Published in final edited form as:

Nat Cell Biol. 2003 March ; 5(3): 249–254. doi:10.1038/ncb940.

A non-proteolytic function of separase links anaphase onset to mitotic exit

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Abstract

Separase is a protease that triggers chromosome segregation at anaphase onset by cleaving cohesin, the chromosomal protein complex responsible for sister chromatid cohesion^{1,2}. After anaphase, cells exit from mitosis, i. e. they complete downregulation of cyclin-dependent kinase activity, undergo cytokinesis and enter G1 of the next cell cycle. Here we show that separase activation at anaphase onset is sufficient to promote release from the nucleolus and activation of the yeast phosphatase Cdc14, a key step in mitotic exit³⁻⁵. The ability of separase to activate Cdc14 is independent of its protease function but may involve promoting phosphorylation of the Cdc14 inhibitor Net1. This novel separase function is co-regulated with its proteolytic activity by the separase inhibitor securin. This helps to explain the coupling of anaphase and mitotic exit: after securin degradation at anaphase onset, separase cleaves cohesin to trigger chromosome segregation and concurrently uses a non-proteolytic mechanism to initiate mitotic exit.

We have studied the contribution of separase activation and chromosome segregation to mitotic progression. We arrested budding yeast cells in metaphase by shutting off expression of the APC activator Cdc20 under control of the methionine regulated *MET3* promoter. Endogenous separase is kept inactive in this arrest by stable securin. Sister chromatid segregation (anaphase) was triggered in these cells by overexpression of separase from the galactose-inducible *GAL1* promoter. As a comparison, we similarly overexpressed the foreign tobacco etch virus (TEV) protease that is also able to trigger anaphase, in the absence of active separase, by cleaving the accordingly engineered cohesin¹. Both separase and TEV protease triggered anaphase with similar efficiency, as measured by appearance of binucleate cells with elongated spindles (Fig. 1a). However, while cells expressing TEV protease stayed dumbbell-shaped in telophase, cells in which separase was expressed started to produce new buds after anaphase (Fig. 1a). This rebudding indicated that some aspects of the cell cycle had resumed. Cyclin degradation after separase overexpression in mitotic cells has been reported previously⁶.

Separase-induced rebudding in metaphase arrested cells depended on the activity of the phosphatase Cdc14 (supplementary Fig. S1). We therefore analysed whether separase might have caused rebudding by initiating mitotic exit through release of Cdc14 from the nucleolus. Indeed, Cdc14 was released and redistributed throughout the nucleus and cytoplasm of cells expressing separase, with kinetics coinciding with or slightly preceding anaphase (Fig. 1b). Cdc14 release was a consequence of separase expression, rather than anaphase, because Cdc14 stayed tightly sequestered in the nucleolus of cells in which anaphase was triggered by TEV protease. Separase-released Cdc14 was active as a phosphatase to dephosphorylate its targets Cdc15 and Sic1 (supplementary Fig. S2). We noticed that although DAPI-stained chromosomes segregated efficiently during TEV

protease-triggered anaphase, nucleoli often failed to separate. This is consistent with a requirement of active Cdc14 for nucleolar separation, as reported previously⁷.

Separase expression caused release of active Cdc14, but only a small fraction of cells completed mitotic exit, as judged by cytokinesis and entry into the following cell cycle (Fig. 1c). This might indicate sister chromatid separation and Cdc14 release are insufficient for complete mitotic exit, and other separase-independent events must normally occur at anaphase onset. Alternatively, separase might be in principle sufficient to initiate mitotic exit, but after depleting the essential APC activator Cdc20, the Cdc14-dependent activator Cdh1 might not be able to counteract all mitotic Cdk activity. To test the latter possibility we lowered mitotic Cdk activity by deleting the non-essential cyclin *CLB5*. This reduces levels of the major mitotic cyclin Clb28 and allows mitotic exit in the absence of Cdc20 (Ref. 9). Figure 1c shows that following separase-triggered anaphase, cells lacking Clb5 completed mitotic exit, underwent cytokinesis, and entered the next cell cycle. *CLB5* deletion did not allow mitotic exit of cells in which anaphase was triggered by TEV protease. This shows that once cyclin levels are reduced separase is sufficient for activation of Cdc14 and subsequent mitotic exit. A first wave of cyclin destruction normally occurs when Cdc20 activates the APC. The dependence of separase-induced mitotic exit on lowered Cdk activity is thus consistent with the essential contribution of Cdc20 to cyclin degradation during mitotic exit^{9,10}.

