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Anaphase specific auto-cleavage of separase

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Abstract Sister-chromatid separation is triggered by a specific proteolytic cleavage of chromosomal cohesins catalyzed by the endopeptidase separase. Prior to anaphase, separase is inhibited independently by affinity binding to securin and by specific inhibitory phosphorylation. Here we show that separase itself is also subjected to proteolytic cleavages at three adjacent sites. The cleavages are auto-catalyzed and occur specifically at anaphase coincident with separase activation. The cleaved fragments remain associated with each other and are catalytically active. Mapping of the cleavage sites reveals that all three sites are conserved in vertebrates underlining a significant function for this regulation. © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Separase; Securin; Chromatid separation; Anaphase; Cysteine protease

1. Introduction

The partition of the replicated genetic material occurs at the metaphase to anaphase transition of the cell division cycle [1]. At this transition, sister chromatids aligned on the metaphase plate separate simultaneously from each other and migrate to opposite poles. Although the cytological description of this transition was first documented over a century ago, the molecular mechanisms that choreograph these chromosome movements are being elucidated only recently. Sister chromatids are physically paired with each other immediately after their replication [2,3]. By doing so, cells avoid having to re-identify sisters later to ensure equal partitioning in mitosis. The establishment and maintenance of cohesion requires a five-subunit cohesin complex [4–6]. The key protein that regulates the dissolution is a cysteine protease known as separase [7]. Activated at anaphase, separase cleaves the cohesin subunit SCC1/MCD1 at two specific sites, a process both necessary and sufficient to trigger the separation of sister chromatids [8].

The regulation of separase activity is apparently critical for the temporal control of sister-chromatid separation. Separase is inhibited by at least two independent mechanisms prior to anaphase. The first inhibitory mechanism is mediated by a small protein inhibitor called securin, which forms a protein complex with separase [9–12]. The second mechanism is achieved by an inhibitory phosphorylation on separase, which appears to be regulated, directly or indirectly, by the CDC2/cyclin B kinase [13]. In this report, we describe a third mechanism that modifies separase via a proteolytic cleavage event on separase itself.

2. Materials and methods

2.1. Constructs and reagents

Various separase mutants were created by site-directed mutagenesis using either the QuickChange kit (Stratagene) or the GeneEditor system (Promega). All constructs were confirmed by DNA sequencing for the presence of the introduced mutations and for the absence of unintended changes. The protease-inactive separase-CS mutant was described previously [13]. The separase-2RA replaces R1486 and R1506 with alanines. The separase-3RA replaces R1486, R1506, and R1535 with alanines. The separase-TEV2 replaces 1504-ILRGSDG-EDSASG-1516 with two TEV cleavage sites, NLYFQGENLYFQ, in addition to the R1486A and R1535A mutations. All constructs were cloned into a pCS2-based expression plasmid designed for amino-terminal fusion with HA_2-, HA_3 or myc_6-tags. Anti-HA (3F10) and anti-c-myc (9E10) were obtained from Roche. The preparation of affinity-purified anti-separase antibody, recombinant securin, and active separase has been described elsewhere [10,13].

2.2. Cell culture, transfection, and synchronization

Both HeLa S3 and 293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. 293T cells were transiently transfected with a calcium phosphate-based method. Both cell lines were synchronized by either a double-thymidine or a thymidine-nocodazole arrest protocol [15].

2.3. In vitro separase activity assay and size exclusion chromatography of active separase

The activity assay was performed as described previously [13] except that for Fig. 4 the immunocomplex on agarose beads was used directly in the cleavage reaction. For each reaction, 5 μl beads were incubated at 37°C with 1 μl in vitro translated and radioactively labeled cohesin$^{SCC1/MCD1}$ in 4 μl cleavage buffer (30 mM HEPES/KOH pH 7.7, 30% glycerol, 25 mM NaF, 25 mM KCl, 5 mM $MgCl_2$, 1.5 mM ATP, 1 mM EGTA). For size exclusion chromatography of active separase, ZZ-TEV4-separase was co-expressed with securin, activated in *Xenopus* extract, and re-isolated on IgG-Sepharose as described [13]. After elution with TEV protease, separase was diluted 1:1 in 30 mM HEPES-KOH pH 7.8, 2 mM DTT, 5 mM EDTA, 5 mM EGTA, 1 M NaCl and separated on a Superose 6 column (PC 3.2/30, Amersham) in the same buffer. Separase eluted with an apparent molecular weight of 600 kDa as judged by anti-separase immunoblot. For the activity assay, the Superose 6 fractions were dialyzed against cleavage buffer in the presence of ^{35}S-labeled cohesin$^{SCC1/MCD1}$ for 2 h at 37°C.

