Studies on Substrate Recognition by the Budding Yeast Separase*

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Sister chromatid cohesion is resolved at anaphase onset when separase, a site-specific protease, cleaves the Scc1 subunit of the chromosomal cohesin complex that is responsible for holding sister chromatids together. This mechanism to initiate anaphase is conserved in eukaryotes from budding yeast to man. Budding yeast separase recognizes and cleaves two conserved peptide motifs within Scc1. In addition, separase cleaves a similar motif in the kinetochore and spindle protein Slk19. Separase may cleave further substrate proteins to orchestrate multiple cellular events that take place during anaphase. To investigate substrate recognition by budding yeast separase we analyzed the sequence requirements at one of the Scc1 cleavage site motifs by systematic mutagenesis. We derived a cleavage site consensus motif (not(FKRWY))(ACFHILMPVWY)(DE)X(AGSV)RX. This motif is found in 1,139 of 5,889 predicted yeast proteins. We analyzed 28 candidate proteins containing this motif as well as 35 proteins that contain a core (DE)XXRX motif. We could so far not confirm new separase substrates, but we have uncovered other forms of mitotic regulation of some of the proteins. We studied whether determinants other than the cleavage site motif mediate separase-substrate interaction. When the separase active site was occupied with a peptide inhibitor covering the cleavage site motif, separase still efficiently interacted with its substrate Scc1. This suggests that separase recognizes both a cleavage site consensus sequence as well as features outside the cleavage site.

The segregation of sister chromatids to daughter cells during mitosis requires a complex series of cellular events to be performed faithfully, which has been studied in some detail in the budding yeast Saccharomyces cerevisiae (1). One of the key events is activation of a site-specific protease, separase, which triggers the resolution of sister chromatid cohesion at anaphase onset. Sister chromatids are kept aligned in metaphase by a protein complex, cohesin, which counteracts the pulling force of the mitotic spindle. Cohesion is lost abruptly at anaphase onset when separase cleaves the Scc1 subunit of the cohesin complex. During meiosis Scc1 in cohesin is replaced by a related subunit, Rec8, which is cleaved by separase during the two subsequent rounds of meiotic chromosome segregation. Premature loss of cohesion leads to chromosome missegregation, thus cohesin cleavage by separase is kept under tight cellular control (2).

Separase is required for processes in addition to sister chromatid separation. These include anaphase spindle stabilization and the coupling of anaphase to mitotic exit during budding yeast mitosis (3–8) as well as the coordination of the chromosome segregation and spindle cycles during meiosis (9). In fission yeast, separase binds to the mitotic spindle and has been implicated in spindle pole body morphology and positioning (10–12). In nematode worms separase is required for correct centrosome positioning during the first asymmetric mitotic division (13) as well as for formation of an intact eggshell of the one-cell embryo (14). One way in which separase could contribute to multiple processes is that target proteins other than cohesin exist and that cleavage of several proteins during mitosis coordinates these events. We demonstrated previously that the yeast kinetochore and spindle protein Slk19 is a substrate for separase cleavage (15). Slk19 is cleaved concomitantly with Scc1 at the metaphase to anaphase transition, and failure to cleave Slk19 leads to defects of the anaphase spindle. Human and Drosophila separases undergo self-cleavage, contributing to the down-regulation of separase activity at the end of mitosis (16–18). Identification of further separase cleavage targets could yield insight into how multiple events are orchestrated during mitosis. Some of the requirements for separase may also be explained through the recent discovery of a second, protease-independent, activity within separase (8).

With the aim of aiding identification of separase cleavage targets we investigated how separase recognizes its substrates. All known budding yeast substrates (Scc1, Rec8, and Slk19) are cleaved at one or two conserved peptide motifs, about eight amino acids in length (see Fig. 1A) (15, 19, 20), and related motifs are found at separase cleavage sites in other organisms (16–18, 21, 22). Separase always cleaves downstream of a conserved arginine residue, mutation of which to a negatively charged amino acid largely abolishes cleavage. The exact sequence requirements for recognition of the cleavage site motif have not been characterized. We therefore performed a systematic mutational analysis of the major separase cleavage site in Scc1. The experimentally derived consensus motif is less stringent than would have been predicted based on sequence conservation, and it can be found in at least 1,100 proteins within the yeast proteome. We show that determinants in addition to the cleavage site are likely to contribute to substrate recognition by separase.

EXPERIMENTAL PROCEDURES

Scc1 Cleavage Site Mutagenesis—The SCC1 gene including its native promoter was cloned into YIplac128 (23), fused to a triple HA epitope at the C terminus. PCR-based mutagenesis was used to introduce the restriction sites BclI and AvrII surrounding the separase cleavage site at amino acid position 268. The SCC1 coding sequence at nucleotides

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780–786 was changed to TGATCGA introducing the BclI site. This created a conservative point mutation of the amino acid sequence (N262Q). Nucleotides 805–812 were changed to CGCTTAGG to introduce the AvrII site, which did not alter the encoded amino acids. After restriction with these two enzymes oligonucleotide linkers encoding the mutant cleavage site motifs were inserted. All constructs were verified by DNA sequencing.

Analysis of Scc1 Cleavage Site Mutants—Scc1 plasmids were linearized by digestion with EcoRV and integrated at the LEU2 locus of strain K7062 (MATa, sec1Δ GAL-SCC1myc18, w303 background) (24). The endogenous SCC1 gene in this strain is under control of the galactose-inducible GAL1 promoter. All mutant Scc1 plasmids supported cell growth on glucose media when the GAL1 promoter was repressed, indicating that the mutant Scc1 proteins complemented the essential function of the protein. Transformants were chosen which exhibited levels of mutant Scc1 expression similar to endogenous wild type Scc1. Protein extracts were prepared from exponentially growing cultures of these strains, and the relative abundance of the two Scc1 cleavage fragments containing the C-terminal HA epitope tag was analyzed by Western blotting using monoclonal antibody 12CA5.

Screening of Potential Separase Substrates—Putative separase substrates were identified by searching the yeast genome for the optimal separase consensus cleavage motif (not(FKRWY)(ACFIHLMPPVWY)(DE)XAGSVIR under the LocusLink program at the Saccharomyces Genome Database). Other putative substrates containing a (DE)X core motif were selected which are known to function in mitotic processes. Candidate genes were identified by their endogenous loci in strain K7428 (MATa, cdc20Δ, cdc24Δ, GAL-SCC1myc18, H9004) (24) by a one-step PCR tagging technique to introduce a C-terminal HA epitope tag. These strains were arrested in metaphase and released synchronously into anaphase using the mitotic separase inhibitor Bio-SVEQGR-amk. Protein extracts were prepared from asynchronously growing cells infected with a baculovirus expressing an Scc1-HA–FLAG-Intein-chitin binding domain construct as described previously (6). Overexpression in budding yeast cells of separase, fused to a FLAG epitope and chitin binding domain, and its purification on chitin beads were as described previously (6, 26). Separase purified on beads was incubated with 6 μm separase inhibitor Bio-SVEQGR-amk (6) for 10 min at 25 °C to saturate inhibitor binding to separase. Beads were then incubated with 500 ng of Scc1 for 10 min at 25 °C. After washing, bound proteins were eluted in SDS-PAGE loading buffer and analyzed by Western blotting.

RESULTS

Analysis of a Separase Cleavage Site Motif by Site-directed Mutagenesis—There are five known separase cleavage sites in budding yeast proteins, two in Scc1, two in Rec8, and one in Slik19, which all are characterized by a conserved amino acid motif (Fig. 1A). A systematic analysis of the significance of this motif for cleavage by separase has not been performed to date. We therefore constructed a series of cleavage site motif mutant Scc1 proteins to assess the contribution of each amino acid in this motif to cleavage by separase. Of the two cleavage sites in Scc1, the second more C-terminally located site is cleaved more efficiently, and we used this site for our analysis (Fig. 1B). The task to express and purify large quantities of active separase for biochemical studies in vitro is still a considerable challenge. We therefore constructed yeast strains expressing the mutant proteins as their sole source of Scc1 and analyzed the pattern of cleavage of these mutant proteins in vivo. Separase cleavage of only one site in Scc1 is sufficient for chromosome segregation such that cells can tolerate even complete inactivation of one cleavage site (19). Cleavage at the remaining wild type site can serve as an internal comparison for the efficiency of cleavage at the mutant site.