We next addressed the mechanism by which separase releases Cdc14 from inhibition in the nucleolus. The mitotic exit network (MEN) is essential for mitotic exit and regulates Cdc14 nucleolar release⁵. To test whether separase acts through the MEN, we expressed separase in metaphase blocked cells carrying a temperature sensitive mutation in the essential MEN kinase Cdc15. While separase induction and anaphase proceeded with somewhat reduced efficiency at 37°C, the restrictive temperature of the *cdc15-2* allele, all cells that underwent anaphase also showed complete nucleolar release of Cdc14, qualitatively indistinguishable from wild type cells (Fig. 2a). This indicates that Cdc14 release by separase is independent of the MEN.

Recently, transient MEN-independent nucleolar release of Cdc14 in early anaphase has been described¹¹. This transient release was not observed in cells mutant in Polo or separase, or lacking Slk19 or Spo12. We therefore tested whether Cdc14 release triggered by separase expression depended on Slk19, Spo12 or Polo. Strains expressing Cdc20 under control of the *MET3* promoter and deleted for *SLK19* or *SPO12* grew very poorly. Instead, we used the spindle poison nocodazole to arrest wild type, *slk19Δ*, and *spo12Δ* strains in metaphase and expressed separase as above. Separase accumulated to similar levels in all strains, and Cdc14 was released from the nucleolus of wild type but not *slk19Δ* or *spo12Δ* cells (Fig. 2b). This indicates that Slk19 and Spo12 are both required for nucleolar release of Cdc14 by separase. The dependence of separase on Slk19 and Spo12 could indicate that these proteins act downstream of separase. However, overexpression of either Slk19 or Spo12 in metaphase arrested cells did not lead to nucleolar release of Cdc14 (supplementary Fig. S3). This suggests that Slk19 and Spo12 cannot act independently of separase, but that they may act in parallel or in a complex with separase.

We then analysed the contribution of Polo to Cdc14 release by separase. Polo is also part of the MEN, and is essential for release of Cdc14 from the nucleolus^{12,13}. We compared Cdc14 release after separase expression in wild type or Polo temperature sensitive *cdc5-1* cells arrested by nocodazole treatment at the restrictive temperature. Release was reduced in the *cdc5-1* mutant but did occur in a significant number of cells after longer incubation (Fig. 2c). This indicates that Polo contributes to Cdc14 release by separase, but also that high levels of separase may facilitate Cdc14 release in *cdc5-1* cells. Overexpression of Polo is

itself sufficient to at least partly release Cdc14 from the nucleolus (Ref. 14-15, and see below), making Polo a possible downstream target of separase. Alternatively, Polo could act in a pathway parallel to separase.

Cdc14 release by separase depended on Slk19, itself a separase cleavage target in anaphase¹⁶. This suggested Slk19 cleavage might trigger Cdc14 release. However, a Slk19 variant that was resistant to cleavage by separase was proficient in supporting Cdc14 nucleolar release in early anaphase (supplementary Fig. S4). Separase might therefore cleave another target protein to promote Cdc14 nucleolar release. Alternatively, Cdc14 release might be a function of separase independent of its proteolytic activity. To differentiate between these possibilities we expressed in metaphase arrested cells a proteolytically inactive point mutant of separase, in which the catalytic cysteine residue was changed to alanine (C1531A)(Ref. 1). C1531A expression released Cdc14 from the nucleolus of metaphase nuclei that remained undivided, with kinetics indistinguishable from expression of proteolytically active wild type separase (Fig. 3a). This confirms that nucleolar release of Cdc14 is independent of chromosome segregation in anaphase, and suggests it might be a function of separase independent of its proteolytic activity. However, overexpressed C1531A competes with endogenous separase for binding to securin, eventually releasing endogenous, proteolytically active, separase. As a consequence, some cells slowly entered anaphase after C1531A expression (Fig. 3a). We therefore analysed endogenous levels of C1531A separase, expressed from the cloned separase promoter, in cells carrying the temperature sensitive separase mutation *esp1-1* (Ref. 17). In these cells, C1531A is released from securin at anaphase onset, and we were able to assess its ability to rescue Cdc14 release that is defective in *esp1-1* cells. Cultures were synchronised in G1 by a α -factor treatment and released into the cell cycle at 35.5°C, a restrictive temperature for *esp1-1*. As expected, *esp1-1* cells failed to separate sister chromatids after securin destruction, and Cdc14 release and cytokinesis occurred with a delay compared to a control strain rescued by wild type separase (Fig. 3b). Proteolytically inactive separase C1531A rescued Cdc14 release with timing indistinguishable from wild type separase, even though chromosomes did not segregate (Fig. 3b). These cells also proceeded with mitotic exit and cytokinesis faster than *esp1-1* cells. This suggests that separase promotes Cdc14 release and mitotic exit independently of chromosome segregation and independently of its protease function.