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2.4. Mapping of the cleavage sites

Myc_6-separase was expressed in 293T cells and arrested at metaphase [13]. To release cells into anaphase, nocodazole was washed away and the culture was returned to the incubator for an additional 2 h. Cells were harvested and lysed with ice-cold lysis buffer (150 mM NaCl, 20 mM Tris pH 8.0, 10% glycerol, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.5% NP-40) supplemented with 1 mM L-microcystin. Next, an S100 was prepared and separase was affinity-purified using anti-c-myc agarose beads (Roche). The beads were washed twice with lysis buffer and twice with lysis buffer in the presence of 500 mM NaCl and 1% NP-40. Separase was eluted at 37°C for 30 min by two incubations with 2 mg/ml myc peptide (Roche) dissolved in lysis buffer. Eluted proteins were separated by SDS–PAGE and detected by silver staining. The corresponding bands were subjected to in-gel digestion and subsequently analyzed by nanoelectrospray using a quadrupole-time-of-flight mass spectrometer. Alternatively, the proteins were transferred onto a Sequi-Blot PVDF membrane (Bio-Rad) and detected by Coomassie staining. The corresponding band was cut out and automated Edman degradation was performed by ProSeq (Boxford, MA, USA).

3. Results and discussion

We first noticed the cleavage of separase when we were analyzing the cell cycle regulation of separase in synchronized HeLa S3 cultures. Separase from cultures at different stages of the cell cycle were enriched by immunoprecipitation followed by anti-separase immunoblot. As a temporal control, the same blot was also analyzed for the levels of securin. Excess amounts of anti-separase beads were used in the immunoprecipitation step to ensure quantitative capture of all separase in the lysate. This was confirmed by the absence of separase in the supernatants (data not shown). As shown in Fig. 1, the level of full-length separase decreases upon entry into the anaphase concomitantly with degradation of securin. At the same time, two bands with molecular weights of approximately 170 kDa and 60 kDa were detected indicating that separase was being cleaved. Since both fragments were detected by the antibody, we expected the cleavage site to reside within the peptide used to raise the antibody (Ser1305 to Thr1573 of the 2120 residues long separase). The identity of the 170 kDa band was confirmed by detection with an antibody raised against the N-terminal 15 amino acids of separase [13]. The anaphase specific cleavage is unlikely an artifact of the nocodazole arrest since most separase remained intact when the drug was not removed, even after incubation for 12 h. Furthermore, cleavage was also observed when a culture went through mitosis in the absence of nocodazole upon release from a double-thymidine arrest (data not shown). Consistent with this observation, human separase is cleaved in *Xenopus* extracts arrested at anaphase [13,14].

To gain further insight into this proteolytic event, we next searched for the protease that is responsible for this cleavage. Separase itself is an endopeptidase. We therefore examined whether the cleavage is self-imposed. By changing the catalytic residue cysteine to a serine, we generated a protease-inactive mutant, separase-CS [8,13]. A HA_2-tag was fused to the amino-terminus of separase-CS to facilitate detection. When this mutant was introduced into 293T cells, the cleavage was largely blocked (Fig. 2A, lane 2). This is in a striking contrast with the wild-type separase (lane 1), of which about 50% was detected in the cleaved form. The residual amount of cleaved separase-CS was presumably due to the activity of the endogenous separase (see below). Therefore, we conclude that the anaphase specific cleavage requires the proteolytic activity of

