Sister chromatids are held together by the ring-shaped cohesin complex, which likely entraps both DNA-double strands in its middle. This tie is resolved in anaphase when separase, a giant protease, becomes active and cleaves the kleisin subunit of cohesin. Premature activation of separase and, hence, chromosome missegregation are prevented by at least two inhibitory mechanisms. Although securin has long been appreciated as a direct inhibitor of separase, surprisingly its loss has basically no phenotype in mammals. Phosphorylation-dependent binding of Cdk1 constitutes an alternative way to inhibit vertebrate separase. Its importance is illustrated by the premature loss of cohesin when Cdk1-resistant separase is expressed in mammalian cells without or with limiting amounts of securin. Here, we demonstrate that crucial inhibitory phosphorylations occur within a region of human separase that is also shown to make direct contact with the cyclin B1 subunit of Cdk1. This region exhibits a weak homology to Saccharomyces cerevisiae Cdc6 of similar Cdk1 binding behavior, thereby establishing phosphoserine/threonine-mediated binding of partners as a conserved characteristic of B-type cyclins. In contrast to the Cdc6-like domain, the previously identified serine 1126 phosphorylation is fully dispensable for Cdk1 binding to separase fragments. This suggests that despite its in vivo relevance, it promotes complex formation indirectly, possibly by inducing a conformational change in full-length separase. The Cdk1 kinase and the protease separase are two key players of mitosis and meiosis, which control proper segregation of chromosomes into newly forming daughter cells. Cdk1 is considered the master regulator of mitosis. Apart from the catalytic subunit, the holoenzyme consists of a regulatory cyclin of the A- or B-types and a small, phosphopeptide-binding Cks subunit, which might recruit the kinase to prephosphorylated substrates (1). High activity of Cdk1 induces mitotic entry and associated processes like DNA condensation, nuclear envelope breakdown, and mitotic spindle assembly. Cdk1 down-regulation, which is required for reversal of these steps during mitotic exit, is primarily due to degradation of B-type cyclins via the ubiquitin-proteasome system. This destruction is mediated by an E3 ligase called anaphase-promoting complex or cyclosome in conjunction with its activator Cdc20, which is kept inactive by the spindle assembly checkpoint until all chromosomes have properly attached to microtubules and aligned at the metaphase plate of the mitotic spindle. In budding yeast the Cdk inhibitors Sic1 and Cdc6 might contribute slightly to down-regulation of Cdk1 activity in late mitosis (2). In case of Cdc6, the N terminus (Cdc6-N) is both necessary and sufficient for interaction with the kinase (3). Interestingly, Cdc6-N contacts the cyclin B subunit of Cdk1 directly but only when phosphorylated at two (or more) of four Cdk1 phosphorylation sites (4).

Separase is the universal trigger of eukaryotic anaphase via proteolytic cleavage of chromosomal cohesin (5–7). This protein complex holds sister chromatids together, probably by embracing them in a ring-like manner (8, 9). To ensure the timely separation of sister chromatids, separase activity must be tightly controlled. Two mechanisms that are not yet fully understood involve interaction with protein phosphatase 2A (PP2A)² and auto-cleavage (10, 11). In contrast, it is clear that separase is kept inactive by mutually exclusive association with securin or Cdk1 prior to anaphase (12). Securin, which is degraded in an anaphase-promoting complex or cyclosome-dependent manner just like cyclin B1, sequesters most of separase in a mammalian metaphase cell. This inhibitor somehow exerts a positive effect on separase as illustrated by a much decreased level/activity and haploinsufficiency of separase in securin−/− cells (6, 7, 13). On the other hand, securin deficiency results in loss of cohesion in metaphase-arrested Cdc20−/− embryos (14). In mice and human cells, however, deletion of just securin does not prevent long term segregation of chromosomes with correct timing and high fidelity (15–17). This unexpected finding has led to a recent appreciation of the Cdk1-dependent inhibition of anaphase. Indeed, mammalian cells containing no or only limiting amounts of securin lose sister chromatid cohesion prematurely when this alternative mode of anaphase inhibition is additionally abrogated by expression of a Cdk1-resistant separase mutant (18, 19). Interestingly, association of separase and Cdk1 in vitro leads to inactivation not only of the protease but also of the kinase (12). This unexpected role of separase as a Cdk inhibitor is independent of its proteolytic activity and seems to be crucial for down-regulation of Cdk1 at

² The abbreviations used are: PP2A, protein phosphatase 2A; CLD, Cdc6-like domain; GST, glutathione S-transferase; DTT, dithiothreitol; BSA, bovine serum albumin; pBPa, l-phenylalanyl-l-phenylalanine; MS/MS, tandem mass spectrometry; WT, wild type; PBD, polo box domain; HA, hemagglutinin; PPase, protein pyrophosphatase; MOP, maltose-binding protein.