Cdc14 release in cells containing wild type or C1531A separase coincided with securin destruction (Fig. 3b), consistent with the idea that release from securin activates separase. Securin has been shown to inhibit the proteolytic activity of separase¹⁸, so we addressed whether the protease-independent function of separase is also regulated by securin. We expressed separase in metaphase cells together with securin. Securin not only prevented ectopic separase from triggering anaphase, but also prevented wild type and C1531A separase from promoting nucleolar release of Cdc14 (Fig. 4a). This indicates that securin regulates both proteolytic and non-proteolytic activities of separase.

The ability of separase to release Cdc14 from the nucleolus depended on Slk19, but not on cleavage of Slk19 by separase. To analyse whether the relationship between separase and Slk19 extends beyond Slk19 cleavage, we performed co-immunoprecipitation experiments from cells arrested in metaphase by depletion of Cdc20 under control of the *GAL1* promoter, and released into anaphase by Cdc20 reinduction¹⁹. During metaphase, only background levels of Slk19 associated with separase. In contrast during anaphase, a complex between separase and Slk19 was formed that persisted after Slk19 cleavage (Fig. 4b). We do not currently know the significance of separase's interaction with Slk19 during anaphase. That this interaction may have physiological consequences is suggested by the observation that separase localisation to the spindle midzone and kinetochores during anaphase depends on Slk19 (supplementary Fig. S5). Interaction of separase with its substrates is prevented by

securin¹⁸, therefore complex formation between separase and Slk19 might only become possible after securin degradation. This raises the possibility that separase acts in a complex with Slk19, and that the interaction between separase and Slk19, rather than Slk19 cleavage, might promote mitotic exit.

We next revisited the relationship between separase and Polo. Cdc14 release by separase depended at least in part on Polo, and recent studies have shown that ectopic expression of Polo itself can cause nucleolar release of Cdc14 and mitotic exit^{14,20,21}. Net1, the nucleolar binding partner and inhibitor of Cdc14, is phosphorylated in a Polo-dependent manner in anaphase, and hyperphosphorylated Net1 shows a weaker affinity for Cdc14 (Ref. 14, 15). Polo expression caused Cdc14 nucleolar release from the nucleolus of either wild type, or of *slk19Δ* or *spo12Δ* cells with similar efficiency (supplementary Fig. S6). Unlike with separase, Cdc14 release by Polo could not be inhibited by securin (supplementary Fig. S6). Thus, Polo acts independently of Slk19, Spo12 and securin, and therefore maybe more directly than separase. Furthermore, this may indicate that separase might be the main, if not the only, target for securin in inhibiting mitotic exit.

Polo may release Cdc14 from inhibition in the nucleolus by phosphorylation of the Cdc14 inhibitor Net1 (Ref. 14,15). Nevertheless, when directly compared with separase expression in metaphase cells, Polo expression seemed less efficient in releasing Cdc14. In contrast to separase expression, some Cdc14 remained associated with the nucleolus in many cells, and Net1 itself appeared dispersed after Polo overexpression (supplementary Fig. S7, and Ref. 14). Furthermore, overall levels of Polo activity do not appear to quantitatively change during mitosis²². One possible explanation for anaphase-specific Polo-dependent phosphorylation of Net1 could therefore be that separase causes a qualitative change to Polo activity. To test this possibility, we analysed the effect of separase on Net1 phosphorylation in metaphase arrested cells. Expression of proteolytically inactive separase caused Net1 species with slower electrophoretic mobility to appear (Fig. 4c), similar to the Polo-dependent phosphoisoforms observed during anaphase¹⁵. We do not know whether Polo itself is responsible for this phosphorylation, or whether this phosphorylation directly causes Cdc14 release. Nevertheless, this shows that separase allows a phosphorylation reaction on Net1 to occur that was not possible in metaphase.