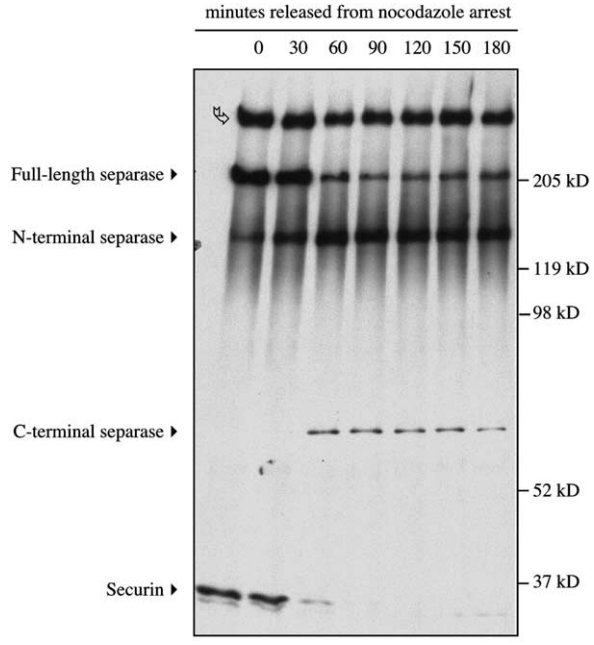

Fig. 1. Cell cycle regulation of separase cleavage in HeLa S3 cells. The dynamics of endogenous separase were analyzed by immunoprecipitation and anti-separase immunoblot. The hollow arrow indicates a cross-reacting band. In addition to the 220 kDa full-length separase, a 170 kDa and a 60 kDa fragment were detected as N-terminal and C-terminal halves of separase, respectively. As a temporal control, the levels of securin were also determined by immunoblot with anti-securin.

separase itself. This is consistent with a simple model that separase cleavage is auto-catalyzed, which is further supported by the mapping of the cleavage sites (see below). We noticed that unlike with endogenous separase in HeLa S3 cells, we rarely detected more than 50% cleavage when separase was transfected in 293T cells. Over-expression and inferior synchronization in the latter case are the likely explanation for this difference.

To characterize the auto-proteolytic reaction, we analyzed whether it occurs via an inter-molecule mechanism. This would predict that wild-type separase is able to cleave protease-inactive separase-CS. To test this model, HA_3-separase-CS and myc_6-separase-WT were co-transfected together with securin into 293T. Lysates from anaphase cells were analyzed by immunoblot with anti-HA. As shown in Fig. 2A, HA_2-separase-CS was cleaved when co-expressed with the myc_6-separase-WT (lane 5) but remained intact when co-expressed with myc_6-separase-CS (lane 3). This result suggests that the cleavage can be catalyzed via an inter-molecule mechanism. It also explains the small amount of cleavage of separase-CS when introduced into 293T cells as these cells also express endogenous separase (Fig. 2A, lane 2). This experiment, however, does not address whether intra-molecule reaction also contributes to the self-cleavage.

Does the cleavage of separase result in dissociation of the fragments? To clarify this issue, we generated active separase by incubation of a separase–securin complex in a *Xenopus* extract to degrade securin. Separase was re-purified from the extract via an N-terminal tag and eluted from the affinity matrix [13]. The eluate was then further purified by gel filtration in 1 M NaCl. As shown in Fig. 2C, the N- and C-terminal separase fragments co-migrated perfectly despite their