To facilitate analysis of Scc1 cleavage, we fused Scc1 to a C-terminal HA epitope tag. Western blotting of extracts from asynchronously growing cells against this epitope revealed three possible bands, corresponding to full-length Scc1 and two C-terminal cleavage fragments (Fig. 1, B and C). In wild type cells only full-length Scc1 and a short cleavage product are visible, corresponding to cleavage at the efficiently used C-terminal site at amino acid 268 (Fig. 1C). The more N-terminal site at amino acid 180 is also cleaved in most of the Scc1 molecules, but the longer fragment thereby produced is processed further by cleavage at the second site (19). Mutation of the conserved arginine at the C-terminal cleavage site to aspartate abolishes separase cleavage at this site, and detection of the C-terminal epitope tag now visualizes the longer Scc1

FIG. 1. Mutational analysis of a separase cleavage site motif in Scc1. A, alignment of the five known separase cleavage sites in budding yeast. The site of cleavage, based on analysis of Scc1 (19), is indicated by an arrow. B, diagram of cleavage site positions within Scc1 and of the protein fragments detectable via the C-terminal HA epitope tag after cleavage. C, analysis of cleavage site motif mutant Scc1. Protein extracts from yeast strains harboring the various Scc1 cleavage site mutants were analyzed by Western blotting against the HA epitope tag. A reduction in protein fragments detectable via the C-terminal HA epitope tag after cleavage.

The Saccharomyces Genome Data Base can be accessed at www.yeastgenome.org.
cleavage fragment (Fig. 1C). We expected that mutations in the second cleavage site that reduce but do not abolish cleavage would generate a mixture of both cleavage products and that their relative intensities would relate to the efficiency of cleavage at the mutant site.

We engineered two restriction sites in the coding sequence flanking the C-terminal cleavage site to facilitate mutagenesis. This introduced a conservative point mutation (N262Q) upstream of the cleavage site motif. We analyzed this mutation as well as two other amino acid changes at this position (N262L and N262H) and found that they had no noticeable effect on cleavage (Fig. 1C). The N262Q change is therefore contained in all further mutant proteins. We constructed 30 mutations in the separase cleavage site motif, corresponding to 29 single amino acid changes and an insertion of one amino acid (Fig. 1C). We then compared the effect that each mutation had on the relative abundance of the two C-terminal Scc1 cleavage fragments.

Result of the Cleavage Site Analysis—We use the convention P1, P2, . . . P6 to indicate amino acid positions upstream of the cleavage site, the numeral indicating the distance from the cleavage site. P1 is the amino acid following the cleavage site. The P6 position is occupied by an invariant serine in all known budding yeast separase cleavage sites (Fig. 1A). The serines at the Scc1 cleavage sites are subject to mitotic phosphorylation by Polo-like kinase, which is thought to regulate cleavage positively (27). It came therefore to our surprise that substitution not only by negatively charged aspartate or glutamate, but also by hydrophobic leucine or phenylalanine was well tolerated and only led to a minute accumulation of the longer cleavage fragment, indicative of less efficient cleavage at the mutant cleavage site (Fig. 1C). Even an arginine, of opposite charge to phosphoserine, had only a small effect. When compared side by side, phenylalanine and arginine appeared somewhat less favored at P6 compared with other amino acids. Therefore, large hydrophobic or positively charged amino acids at P6 may not be optimal for recognition of this site by separase.

P5 is a small hydrophobic residue in most of the known cleavage sites, although a histidine is found at one site in Rec8. Introduction of a large hydrophobic phenylalanine or tyrosine at this position did not affect cleavage. Polar serine or charged glutamate or arginine in contrast strongly reduced or even abolished cleavage at this site. The hydrophobic character of the amino acid at P5 is therefore important for cleavage by separase.

The P4 position is filled by a negatively charged amino acid in all known cleavage sites, in most cases glutamate. Removal of the negative charge by mutation to glutamine significantly interfered with cleavage, and changes to a polar serine or threonine, hydrophobic leucine, or positively charged arginine prevented cleavage entirely. The negatively charged character of P4 is therefore indispensable for cleavage by separase.

P3 shows no conservation. Various hydrophobic, polar, or positively charged amino acids are found in the natural cleavage sites. We tested also whether negatively charged aspartate was tolerated at this position, and this was indeed the case. Thus, there is no evidence for any preference at P3.