8 This work was supported by a grant from the Deutsche Forschungsgemeinschaft Emmy-Noether Programm (to O. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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the end of female meiosis I. Antibodies that selectively block the binding of Cdk1 to separase prevented extrusion of the first polar body (PBE1) in frog and mouse oocytes. Meiosis I could be rescued, by independently inhibiting Cdk1 at the same time (20). Contradicting these data, a conditional knock-out of separase in mouse oocytes was reported to block PBE1 but not Cdk1 inactivation (21). For both studies to be true nonetheless, one has to assume that an alternative, compensatory mode of Cdk1 inactivation is switched on if, and only if, separase is absent. Furthermore, separase would have an additional, Cdk inhibitor-independent role in PBE1.

Given these clear and unclear functions of great cellular importance, a thorough biochemical understanding of the separase-Cdk1 complex appears instrumental. It has been previously shown that Cdk1 binds human separase only when the latter is phosphorylated on serine 1126 and contains an intact, so-called Cdc6-like domain (CLD) (12). As its name implies, this sequence stretch of separase exhibits a very weak, yet intriguing homology toward the N terminus of *Saccharomyces cerevisiae* Cdc6. This suggested that human separase and yeast Cdc6 might bind Cdk1 in the same manner. Here, we demonstrate that this as yet highly speculative model is, in fact, correct. Like the N terminus of Cdc6, the CLD of separase is phosphorylated and in direct contact with cyclin B1. Our *in vitro* and *in vivo* studies also help to clarify the mechanistic role of the crucial serine 1126 phosphorylation in establishing the separase-Cdk1 complex.

**EXPERIMENTAL PROCEDURES**

*Antibodies and Affinity Matrices*—Antibodies against the N terminus (see Fig. 2A) and an internal fragment (immunoprecipitation for supplement Fig. S1) of human separase as well as against the C terminus of Cks1/2 were described (12, 22). Rabbit anti-GST was raised against GST-Plk1 and purified using a GST affinity column. Human cyclin B1 antibody from mouse (see Fig. 3) and rabbit (see Fig. 4) were from Upstate (05-373) and Santa Cruz (sc-549), respectively. Rabbit anti-Myc, mouse anti-Myc, and mouse anti-β-tubulin were from Santa Cruz (sc-789), Upstate (05-724), and (Developmental Studies Hybridoma Bank), respectively. The following affinity matrices were used: protein G-Sepharose (GE Healthcare, 17-0618-01), IgG-Sepharose (GE Healthcare, 17-0969-01), glutathione-Sepharose (GE Healthcare, 17-5132-02), amylose-resin (New England Biolabs, E0021L), and (Developmental Studies Hybridoma Bank), respectively. The following affinity matrices were used: protein G-Sepharose (GE Healthcare, 17-0618-01), IgG-Sepharose (GE Healthcare, 17-0969-01), glutathione-Sepharose (GE Healthcare, 17-5132-02), amylose-resin (New England Biolabs, E0021L), and nitrotetraacetic acid-agarose (Qiagen, 1018240), and anti-Myc-agarose (Santa Cruz, sc-40 AC).

**Pulldown Experiments**—Bacterially expressed separase fragments immobilized via N-terminal GST or MBP tag were incubated at 18°C in a 5–10-fold larger volume of *Xenopus* anaphase extract containing 17 ng/µl His<sub>6</sub>-hCyclinB1-Δ90 (high-Δ90-extract (22)) for 1 h. The beads were washed five times with XB (100 mM KCl, 0.1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 10 mM Hepes/KOH, pH 7.7, 50 mM sucrose, pH 7.7) containing 0.01% Triton X-100 and then eluted with 50 mM glutathione or 50 mM maltose in XB, 0.01% Triton X-100, 2 mM DTT for analysis. For Fig. 3, 10 µl of glutathione or amylose beads carrying fragments of human separase, fly three rows, or yeast Cdc6 were reisolated from *Xenopus* egg extract as above, equilibrated in PPase buffer (50 mM Tris/HCl, pH 7.6, 0.1 mM EDTA, 2 mM DTT, 0.01% Triton X-100), and incubated with 2 µg of λ-phosphatase (New England Biolabs, P0753L), 10 µg of BSA, or buffer in 20 µl for 1 h at 18°C. After collecting the supernatant, the beads were extensively washed with PPase buffer and finally eluted as above.

For purification of Myc<sub>6</sub>-Tev2-separase transgenic HEK-293 Flp-In T-Rex lines were arrested in the S phase by treatment with 4 mM deoxothymidine for 20 h, released for 4 h, and then treated with nocodazole (0.2 µg/ml) and doxycycline (2 µg/ml) for 14 h. Separase was affinity-purified from native lysates of 6 × 10⁷ cells each (lysis buffer: 20 mM Tris/HCl, pH 7.7, 100 mM NaCl, 10 mM NaF, 20 mM β-glycerophosphate, 5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 5% glycerol, 1 mM EGTA, 1× complete protease inhibitor mix (Roche Applied Science), 1 µM microcystin LR) over 5 µl of anti-Myc-agarose. The beads were extensively washed in lysis buffer containing additional 50 mM NaCl, eluted by TEV protease treatment, and finally analyzed by SDS-PAGE and Coomassie staining or Western blotting.

**Kinase Assay and Far Western Analysis**—1.5 µg GST-separase fragments in 2.5 µl of XB were combined with 2.5 µl of XB containing 10 µM ATP, 20 mM MgCl<sub>2</sub>, 1 µM microcystin-LR, 0.2 µl [γ-³²P]ATP (10 µCi/µl) and active Plk1 purified from SF9 cells (gift from Ingo Görlich). The reaction was performed at room temperature for 30 min.

Separase-agarose complexes were isolated from transfected 293T cells as described (22), resolved by SDS-PAGE, and transferred onto polyvinylidene difluoride membrane. The blocked membranes were incubated for 3 h at room temperature with 1 µg/ml bacterially expressed GST-PBD (amino acids 326–603) from human Plk1 in TBS containing 0.01% Tween 20 and 1% BSA. Subsequently, bound PBD was detected by standard anti-GST immunoblot.

**Site-specific l-p-Benzoylphenylalanine-mediated UV Cross-linking**—Wild type separase fragment (amino acids 1192–1410) or corresponding TAG point mutants generated by GeneEditor *in vitro* site-directed mutagenesis (Promega) were cloned into a pMAL derivative encoding an N-terminal MBP-Tev2-2 as well as a C-terminal (His<sub>6</sub>-FLAG)₉ tag (23, 24). Expression constructs were transformed into competent BL21 cells already carrying plasmid pSup-MjBpARS-6TRN (24) (gift from P. G. Schultz). The cultures were grown in LB containing 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, and 2 mM l-p-benzoylphenylalanine (pBpa; Bachem, F-2800). Expression was induced at an A<sub>600</sub> of 0.8 with 1 mM isopropyl β-D-thiogalactopyranoside and carried out for 3 h at 37°C.

Following denaturing purification (Tris/HCl, pH 7.6, 8 M urea, 300 mM NaCl, 10 mM imidazol, 0.02% Triton X-100) over nitrotetraacetic acid-agarose, fusion proteins were renatured *in situ* with phosphate-buffered saline, 400 mM NaCl, and 0.02% Triton X-100 and eluted in the same buffer plus 250 mM imidazol. Soluble fusion proteins were then bound to a limiting amount of amylose resin, of which 30 µl were incubated in 300 µl of high-Δ90-extract for 1 h at 18°C. After two washes with XB, 0.01% Triton X-100, and three washes in PPase buffer lacking DTT, 20 µl of beads were eluted in 120 µl of PPase buffer (without DTT) containing 50 mM maltose, whereas the remainder (10 µl of beads) was eluted in 60 µl of PPase buffer (without
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DTT) containing 30 units of TEV-protease. Where indicated, eluates were UV-treated using a mercury arc lamp (Lot-Oriel, 500 W, 300–700 nm spectrum, for 15 min at 4 °C) in aliquots of 60 µl. Finally, all of the samples were subjected SDS-PAGE, followed by Coomassie staining (1/10th of each sample) and immunoblotting (1/10th).

Myc- Separase Expressing Flp-In T-Rex Cell Lines—Isogenic HEK-293 Flp-In T-Rex cells carrying inducible Myc-tagged separase transgenes were generated by Flp-recombinase mediated, site-specific genomic integration as described (19, 25). Separase expression was induced with 2 µg/ml doxycycline for 14 h (chromosome spreads) or 24 h (cell cycle profiling) in the presence of 0.2 µg/ml nocodazole, where indicated. For determination of DNA contents 10^6 cells were fixed in 70% ethanol (−20 °C), washed with phosphate-buffered saline containing 0.1% BSA, and resuspended in 69 µM propidium iodide, 100 µg/ml RNase A in 38 mM trisodium citrate. After incubation at 37 °C for 30 min, the cell cycle profiles were analyzed on a FACSCalibur (Becton Dickinson) using CellQuest Pro software.

For chromosome spreads, stepwise addition of 0.25 and 2 ml hypotonic buffer (40% Dulbecco’s modified Eagle’s medium, 400 ng/µl nocodazole) to 10^6 cells were followed by a 5-min incubation. The cells were then pelleted and fixed by stepwise addition of 0.25, 0.25, and 2 ml of Canoy’s solution (methanol: acetic acid = 3:1), followed by a 30-min incubation at 4 °C. After washing with Canoy’s solution, 10 µl out of a total volume of 0.25 ml of cell suspension were put onto a precooled coverslip and dried at 60 °C. The specimens were then stained with Giemsa (Fluka, 11700) for 10 min at room temperature, washed in water, sealed, and analyzed by bright field microscopy.

Mass Spectrometry—Coomassie-stained protein bands were cut and in-gel digested by a mixture of trypsin (15 ng/µl) and GluC (30 ng/µl) (both enzymes from Roche Applied Science) essentially as described (26). After 16 h, peptides were extracted using 10% formic acid in 30% acetonitrile and dried. For pairwise quantitation experiments, one of the samples was digested in buffer made from 18O water (95 atom %; Aldrich) essentially as described (27). Peptide separations in nano liquid chromatography MS/MS experiments were performed on a LCQ Deca (Thermo Finnigan, London, UK). For phosphorylation analyses, the phosphopeptides were enriched using titanium dioxide microcolumns (28). The samples were loaded onto a 2.5-cm-long 100-µm ID precolumn packed with 5 µM AQUASIL C18 phase (Maisch GmbH, Ammerbuch, Germany) and separated on a 13-cm 75-µm column packed with 3 µM reversed-phase material (ReproSil-Pur C18-AQ; Dr. Maisch GmbH, Ammerbuch, Germany) into a pulled fused silica capillary with a tip of 8 µm (NewObjective, Woburn, MA). The peptides were separated by a stepwise 60-min gradient of 0–100% between buffer A (2% acetonitrile, 0.5% formic acid) and buffer B (80% acetonitrile, 0.5% formic acid) at a flow rate of 170 nl/min.

The mass spectrometer (Q-TOF Ultima, Waters, Manchester, UK) was operated in data dependent MS/MS mode to automatically switch between 1.5-s MS survey and 2.0-s MS/MS fragmentations of the four most abundant precursor ions. Data base searches were performed using the Mascot (Matrixscience, London, UK) software package with carbamidiomethyl cysteine as a fixed modification and oxidized methionine and phosphorylation as variable modifications. Precursor and fragment ion tolerances of 0.15 Da were used for the data base searches, and all of the identified phosphorylation sites were validated by direct visual inspection of MS/MS spectra.

RESULTS

A sequence homology to the cyclin B1–binding N terminus of S. cerevisiae Cdc6 defined the CLD of human separase (amino acids 1342–1404) (12) and spurred the speculation that Cdc6 and separase might bind the regulatory subunit of Cdk1 in the same phosphorylation-dependent manner (4). However, doubts about this model are raised by the facts that the homology is very weak and that serine 1126 of separase, the only residue whose phosphorylation is known to be essential for separase-Cdk1 complex formation (22), lacks an equivalent in Cdc6. For the model to be true nonetheless, the following parallels to Cdc6-cyclin B1 have to be fulfilled: 1) Only phosphorylated CLD should mediate the interaction of separase with Cdk1. 2) Phosphorylated CLD should be sufficient for binding of Cdk1. 3) Phosphorylated CLD should entertain direct contacts with the cyclin B1 subunit of Cdk1. 4) Phosphorylated serine 1126 should have an indirect role in binding of the kinase. Here, we combine biochemical and cell biological approaches to rigorously test these predictions.

Phosphorylations within the Cdc6-like Domain of Separase Are Critical for Cdk1 Binding—Asking whether residues within the CLD might be phosphorylated in vivo, we performed mass spectrometric analysis of endogenous separase immunoprecipitated from mitotically arrested HeLaS3 cells. Indeed, we identified three phosphorylation sites within the CLD: Threonine 1346 (22), threonine 1363, and serine 1399 (supplement Fig. S1). Interestingly, Thr1346 and Ser1399 align with Thr7 and Thr39 of S. cerevisiae Cdc6, both phosphorylation sites that are crucial for Cdk1 binding (see Fig. 4 in Ref. 12).

We changed the three phosphorylation sites identified within the CLD to alanine and analyzed the resulting separase mutant (CLD-AAA) by immunoprecipitation from HEK-293 cells. Indeed, although it bound more Cdk1 than separaseS1126A (1126-A), its Cdk1 binding capacity was much decreased compared with wild type (Fig. 1A).

To test whether these CLD phosphorylations contribute to Cdk1-dependent inhibition of separase in vivo, we applied an approach developed by Holland and Taylor (19). They had shown that Cdk1-dependent inhibition becomes crucial when securin is titrated out by induced overexpression of separase in transgenic HEK-293 Flp-In T-Rex cells. Confirming previous findings, we found that doxycycline-induced overexpression of separase-S1126A led to an increase of the G2/M cell population within 24 h, whereas doxycycline-induced overexpression of wild type separase left the cell cycle profile basically unchanged (Fig. 1, B and C). This observation can be explained by 1126-A-specific premature separation of sister chromatids followed by spindle assembly checkpoint-dependent mitotic arrest. Analysis of the CLD-AAA mutant in the same system demonstrated that it, too, elicited a pronounced accumulation of mitotic cells from 24 to 37% within 24 h of doxycycline induction, although the effect was again less dra-
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FIGURE 1. CLD phosphorylation is necessary for efficient Cdk1-dependent inhibition of separase in human cells. A, preventing CLD phosphorylation impairs the ability of separase to associate with Cdk1. Overexpressed Myc<sup>+</sup>-separases were immunopurified from nocodazole-arrested HEK-293 Flip-IN T-Rex cells and analyzed by Coomassie-staining (top right panel) and Western blotting for Cdk1 subunits. B, preventing CLD phosphorylation elicits a G<sub>2</sub>/M arrest that is reversed by phosphorylation-mimicking mutations. Shown on the left are DNA content profiles of cells 24 h after induction of separase transgenes. Equal expression levels of Myc<sup>+</sup>-separase-WT and -mutants upon doxycycline addition (Dox) were confirmed by immunoblotting (right). Anti β-tubulin staining served as a loading control. C, average ratios of G<sub>2</sub>/M over G<sub>1</sub> cells (± S.D.) from three independent separase overexpression experiments like the one shown in B. D, preventing CLD phosphorylation causes premature separation of sister chromatids that is reversed by phosphorylation-mimicking mutations. Giemsa-stained chromosome spreads from doxycycline- and nocodazole-treated cells (14 h) were analyzed quantitatively for their degree of separation (100 spreads each). The bars on these representative images correspond to 10 μm. Thr<sup>1346</sup>, Thr<sup>1363</sup>, and Ser<sup>1399</sup> of human separase were changed to Ala or acidic residues to give CLD-AAA or -EED, respectively.

Cks1/2 is Fully Dispensable for Separase-Cdk1 Complex Formation—How does phosphorylation of serine 1126 enable formation of the separase-Cdk1 complex? Given that binding of B-type cyclins to Cdc6 requires its N terminus to be phosphorylated at multiple, closely juxtaposed sites (4), we considered it unlikely that this isolated phosphorylation site, which even lacks an equivalent in Cdc6, would entertain direct contacts with cyclin B1. However, cyclin B1 is not the only subunit in Cdk1 with the ability to bind partners in a phosphorylation-dependent manner. The small Cks proteins contain an anion-binding pocket and have been shown to interact with phosphorylated peptides (29). To test whether binding of Cks1/2 to phosphorylated serine 1126 might contribute to stable association of Cdk1 with separase, we capitalized on the existence of a Cks-binding-deficient Cdk1 mutant. Myc<sup>+</sup>-tagged Cdk1-WT or -F214G were immunopurified from transfected, nocodazole-arrested 293T cells and probed for association with Cks1/2 and separase. Although Cks1/2 co-purified with wild type but not mutant kinase, equal amounts of separase were detected in both samples (Fig. 2A). This interaction was specific, because separase did not bind to anti-Myc beads in a control, in which the overexpressed Cdk1-WT had been untagged. The fact that Cks-less Cdk1 is fully competent of binding separase strongly argues against Cks1/2 being in direct contact with phosphorylated serine 1126.

**A Ser<sup>1126</sup>-Plk1-CLD Cascade**—Once formed, the separase-Cdk1 complex withstands treatment with phosphatase (12), despite the dephosphorylation of serine 1126 under these conditions (19). This transient requirement could indicate that phosphorylated serine 1126 is not itself part of the Cdk1-binding site but rather exerts an indirect effect. Consistent with this assumption is a model that predicts phosphorylated serine 1126 to act through recruitment of polo-like kinase, which could then phosphorylate the CLD and activate it for binding of Cdk1. It is now firmly established that polo-like kinase typically binds via its polo box domain (PBD) to substrates that had been prephosphorylated by priming kinases like Cdk1 (30, 31). Indeed, serine 1126 and surrounding residues perfectly match the consensus-binding site for the PBD (31). Moreover, based on their neighboring amino acids, threonine 1363 and serine 1399 fit quite well to the consensus phosphorylation site for Plk1 substrates (32). To put this model to a test, we isolated various separases from transfected, nocodazole-arrested 293T cells and analyzed their recognition through bacterially expressed GST-
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According to the “polo model” serine 1126 phosphorylation would assist separase-Cdk1 complex formation exclusively by mediating CLD phosphorylation. If it was correct, then mimicking constitutive CLD phosphorylation should overcome the requirement of serine 1126 phosphorylation with respect to inhibition of separase by Cdk1. The aforementioned CLD-EED mutant likely resembles constitutive phosphorylation because, in contrast to CLD-AAA, it does not elicit a mitotic arrest in HEK-293 cells. However, the EED mutation could not rescue the phenotype caused by 1126-A when both mutations were combined in vivo. Upon their overexpression, separate-1126-A as well as separate-1126-A/CLD-EED efficiently arrested cells in mitosis (supplement Fig. S2, A and B). This illustrates that the double mutant is still fully resistant to Cdk1-dependent inhibition and, further, that the S1126A mutation is dominant. As another test of the “polo model,” we asked more directly whether the S1126A mutation led to a decrease in CLD phosphorylations. Following their co-expression, HA-tagged separate-WT and Mhc-tagged separate-1126-A were affinity-purified from nocodazole-arrested 293T cells on anti-HA and anti-Mhc agarose, respectively (data not shown). The subsequent in-gel trypsin digestion was performed in heavy water in case of separate-WT, thereby specifically labeling only its peptides with $^{18}$O (27). Both samples were then combined and subjected to quantitative mass spectrometry. This analysis revealed that serine 1399 phosphorylation was unaffected by serine 1126 phosphorylation (supplement Fig. S3). Taken together, these results provide strong in vivo evidence against the hypothesis that serine 1126 phosphorylation promotes separase-Cdk1 complex formation via recruitment of Plk1.

Cdk1 Binding to Separase Fragments Provides Evidence for an Indirect Role of Ser1126 Phosphorylation—We gained unexpected new insights about the role of serine 1126 phosphorylation when we developed a simple in vitro assay of Cdk1 binding to separate fragments. Here, bacterially expressed, GST-tagged separate fragments were incubated in mitotic Xenopus egg extract, reisolated, and analyzed by Coomassie staining as well as immunoblotting. As hoped, Cdk1 bound to serine 1126- and CLD-containing separate fragments, whereas it did not associate with GST alone (Fig. 3A, lanes 1, 3, 5, and 11). It is known that separate loses its ability to associate with Cdk1 both in vitro and in vivo when the most conserved residues of the CLD (amino acids 1391–1402) are deleted (12, 19). This small internal deletion largely abolished also the binding of Cdk1 to the separate fragment (lane 2), thereby validating our assay. Furthermore, as predicted from our in vivo data, the CLD-AAA mutation was sufficient to abrogate association with Cdk1 (lanes 4 and 10), whereas the CLD-EED mutant retained full kinase binding ability (lane 12). Underscoring the importance of threonine 1363 and/or serine 1399 phosphorylation, additional mutation of these sites abolished residual Cdk1 binding of a T1346A-carrying fragment (lanes 8 and 9). Surprisingly, mutating serine 1126 to alanine did not compromise the ability of separate to associate with Cdk1 in this assay (lane 6). Even an N-terminally shortened fragment, which started only ~70 residues downstream of serine 1126 (amino acids 1192–1440), retained full Cdk1 binding capacity (lane 7). These data provide further argument against serine 1126 being part of the kinase-
binding site of separase. Instead, they suggest that phosphorylation at this crucial site induces a conformational change that is necessary to render full-length separase competent for Cdk1 binding (Fig. 4B).

The CLD Directly Contacts Cyclin B1—The specific binding of Cdk1 to short separase fragments enabled us to ask which subunit of Cdk1 entertains direct physical contact with the CLD by applying a covalent cross-linking approach developed by Schultz and colleagues (23). Here, an orthogonal aminoacyl-tRNA synthetase/tRNA pair recognizes the amber codon TAG by Schultz and colleagues (23). Here, an orthogonal aminoacyl-tRNA synthetase/tRNA pair recognizes the amber codon TAG corresponding to the three distinct C-terminal residues of Thr specifically associated with the Cdk1 binding site of separase fragments (amino acids 1192–1410) and Cdk1 from mitotic Xenopus egg extracts were eluted from amylose beads by maltose (lanes 1–10) or Tev- protease (lane 11) and UV-irradiated where indicated. A specific covalent adduct above background formed only between cyclin B1 and a separate probe containing pBpA at position 1401 (lanes 8 and 10), although all of the pBpA mutants retained full cyclin B1 binding capacity (middle panel). Note that the cyclin B1-separase adduct and free separate bait experience the same downshift by approximately 45 kDa upon Tev-protease-mediated removal of the MBP tag (lanes 10 and 11). This also note that a specific covalent adduct above background could not be observed between Cdk1 and any pBpA-separase mutant (data not shown). B, model of stepwise assembly of the separase-Cdk1 complex.

Phosphorylation-dependent Binding of Cdk1 Is a Conserved Feature of Separases—The bipartite separase of Drosophila melanogaster, which consists of Sse and Thr, is linked genetically to Cdk1 (34). However, direct binding between these two key cell cycle players has not yet been demonstrated in this organism. The specific binding of Cdk1 to short fragments of human separase inspired us, therefore, to screen fragments from D. melanogaster separase for their ability to physically interact with Cdk1. Indeed, a GST fusion protein comprising the C-terminal 370 amino acids of Thr specifically associated with Cdk1 from mitotic Xenopus egg extracts (Fig. 3B, lane 11). If this interaction of fly separase with Cdk1 resembles that of human separase and yeast Cdc6 with Cdk1, then it should also occur in a phosphorylation-dependent manner. We wanted to
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know whether a putative phosphorylation dependency of the Thr-Cdk1 complex formation could be investigated without the need of lengthy mapping and mutation of specific phosphorylation sites. To this end, complexes of Cdk1 with a CLD-containing fragment of human separase or the N-terminal ~50 amino acids of S. cerevisiae Cdc6 were treated with either λ-PPase or unspecific protein (BSA). This led to elution of Cdk1 only in the PPase-containing samples but not in the controls (lanes 3–10). As predicted, λ-PPase treatment was insufficient to dissociate Cdk1 from a CLD-EED separase fragment (lanes 1 and 2). These results not only substantiate the phosphomimetic character of the CLD-EED mutation but also show that crucial phosphate groups within Cdk1-binding fragments of human separase and yeast Cdc6 remain accessible even after association with the kinase. When Cdk1-Thr was treated with PPase accordingly, Cdk1 indeed eluted also from the insect separase (lane 12). However, it remained stably bound in the controls (lanes 11 and 13). Based on this result, we propose that direct interaction with Cdk1 is a conserved characteristic of metazoan (and possibly all) separases.

DISCUSSION

The separase-Cdk1 complex not only plays an important role in the regulation of sister chromatid separation (12, 18, 19), its formation likely contributes also to the down-regulation of Cdk1 at the end of meiosis I (20). Based on our studies we can now develop a model of how this crucial complex is formed (Fig. 4B): First, separase is phosphorylated on serine 1126 and on several residues within the CLD. Although Cdk1 targets serine 1126 and threonine 1346, Plk1 might phosphorylate serine 1399 (and possibly threonine 1363). Of these phosphorylations, the one at serine 1126 likely induces a conformational change in separase, priming it for the final step, namely the stable association with Cdk1. This occurs primarily via recognition of the phosphorylated CLD of the protease by the cyclin B1 subunit of the kinase.

Apart from the additional level of regulation by serine 1126 phosphorylation and conformational change, separase-Cdk1 closely resembles the Cdc6-Cdk1 complex (Refs. 4 and 12 and this study): 1) S. cerevisiae Cdc6 and human separase share a region of weak homology, which is crucial for Cdk1 binding in either case. 2) Cdk1 binding is greatly enhanced by multiple phosphorylations within these related sequence stretches. Moreover, two sites important for complex formation in either case are conserved in position: Thr7 and Thr39 of S. cerevisiae Cdc6 align with Thr1346 and Ser1399 of human separase. Another important phosphorylation site of Cdc6, Ser43, aligns with Asp1463, which might mimic constitutive phosphorylation of separase at this position. 3) The regulatory B-type cyclin alone is sufficient to bind separase or Cdc6 and does so by making direct contact with the phosphorylated region of homology. It has been proposed that the Cks subunit recruits Cdk1 to prephosphorylated substrates (1). Our studies on separase-Cdk1 together with the work from the Diffley group on Cdc6-Cdk1 now firmly establish cyclin B as another subunit of Cdk1, which links this crucial mitotic kinase to phosphorylated binding partners (Refs. 4 and 12 and this study). However, whereas Cks-mediated recruitment is suspected to enable further phosphorylations by Cdk1, cyclin B-mediated binding of Cdk1 to both separase and Cdc6 results in inhibition of the kinase (2, 3, 12). Taken together, the questions arise whether the homology between separase and Cdc6 might enable the bioinformatic identification of novel cyclin B-interactors and whether these, too, might be inhibitors of Cdk1.

Herein, we have identified Ser1399 of human separase as a novel phosphorylation site crucial for Cdk1 binding. Its importance is underscored by the finding that a separase lacking all other CLD phosphorylation sites is inhibited almost normally by Cdk1 when Ser1399 is present but becomes largely resistant to Cdk1 when this last phosphorylation site is also removed. Interestingly, serine 1399 is the only site within a 500-amino acid fragment of separase that is phosphorylated by polo kinase in vitro. Although we excluded the possibility that phosphorylated Ser1126 serves as a landing pad for Plk1, it remains possible that Plk1 phosphorylates Ser1399 after being recruited via its PBD to another, hitherto unidentified phosphorylation site within separase. The binding of wild type but not mutant PBD to phosphorylated separase-securin is consistent with this speculation.

The proposed conformational change in separase upon phosphorylation of serine 1126 does not activate phosphorylation of the CLD by the corresponding kinases because a S1126A mutation leaves the degree of serine 1399 phosphorylation unchanged. Although other explanations are possible, we instead favor that it allows cyclin B1 to gain access to the phosphorylated CLD by relieving a steric hindrance (Fig. 4B). This explanation is supported by the dispensability of serine 1126 for the binding of Cdk1 to separase fragments, in which the CLD is likely to be freely accessible.

Recent results from Taylor and co-workers (10) indicate that PP2A might stimulate the proteolytic activity of separase, at least indirectly. Because the PP2A-binding site on separase is closely juxtaposed to the CLD and because Cdk1-binding to separase depends on phosphorylation, an obvious model is that PP2A dephosphorylates separase, thereby acting as an antagonist of Cdk1-dependent inhibition. However, deleting the PP2A-binding site does not obviously result in an increased association of Cdk1 with separase (10). Vice versa, mutating the CLD does not affect PP2A binding to separase in prometaphase (10). Both observations argue against the above possibility. Thus, how PP2A regulates separase and which phosphatases, if any, affect separase-Cdk1 complex formation remain unresolved issues.

Why do separase-Cdk1 complexes form according to such a complicated mechanism? In particular, why were Ser1126 phosphorylation and subsequent conformational change added to separase-Cdk1 as opposed to Cdc6-Cdk1 formation? Two possible answers come to mind: 1) The additional step might integrate an additional level of regulation. Because Ser1126 seems to be phosphorylated by Cdk1 (see below) just as the critical sites in Cdc6 are, this leaves the step of conformational change for added regulation. The mitotic rotamase Pin1 induces conformational changes in substrate proteins by catalyzing a peptidyl-
prolyl isomerization from cis to trans (35). Given that it has a strong preference for a phosphorylated serine N-terminal to the isomerized proline bond (36) and given that serine 1126 phosphorylation matches this consensus, it remains an attractive possibility that Pin1 might be required for the Cdk1-dependent inhibition of separase. 2) Phosphorylated Ser<sub>1126</sub> might have a second function that is closely coupled to separase-Cdk1 complex formation. For example, separase might catalyze degradation of cyclin B1 via a meiosis I-specific SCF-complex that recognizes phosphorylated serine 1126. This highly speculative model is inspired by a number of observations; SCF complexes have been shown to see to the destruction of other cyclins (37, 38). Some F-box proteins predicted to recruit phosphorylated proteins to SCF are specifically expressed only during meiosis. This might explain why separase seems to be an efficient inhibitor of Cdk1 at the end of meiosis I (20) but to have no role in exit from mitosis. