit (Gorr et al. 2005).

Through a genome-wide screen for protein interactions, separase was revealed to be related to genes involved in different functions including transposition, DNA repair, sister chromatid segregation and stress response regulation (Ho et al. 2015). Among them, Ty1 integrase was identified as a separase binding partner in different stages of the cell cycle (Ho et al. 2015). This Ty1 integrase-separase interaction functions to remove cohesin and target Ty1 integrase into the genome for retrotransposition.

The diverse functional roles of separase also include membrane trafficking, distinct from cell division, as separase is located on the vesicle membranes of meiosis-specialized cortical granules (CGs) in *C. elegans* (Bembenek et al. 2007; Bai and Bembenek 2017). In other species including human and *Drosophila*, separase is associated with membranes and plays roles in membrane traffic, protein secretion, and Golgi organization (Bacac et al. 2011; Bard et al. 2006).

Regulation of Separase

Due to the irreversibility of cohesin resolution and other functions, multiple levels of regulation exist to control separase activity tightly. The first and best-known regulator is the inhibitory chaperone securin. To ensure timely activation of separase for chromosome segregation, securin interacts with separase and potently inhibits its proteolytic activity until the onset of anaphase when securin is targeted by the APC/C complex via its N-terminal KEN/D-boxes (Fig. 4.1) and destroyed by the proteasome pathway (Ciosk et al. 1998; Zou et al. 1999; Kumada et al. 1998). Studies also revealed that securin inhibits separase by blocking the access of substrates into the active site of separase (Waizenegger et al. 2002) and this was further supported

by an amino acid substitution on securin that turned it into a substrate (Nagao and Yanagida 2006). This association is conserved in eukaryotes including fungi, plants and animals (Cromer et al. 2019). On the other hand, securin functions as a chaperone in a positive way to help solubilize and stabilize separase (Hornig et al. 2002). Securin binds to nascent separase when it reaches a size of 627 amino acids to assist its proper folding (Hellmuth et al. 2015a). Moreover, in yeast, securin guides the nuclear localization of separase (Agarwal and Cohen-Fix 2002; Jensen et al. 2001) and mediates the proper loading onto the spindle for accelerating spindle elongation (Jensen et al. 2001). This localization process depends on the phosphorylation of three residues of securin (Ser277/Ser292/Thr304) by Cdc28/Cdk1 which also enhances the interaction with separase (Agarwal and Cohen-Fix 2002). Under replication stress conditions, this complex formation and spindle elongation can be repressed through dephosphorylation by PP2A-Cdc55, a key phosphatase of cell cycle progression (Khondker et al. 2019).

Another widely recognized regulator in higher eukaryotes is the Cdk1-cyclin B1 complex, which controls separase activity in both a positive and negative manner like securin. Phosphorylation of separase by Cdk1 was first observed in frog as an additional way to curb its activity independent of securin (Stemmann et al. 2001). Ser1126 of human separase is phosphorylated during metaphase and is dephosphorylated upon anaphase onset for activation. Further investigation showed that this inhibition could sufficiently compensate the inhibition by securin, and it is achieved by cyclin B1 binding to the CLD of separase after the Thr1346/Ser1399 residues in this domain are phosphorylated (Gorr et al. 2005; Boos et al. 2008). This observation is also confirmed by the fact that a normally executed mitosis was seen in securin knock-out human cells (Pfleghaar et al. 2005). Similar to securin, cyclin B1 is also a target of the APC/C machinery (King et al. 1995; Hagting et al. 2002) and its destruction is critical for separase activation during the transition of metaphase to anaphase. Moreover, securin and Cdk1-cyclin B1 interact with separase in a mutually exclusive manner (Gorr et al. 2005). Intriguingly, Ser1126 phosphorylation causes destabilization and aggregation of separase leading to an inactive state. Resembling securin, the Cdk1-cyclin B1 complex associates with separase to stabilize but inhibit the activity until the onset of anaphase (Hellmuth et al. 2015a). Recently, Cdk1-cyclin B2 complex was reported to inhibit separase activity in meiosis I in mouse oocyte (Li et al. 2019).

Another regulatory factor that has direct interactions with separase is the phosphatase PP2A, a central player of cell cycle progression. In mammals, a 55-amino acid segment next to the auto-cleavage sites in the regulatory segment of separase forms a complex with a specific subtype of heterotrimeric PP2A through its regulatory subunit B56 (Holland et al. 2007). Interestingly, distinct from the mutually exclusive manner in the interaction of separase with securin and cyclin B1, PP2A can form a heterotrimeric complex with separase and securin but not Cdk1-cyclin B1. This interaction is abolished by the auto-cleavage in the regulatory segment of human separase (Holland et al. 2007). The bound PP2A can dephosphorylate to stabilize separase-associated securin, thus to delay the APC/C-mediated degradation to regulate separase activity for abrupt chromosome segregation (Hellmuth et al. 2014). In

yeast, separase interacts with and inhibits PP2A containing Cdc55, a different type of regulatory subunit, to allow Scc1 phosphorylation to initiate mitotic exit (Yaakov et al. 2012; Queralt et al. 2006). On the other hand, the dephosphorylation activity of PP2A^{Cdc55} on securin disrupts the separase-securin complex, in which phosphorylation of three sites in securin enhances complex formation (Agarwal and Cohen-Fix 2002) to inhibit spindle elongation during replication stress (Khondker et al. 2019).

Post-translational modifications on separase also control the function of separase at multiple levels. The auto-cleavage of separase is among the first to be elucidated and this modification only exists in vertebrates. Removal of securin allows separase to undergo auto-cleavage at three sites (Arg1486/Arg1506/Arg1535) in the regulatory segment, and the N- and C-terminal cleavage products remain associated with each other (Waizenegger et al. 2002; Zou et al. 2002). Interestingly, the cleavage does not affect its interaction with securin, and the cleavage process is not required for separase activation or the proteolytic activity (Waizenegger et al. 2002; Zou et al. 2002). However, the cleavage was reported to coordinate mitosis entry and progression by affecting the spindle assembly and metaphase chromosome alignment (Papi et al. 2005). Another study showed that the auto-cleavage influences the separase-PP2A complex formation and centromeric cohesion in human cells (Holland et al. 2007).

Phosphorylation and dephosphorylation of separase and its inhibitors and substrates are crucial for the regulation of its other physiological functions. As mentioned above, phosphorylation by Cdk1 on Ser1126 as well as Ser1399 within the CLD in mammals is important for the inhibition (Boos et al. 2008) and stabilization (Hellmuth et al. 2015a) by the Cdk1-cyclin B1 complex. In mouse, this phosphorylation is critical for germ cell development, early embryogenesis (Huang et al. 2009) as well as oogenesis (Xu et al. 2011). Separase in budding yeast is phosphorylated by Cdk1 on the regulatory segment (Thr1014/Ser1027/Thr1034), and this phosphorylation is necessary for separase activation and anaphase onset. PP2A-Cdc55 and Slk19 proteins function along with Cdk1-mediated phosphorylation to inhibit separase activity in addition to securin in yeast (Lianga et al. 2018).

A recent intriguing study revealed another level of regulation of human separase. Phosphorylated human separase is a target of Pin1 which is a phosphorylation-specific peptidyl-prolyl cis/trans isomerase and critical for mitosis regulation (Hellmuth et al. 2015b). Pin1 mediated isomerization on the phosphorylated Ser1126-P1127 motif is required for the binding and inhibition of the Cdk1-cyclin B1 complex. This isomerization relies on Pin1′s docking onto the phosphorylated Ser1153-Pro1154 site via its WW domain (Hellmuth et al. 2015b). This conformational change of separase not only removes the possibility of rebinding by residual securin but also shortens the half-life of separase to ensure a rapid termination of the proteolytic activity for mitosis exit and cohesin reloading (Hellmuth et al. 2015b).

Genome-wide screenings for regulators of separase have also been carried out and reported. In *C. elegans*, separase is crucial for cortical granule exocytosis and cytokinesis. Genetic screening for factors related to this function identified the protein phosphatase 5 (PPH-5) as an extragenic suppressor of separase, which affects the localization of separase on vesicles and suppresses exocytosis and cytokinesis, implying a separase regulatory pathway involving phosphorylation (Richie et al. 2011). A yeast

genetic screen identified new factors that interact with separase and discovered functional relevance of separase in a few cellular processes including retrotransposition and the involved factor Ty1 integrase (Ho et al. 2015).

In chronic myeloid leukemia (CML) patients treated with the TK inhibitor drug Imatinib, the separase protein level is reduced while the proteolytic activity increased in BCR-ABL-positive cells, suggesting a feedback regulation mechanism in tumor cells (Haass et al. 2015b). Further studies showed that separase expression is controlled by the transcription factor c-Myb in CML cell lines (Prinzhorn et al. 2016), indicating separase regulation at the transcriptional level.

Separase-Related Diseases and Drug Development

Given its essential roles in chromosome segregation and DNA damage repair, separase is tightly regulated and its malfunction causes aneuploidy and genome instability leading to severe consequences including cancers in higher vertebrates. Overexpression of separase induces aneuploidy and is often observed in a variety of human cancers including breast, bone, brain, and prostate cancers, thus separase was defined as an oncogene (Pati 2008). For example, separase was reported to be overexpressed in more than 50% of human breast cancers (Mukherjee et al. 2014b; Zhang et al. 2008). The overexpression level of separase highly correlates with tumor progression and reduced survival (Meyer et al. 2009; Pati 2008). Thus, separase is an attractive cancer drug target. Substrate-mimic inhibitors have been reported (Waizenegger et al. 2002). In order to find inhibitors of separase, in vitro (Pfleghaar et al. 2005; Basu et al. 2009) and cell-based (Haass et al. 2015a; Henschke et al. 2019) separase activity assays have been developed. Discovery of small-molecule inhibitors using these assays has been reported (Zhang et al. 2014; Henschke et al. 2019).

Structures of the Separase-Securin Complex

In order to understand how separase functions and how it is regulated at the molecular level, structural information on separase and its complex with securin has been actively pursued since its discovery. Low-resolution negative-stain electron microscopy studies of the human separase-securin complex provided the first glimpse of its overall structure, which adopts an elongated whale-like shape and the blurred tail density reflects its flexible nature (Viadiu et al. 2005). Detailed structural information was not available until the publication of a high-resolution crystal structure of the C-terminal catalytic region of separase in complex with a substrate-mimic peptide inhibitor (Lin et al. 2016), revealing details of the active site and how the substrate is recognized. The structure also showed that, instead of two tandem caspase domains

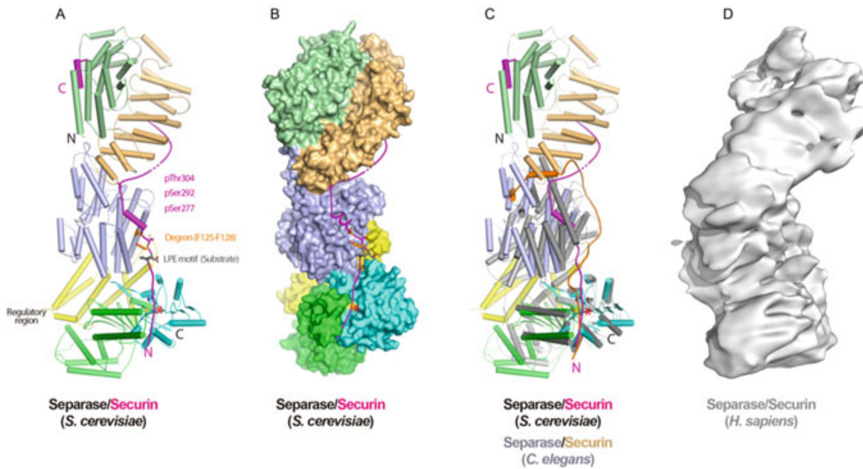

Fig. 4.2 Structures of separase-securin complexes. **a** Budding yeast separase-securin complex structure is represented as a cylindrical cartoon and color-coded as in Fig. 4.1. The active site is labeled with a red star. Modification sites on securin SIS are shown as sticks and are colored and labeled differently. **b** Surface representation in a different view showing the long securin-binding groove in different domains of separase. **c** Superimposition of the structures of yeast and *C. elegans* separase-securin complexes showing that the *C. elegans* separase lacks domains I and II. Colors of different chains are indicated. **d** Low-resolution EM density map of human separase-securin complex (EMDB ID: EMD-3584)

predicted based on sequence analysis, the C-terminal region consists of two subdomains with one resembling the caspase domain and the other sharing no similarity to any known structures (Lin et al. 2016).