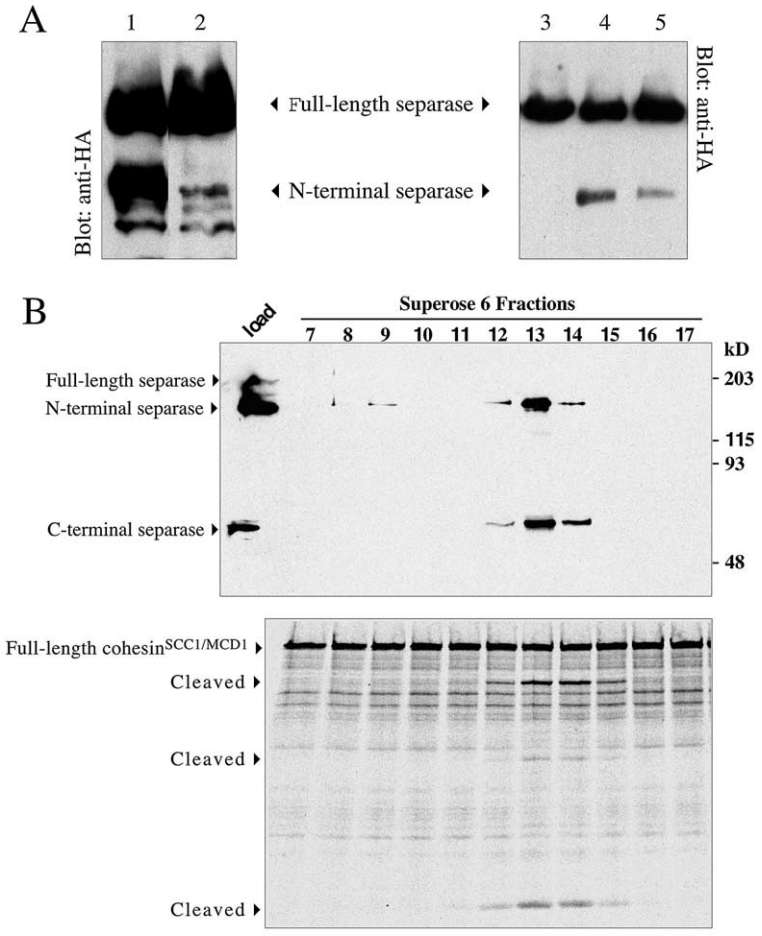

Fig. 2. Characterization of separase cleavage. A: Crude lysates from anaphase 293T cells co-transfected with active HA_2-separase-WT (lane 1) and proteolytic inactive HA_2-separase-CS (lane 2), HA_2-separase-CS and myc_6-separase-CS (lane 3), HA_2-separase-WT and myc_6-separase-CS (lane 4), and HA_2-separase-CS and myc_6-separase-WT (lane 5) were probed with anti-HA to detect full-length separase and its N-terminal cleavage fragment. B: The N- and C-terminal fragments of cleaved separase remain stably associated. N-terminally tagged, active separase was affinity-purified from *Xenopus* extract and further separated by size exclusion chromatography. Resulting fractions were probed for separase fragments and cohesin$^{SCC1/MCD1}$ cleavage activity.

large differences in size (upper panel). Furthermore, the respective fractions exhibited separase activity as judged by a standard cohesin$^{SCC1/MCD1}$ cleavage assay (lower panel) [13]. The fact that the separase cleavage fragments co-purified by affinity and size exclusion chromatography in the presence of high salt concentrations demonstrates that, following autocleavage, the separase fragments stay bound to each other in a stable complex. No full-length separase was detectable in the corresponding fractions, which allows us to conclude that the interaction of the fragments is not bridged by uncleaved separase.

We wanted to map the cleavage site of separase. To this end, we expressed myc_6-separase-WT in 293T cells. Co-expression with securin was performed since expression of separase alone leads to a reduction in the levels of both expression and self-cleavage activity (Fig. 3A, lane 1). This observation is consistent with the notion that securin also plays a positive role in regulating separase stability and activity in addition to its inhibitory function [16]. After release of the transfected cells from a nocodazole arrest, separase was affinity-purified from a crude extract using anti-c-myc beads. After extensive washing with up to 0.5 M NaCl and 1% NP-40, bound proteins were eluted by incubation with myc peptide. As expected, the C-terminal 60 kDa fragment of separase co-eluted with the N-terminal myc_6-tagged 170 kDa fragment. The C-terminal fragment was analyzed by Edman degradations. The sequence obtained (GSDGEDSASGG) matches perfectly with amino acids 1507–1517 of separase. Intriguingly, this sequence is preceded immediately with E-I-L-R, a motif that has been reported to be the consensus site (E-X-X-R) for separase [8]. Therefore, the cleavage was mapped after Arg1506. In parallel, the C-terminal fragment was also analyzed by mass spectrometry. Eleven peptides were identified (data not shown). Consistently, all peptides reside C-terminal of Arg1506 and the most N-terminal peptide identified starts at T1519, 12 residues away from the mapped site.

In an attempt to generate a non-cleavable mutant, we changed Arg1506 to Ala. As shown in Fig. 3B, lane 3, separase-R1506A was still cleaved indicating the existence of a second cleavage site. Using separase-R1506A we tried again automated Edman degradation to map this site. However, among 11 cycles, only four amino acids (xIxxEELxxxx) were unambiguously identified. The mapped sequence was consistent with a cleavage after Arg1486, which is again part of a consensus site (E-I-M-R). The ambiguity in this mapping experiment could result from a third site that was

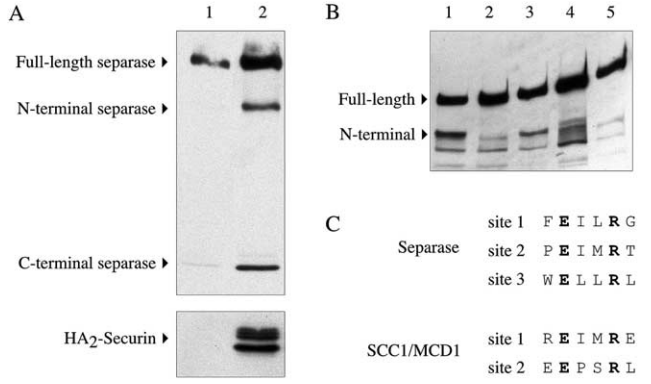

Fig. 3. Mapping of the cleavage sites of separase. A: Myc_6-separase was co-transfected with either HA_2-securin (lane 2) or pCS2-HA_2 vector (lane 1). The expression levels were determined by immunoblot with anti-c-myc. The activity of separase was indicated by the amount of the self-cleavage products. Immunoblot with anti-HA was performed to confirm the expression of transfected securin. B: HA_3-tagged separase-WT (lane 1), -CS (lane 2), -R1506A (lane 3), -2RA (lane 4), or -3RA (lane 5) were prepared from transfected 293T cells in anaphase. The full-length and cleaved N-terminal fragments were detected by anti-HA. C: Sequences comparison of known vertebrate separase cleavage sites. The invariable E and R are in bold.

cleaved at a comparable efficiency as the site at R1486. This was confirmed by that fact that mutations at both R1506 and R1486 still fail to block the cleavage of separase (Fig. 3B, lane 4). A survey of the nearby sequence indeed revealed another potential site at Arg1535 (E-L-L-R). Only when all three arginines were mutated (separase-3RA) was separase cleavage almost completely blocked (Fig. 3B, lane 5). Still, several remaining faint bands suggest the existence of yet more cryptic sites. The fact that all three major cleavage sites bear the consensus recognition motif of separase is consistent with the notion that the cleavage is catalyzed by separase directly. Remarkably, in all three cleavage sites of human separase and two reported cleavage sites on human cohesin$^{SCC1/MCD1}$ [17], there is at least one hydrophobic residue between the conserved E and R (Fig. 3C).

To investigate whether the self-cleavage plays any role in regulating chromatid separation, we first compared the activity of separase in its intact form with that in the cleaved form. We modified the separase cleavage sites by changing both Arg1486 and Arg1535 to Ala and replacing the sequences including Arg1506 with two TEV protease cleavage sites. The Arg1506 site was replaced with TEV cleavage sequence because this site seems to be the primary cleavage site in vivo based on the mapping results described above. The resulting separase-2TEV behaved similarly to separase-3RA when