We show here that separase at the same time as triggering anaphase by cohesin cleavage, independently of its protease activity triggers release and activation of the phosphatase Cdc14, thereby initiating mitotic exit (Fig. 5). The participation of Slk19 in the mitotic exit pathway is intriguing because we previously identified Slk19 as a cleavage target of separase in anaphase¹⁶. Slk19 and its cleavage in anaphase are required for the formation of a stable anaphase spindle. However, spindle instability itself, as observed e.g. in *ase1Δ* cells²³, does not delay mitotic exit (supplementary Fig. 8). Instead, Slk19, but not its cleavage, is required for separase-triggered Cdc14 release, and we show that separase forms a complex with Slk19 in anaphase.

Little is known about the molecular function of Spo12. Our analysis places Polo most downstream in Cdc14 activation. However, the overall activity of Polo does not apparently change at the metaphase to anaphase transition²². The measurement of Polo activity was made using an artificial substrate, and Polo activity *in vivo*, e. g. against Net1, might be regulated in a separase-dependent fashion. Alternatively, separase might influence the accessibility of Net1 to phosphorylation. We cannot exclude that Polo acts in parallel to separase in promoting Net1 phosphorylation, and that separase may influence the phosphorylation status of Net1 via another mechanism.

Cells containing the *esp1-1* mutation at the restrictive temperature are deficient in early anaphase release of Cdc14, yet they only show a delay in mitotic exit of about 20 minutes, after which MEN-dependent Cdc14 activation sets in¹¹. On the other hand, undegradable securin not only efficiently prevents sister separation but it also permanently blocks mitotic exit²⁴. Furthermore, after TEV-protease triggered anaphase, a situation in which securin dominates over endogenous separase, cells remain stably arrested in telophase (Fig. 1c). We cannot exclude that securin blocks mitotic exit by a mechanism in addition to separase inhibition, even though securin did not prevent Cdc14 activation by Polo. Alternatively, undegradable securin might be more efficient in inactivating separase than the *esp1-1* mutation. *esp1-1* cells fail in anaphase but some separase protease activity is detectable in these cells even at the restrictive temperature¹⁶, indicating *esp1-1* is not a null allele. It will therefore be important to analyse mitotic exit in the complete absence of separase.

While separase, Spo12, and Polo are conserved in probably all eukaryotes, orthologs of Slk19 still remain to be identified in organisms other than budding yeast. There are two human homologs of Cdc14 phosphatase, at least one of which has been shown to be able to activate the human APC activator Cdh1, and one of which localises to the nucleolus in interphase^{25,26}. The regulation of human Cdc14 activity is still largely unexplored. It is tempting to speculate, that also human separase, released from securin at anaphase onset, provides a signal not only for chromosome segregation, but also for activation of Cdc14. This model (Fig. 8) implies that mitotic exit is initiated concomitantly with anaphase, and therefore that anaphase onset, rather than anaphase completion^{27,28}, may give the signal to progress out of mitosis.

Methods

All strains were derivatives of W303 or were backcrossed against this background. Epitope tagging of endogenous genes and gene deletions were performed by gene targeting using PCR products^{29,30}. Expression of separase or TEV protease to trigger anaphase in metaphase cells arrested by Cdc20 depletion under control of the *MET3* promoter was as described¹, as were the conditions for growth and release of synchronous cultures from arrest by α -factor¹⁶. Arrest in metaphase by Cdc20 depletion under control of the *GAL1* promoter and release into anaphase by Cdc20 reinduction was as described¹⁹, and immunoprecipitation of endogenous separase from those cells was essentially as described¹⁸, but using α -HA antibody 12CA5 bound to protein A sepharose. For expression of wild type or C1531A mutant separase at endogenous levels the ORFs (Ref. 1) were cloned into YIplac128 (Ref. 31) under control of the separase promoter in 520 bp genomic upstream sequence. Expression levels similar to endogenous separase were confirmed by a protein A-tag at the C-terminus of separase. Polo was cloned under control of the *GAL1* promoter into YIplac211 using PCR, including myc epitopes for detection at the C-terminus. The Cdc14-GFP construct was used as described²¹. Protein extracts to analyse Net1 phosphorylation were prepared as described³². Indirect immunofluorescence and all other techniques were as described¹⁶. Antibodies used were α -HA clone 12CA5, α -myc clone 9E10, α -FLAG clone M2 (Sigma), α -tubulin clone YOL1/34 (Serotec), α -Nop1 clone A66 (Ref. 33), α -securin serum sc-9076 (Santa Cruz Biotechnology), and α -GFP serum from K. Sawin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank J. Aris, O. Cohen-Fix, L. Johnston, K. Sawin, and A. Toh-e for reagents, S. Weitzer for cloning Polo, A. Amon, J. Cooper, J. Hayles, M. Pardo, E. Roldan, T. Toda and all members of the laboratory for discussions and comments on the manuscript.