The P2 position is taken by the smallest amino acid, glycine, in all known budding yeast cleavage sites. Replacement with the next smallest amino acids, alanine, serine, and valine made little difference to the efficiency of cleavage. Larger amino acids, proline, threonine, phenylalanine, and leucine caused an increasing impediment to cleavage. It is unclear why the very large phenylalanine was tolerated somewhat better than medium sized leucine. In any event, a small amino acid at P2 seems important for efficient cleavage.

The P1 residue is crucial for substrate recognition by many proteases. It is an arginine in all known separase cleavage sites, not only in budding yeast but in all organisms so far studied. Mutation of this arginine to aspartate or glutamate has been used to block cleavage of both Scc1 and Slk19 efficiently, although residual slow cleavage occurred when similar mutations were introduced into Rec8 (15, 19, 20). When we placed an uncharged glutamine at P1 of the Scc1 cleavage site, cleavage was blocked almost as effectively as by aspartate. Mutation of the arginine to likewise positively charged lysine resulted in accumulation of the longer Scc1 cleavage product, although some cleavage still occurred at the mutant site. This suggests that a positive charge at P1 is essential and that arginine in particular is an important determinant for substrate recognition by separase.

The P1’ position is arginine or lysine in the Scc1 and Rec8 cleavage sites and serine in Slk19. The amino acid at P1’ will form the new N terminus of the C-terminal cleavage product and will according to the N end rule determine its stability (28). Both arginine and lysine render the Scc1 and Rec8 fragments very short lived, whereas serine ensures stability of the Slk19 cleavage product (15, 20, 29). Mutation of P1’ to large or small hydrophobic phenylalanine or leucine, to negatively charged glutamate, or to polar glutamine did all not influence the efficiency of cleavage. This is consistent with the idea that the P1’ residue in separase cleavage sites does not contribute to recognition by separase but helps to determine the stability of the respective cleavage products (29).

Lastly, we analyzed whether the spacing of critical amino acids within the cleavage site motif is important. We inserted a glycine between P4 and P3, which increased the distance between the important hydrophobic/negative amino acid pair at P5/P4 and the arginine at P1. This led to a strong impediment of cleavage, indicating that the distance between P4 and P3 cannot be changed without losing efficient cleavage site recognition by separase.

A Separase Cleavage Site Consensus—The above analysis revealed a strict requirement at P4 for a negatively charged amino acid and at P1 for arginine, forming a core (DE)XXR motif at separase cleavage sites. The residues at P6, P5, and P2 also influenced cleavage efficiency albeit to a lesser extent. We studied the effect of amino acids with representative characteristics at these positions, from which we extrapolated to derive a consensus motif for optimal recognition by separase. Phenylalanine and arginine, a positively charged and a large hydrophobic amino acid, were least tolerated at P6, therefore we excluded these and related amino acids (FKRWY) from the consensus. At P5, small or large hydrophobic amino acids work best, whereas a hydrophilic (S) or charged amino acid (ER) prevented cleavage. Histidine is found at this position in one of the budding yeast Rec8 cleavage sites, which was therefore also included in the consensus, as well as two other weakly polar amino acids cysteine and methionine. At the P2 position four small amino acids (AGSV) were all tolerated well, whereas somewhat larger ones (PTL) caused a decrease in cleavage. The consensus therefore only includes the four smallest. We could not find evidence for specificity at either the P3 or P1’ position. Taken together an optimal motif at the analyzed cleavage site in Scc1 may be described as (notFKRWY)(ACFHLIMPVWY)/DE/X(AGSV)RX, where X denotes any amino acid and “/” is the site of cleavage.

A Search for New Separase Substrates—We utilized this separase cleavage site consensus in an attempt to identify novel separase cleavage targets in budding yeast which could help to explain the multiple roles of separase during cell division. The above described consensus motif for cleavage by separase...
The epitope-tagged proteins were arrested in metaphase by proteins. The candidates studied included microtubule- or kine-
teins that only contained the core (DE)XXR motif and that were optimal cleavage site consensus, we also included several pro-
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mitotic progression. Candidate proteins were HA epitope tagged to facilitate detection.

Western blot analysis of selected putative separase target proteins. 

Extracts were prepared at 10-min intervals from cultures undergoing synchronous metaphase to anaphase transition after release from a GAL1 promoter-regulated CDC20 (19). The approximate timing of mitotic events was as indicated above the time scale. Three types of protein behavior were noted: A, no change; B, change in electrophoretic mobility during mitotic progression; C, decrease in protein abundance during mitotic progression.

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**Discussion**

We present here the first systematic experimental analysis of the cleavage site motif recognized by budding yeast separase. Cleavage sites in the known separase target proteins in budding yeast are characterized by a strikingly conserved motif, SX(XE)GR/X. We now found that some of the conserved amino acids in this motif are not essential for recognition by separase. Our experimentally derived consensus for cleavage by separase is considerably more relaxed, (not(FKRWY))(ACF-HILMPVWY)(DE)(XAGSV)RX. Even though all of the known cleavage sites contain an invariant serine at the P6 and glycine at the P2 positions, both of these amino acids could be replaced by a number of others without noticeable effect on cleavage. This raises the question as to why these amino acids are so strictly conserved at naturally occurring separase cleavage sites. One reason could be that our *in vivo* assay was not sensitive enough to detect small decreases in the efficiency of cleavage and that there might be strong selective pressure to maintain an optimal rate of substrate cleavage during anaphase. It is notable that the cleavage site in Slk19 perfectly matches those in Scc1 even though, in contrast to Scc1, cleavage of Skl19 is not essential for cell viability (15). An alternative explanation for the strict conservation of the cleavage site motif might therefore be that recognition by separase is not its only function. Some amino acids in the motif are clearly critical for cleavage by separase, but others might be more important for another, so far entirely hypothetical, role. It will be interesting to analyze possible phenotypes of cells in more detail which contain mutations in those conserved cleavage site residues that are of little importance for recognition by separase.

The experimentally derived budding yeast cleavage site consensus also allows comparison with separase cleavage site motifs found in other organisms. The motifs are highly conserved in the Scc1, Rec8, and Skl19 homologs in a number of *Saccharomyces* species (38, 39), although serine in P6 and the hydrophobic P5 show less conservation at predicted Rec8 cleavage sites. Fission yeast separase cleaves at sequences very similar to those found in budding yeast, but again the P6 serine varies at a predicted Rec8 site (1, 21). In higher eukaryotes, an EXXR motif appears to be the main conserved feature at separase cleavage sites (16–18, 22). Our analysis shows that the core (DE)XXR element within the cleavage site motif bears most weight for recognition by separase also in *S. cerevisiae*. This therefore suggests that cleavage site recognition by separase follows a fundamentally similar principle in all organisms. Another critical amino acid for cleavage site recognition by budding yeast separase is the hydrophobic residue at the P5 position. This preference is not apparent in higher eukaryotic cleavage sites where charged, polar or hydrophobic residues fill
this position. The absence of a hydrophobic P5 residue in the two human Sccl cleavage sites could be part of the reason why budding yeast separase does not accept human Sccl as a substrate (16). Vice versa, budding yeast Sccl seems to fulfill the human EXXR/X consensus but fails to be cleaved by human separase. The P2 and P3 residues in human separase cleavage sites are often hydrophobic, but whether this contributes to substrate recognition by human separase and could explain discrimination against budding yeast Sccl remains to be elucidated.

We have also provided evidence that separase interacts with its substrate Sccl at places outside the cleavage site motif. When separase was bound by a cleavage site derived peptide inhibitor it was still able to interact with Sccl. Furthermore, the C-terminal Sccl fragment after separase cleavage, which no longer contains a cleavage site motif, still interacted with separase. This suggests that separase interacts with Sccl via contacts in addition to the cleavage site peptide. We do not know how important the contribution of such an interaction is to substrate recognition by separase. Given that the separase substrate interaction appeared almost undiminished after separase was bound by the peptide inhibitor, the interaction might be significant. It will be of interest to define the regions between separase and its other substrates, and knowledge of a substrate interaction motif different to the cleavage site consensus but fails to be cleaved by human separase. The P2 and P3 residues in human separase cleavage site peptides were found to be hydrophobic. The absence of a hydrophobic P5 residue in the disc mix. 3.1. Separase Substrate Recognition

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