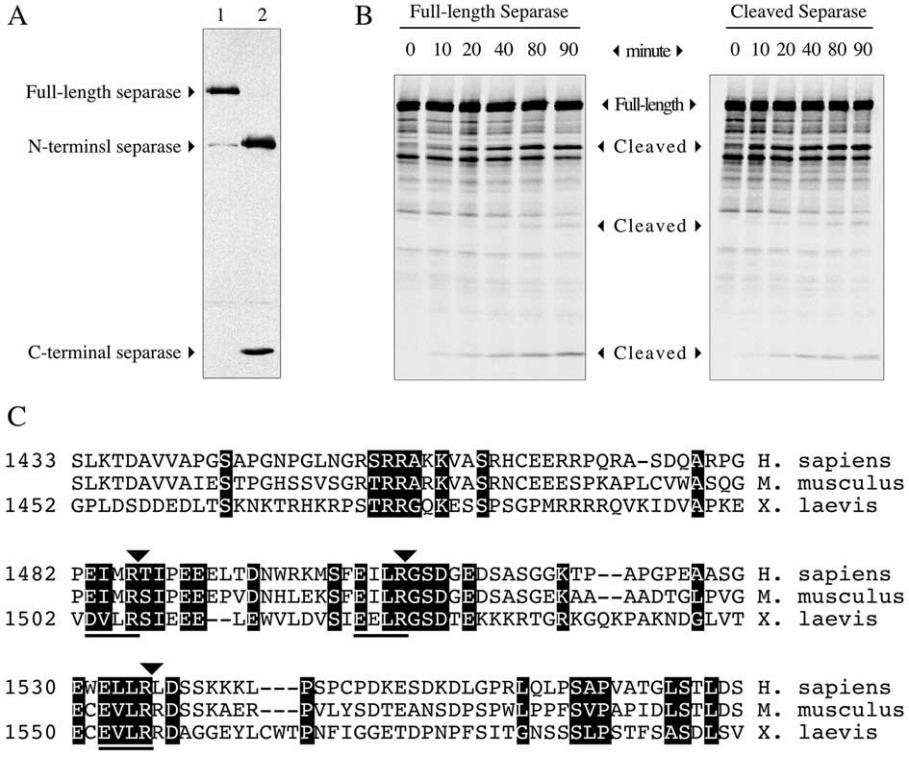

Fig. 4. Separase self-cleavage sites are conserved in vertebrates but self-cleavage has no effect on separase activity in vitro. A: Separase cleavage can be mimicked by cleavage of separase-TEV2 with TEV protease. HA_3-separase-TEV2 was immunopurified on anti-HA matrix and incubated with recombinant TEV protease (lane 2) or TEV buffer only (lane 1) for 30 min at room temperature. The extent of cleavage was examined by immunoblot with anti-HA. B: Comparison of the kinetics of cohesin$^{SCC1/MCD1}$ cleavage by full-length and cleaved separase. C: Alignment of human, mouse, and *Xenopus* separase sequences including the cleavage sites. The human sequence was taken from the cDNA clone KIAA0165. The mouse sequence is derived by combining sequences of EST clones. The *Xenopus* separase sequence was derived from a PCR clone amplified from an egg cDNA library. The numbers on the left side are the positions of the corresponding amino acids. No number is provided for the mouse separase since the full-length sequence is not available. Conserved residues among all three species are shaded in black. The cleavage sites are indicated by the arrows and the conserved E-X-X-R motifs are underlined.

transfected in 293T cells (Fig. 3B, lane 5). As before, we purified separase-TEV2 from transfected, nocodazole-arrested 293T cells and securin was removed in *Xenopus* extract. The resulting separase preparation was split. Half was incubated with recombinant TEV protease to generate cleaved separase (Fig. 4A, lane 2). The other half was mock-incubated with buffer only to leave separase intact (Fig. 4A, lane 1). Finally, the activities of both preparations were compared with each other in the cohesin$^{SCC1/MCD1}$ cleavage assay. As shown in Fig. 4B, the proteolytic activity of cleaved separase is virtually identical to that of full-length separase. The same result was obtained when we compared cleaved separase-WT with intact separase-3RA in a similar experiment (data not shown). These results demonstrated that – at least in vitro – cleavage has no effect on separase activity.

If the limited proteolysis of separase had no function at all, one would not expect the cleavage sites to be conserved, especially as separase of different species is highly divergent outside the catalytic C-terminal domain. We have cloned the gene for *Xenopus* separase and determined the sequence around its putative cleavage sites. Sequence comparison with the existing sequences of human and mouse separase revealed that all three cleavage sites are conserved in an otherwise largely diverse region (Fig. 4C). This evolutionary conservation in vertebrates indicates that these sites and the cleavage events themselves are important. The above results do not rule out the possibility that other aspects of separase function that are not probed by our in vitro activity assay are affected by the self-cleavage. For example, cleavage could result in a change of the sub-cellular localization of separase. Alternatively, cleavage might result in destabilization of separase similar to the situation with *Saccharomyces cerevisiae* cohesin SCC1/MCD1 [18]. Further studies are necessary to reveal the specific cellular function of the separase auto-cleavage.

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