Soon after that report, the first high-resolution X-ray crystal structure was published for the near full-length separase-securin complex from budding yeast (Luo and Tong 2018, 2017) (Fig. 4.2a), revealing how the domains of separase are organized and how securin interacts with separase. Yeast separase adopts an elongated conformation with the longest dimension of over 160 Å and it can be divided into six domains. The C-terminal region contains a substrate-binding domain (SD, or PPD) and a catalytic domain (CD, or APD). The structure of this region is similar to the previous structure (Lin et al. 2016) except for the L4 loop in the CD, which forms a two-stranded β-sheet. The N-terminal helical region consists of four domains, designated from I to IV (Figs. 4.1 and 4.2a). Domains I and II have HEAT repeat-like structures and are arranged into a head-to-tail complex. Domain III is a TPR-like super-solenoid structure, quite different from what was predicted as ARM/HEAT repeats. Domain IV is composed of several helices and bridges the helical region and the C-terminal region. The regulatory segment is likely not far from the active site, and could affect the activity upon its modification.

The C-terminal separase-interaction sequence (SIS) of securin (Fig. 4.1) assumes a mostly extended conformation, with only a few ordered secondary structure elements, and interacts with all domains of separase as a chaperone (Fig. 4.2b). The N-terminal

part of SIS acts as a pseudo-substrate and inserts into the substrate-binding site and catalytic site of separase. It is not cleaved by separase as its binding mode in the active site is not compatible with catalysis, with the scissile carbonyl group 6 Å away from the catalytic Cys residue. The C-terminal part binds along separase with the very C-terminus sitting in a cleft formed by domain I, and this observation might explain why securin binds nascent separase only after it reaches a certain length (Hellmuth et al. 2015a). A degron motif in securin (Phe125/Phe128 in humans) or cyclin B1 is only accessible on free securin or cycline B1, to ensure the degradation of excess securin or cyclin B1 proteins through the proteasome pathway in prometaphase to promote timely onset of anaphase (Thomas et al. 2019; Levasseur et al. 2019; Hellmuth et al. 2014). This securin degron motif is buried in the interface with separase in the structure.

The structure of the *C. elegans* separase-securin complex was determined by cryo electron microscopy (Boland et al. 2017). Compared with the complex from other species, this atypical separase is smaller in size owing to a shorter helical region. Superimposed with the yeast separase-securin structure, their C-terminal regions align well and securin is located in the active site in a similar manner (Fig. 4.2c). However, the helical domain lacks the counterparts of domains I and II in the yeast structure, and thus the securin interaction is different in this region. In the same work, a low-resolution cryo-EM map of the human separase-securin complex was also reported. Its shape is reminiscent of the yeast complex, indicating the structural conservation for this important protease through evolution (Fig. 4.2d).

Summary

Separase is a large cysteine protease, with an N-terminal helical region, a C-terminal catalytic region, and an unstructured regulatory segment in between. Separase has diverse functions including chromosome segregation, DNA damage repair, spindle elongation, and centrosome replication. Its activity is tightly controlled with multiple-layer regulation through inhibitors and modifications. Among them, the inhibitory chaperone securin is the most important regulator and the most extensively studied. Securin binds to separase cotranslationally and stabilizes separase until the anaphase onset, when securin is destroyed by the proteasome and separase activity is released. Dysregulation of separase causes genome instability leading to diseases including cancers in humans, making separase a potential target for discovery of inhibitors for cancer therapy. The structural information for the separase-securin complex from several different organisms provides great insights into this enzyme and molecular mechanisms for the inhibition by securin.

However, there are still many unanswered questions and further studies are needed. In human separase, how auto-cleavage, phosphorylation, and isomeric conformational change regulate the activation is still elusive. Phosphorylation of yeast separase is also reported to be critical for activation (Lianga et al. 2018), and how this activation is different from human separase is also an interesting topic for more

research. Protein factors such as PP2A, Cdk1-cyclin B1, Pin1, Slk19 associate with separase mostly through the regulatory segment. Elucidating the molecular details for these interactions and their roles is also critical for us to better understand separase regulation.

The exact recognition mechanism for the C-terminal part of the cleavage site is not known, although a recent study identified an extra binding motif LPE on Scc1 that is similar to a site on securin, which implies the substrate might bind along the securin binding surface (Rosen et al. 2019). The L4 loop in the CD appears to be flexible when alone but ordered in the complex with securin, and the functional role (if any) of this loop needs to be elucidated as well. The additional structural information could also help the discovery of small-molecule inhibitors of separase.

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