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Figure 1.

Separase promotes mitotic exit. **a**, Rebudding of mitotic cells by separase (Esp1), but not TEV protease, expression. Y393 (*MET3-CDC20 GAL-ESP1*) and Y353 (*MET3-CDC20 scc1Δ SCC1^{TEV} GAL-TEV*) were arrested in metaphase and expression of separase or TEV protease was induced. **b**, Separase expression triggers release of Cdc14 from the nucleolus. The nucleolus was counterstained by the nucleolar marker Nop1 (Ref. 33). As in **a**, but using Y931 and Y930 containing Cdc14-GFP (Ref. 21). **c**, Separase triggers mitotic exit, as visualised by FACS analysis of DNA content, in cells lacking Clb5. As in **a**, but with Y502 and Y504 deleted for *CLB5*.

Figure 2.

Analysis of nucleolar Cdc14 release by separase. **a**, Cdc14 release by separase is independent of the MEN. Y744 (*MET3-CDC20 GAL-ESP1 CDC14-HA*) and Y804 containing the *cdc15-2* allele were arrested in metaphase and shifted to 37°C for 90 min prior to separase induction. **b**, Cdc14 release by separase depends on Slk19 and Spo12. Y828 (*GAL-ESP1 CDC14-HA*) and Y862, Y863 deleted for *SPO12* and *SLK19* respectively were arrested in metaphase by nocodazole treatment and separase was induced. **c**, Cdc14 release by separase depends on Polo. Y828 and Y976 containing the *cdc5-1* mutation were shifted to 34°C and arrested in metaphase by nocodazole treatment for 2.5 hours prior to separase induction.

Figure 3.

Release of Cdc14 is independent of separase protease activity. **a**, Separase proteolytic activity is not required for Cdc14 release. Y864 (*esp1-1 MET3-CDC20 GAL-ESP1 CDC14-HA*) and Y865 expressing the catalytically inactive separase mutant C1531A were arrested in metaphase and expression was induced. **b**, Proteolytically inactive separase C1531A restores mitotic exit in *esp1-1* cells. Y908 (*MATa esp1-1 Securin-myc CDC14-HA*) and Y953 expressing wild type, and Y954 expressing C1531A separase from the separase promoter were synchronised in G1 using α -factor and released at 35.5°C.

Figure 4.

a, Securin (Pds1) inhibits separase in Cdc14 release. Y864, Y865 and Y932, Y933 containing *GAL-PDS1* were arrested in metaphase and expression of separase and securin induced. **b**, Separase interacts with Slk19 after cleavage. Y894 (*cdc20 Δ GAL-CDC20 ESP1-HA SLK19-myc*) and Y895 control were synchronised at the metaphase to anaphase transition for co-immunoprecipitation. **c**, Separase promotes Net1 phosphorylation. Y970 (*esp1-1 MET3-CDC20 GAL-ESP1(C1531A) NET1-HA*) was arrested in metaphase and proteolytically inactive separase was or was not induced.

Figure 5.

Model for anaphase and mitotic exit. Once separase is free from securin it cleaves cohesin to trigger chromosome segregation. Separase also cleaves Slk19, that after cleavage stays associated with separase. In a reaction that depends on Spo12, the separase/Slk19 complex may promote Polo-dependent hyper-phosphorylation of Net1. Phosphorylated Net1 loses its affinity to Cdc14 that is released to initiate mitotic exit. The possibility that other substrates of Polo, like Bfa1 (Ref. 20), may also be subject to this regulation remains to be tested.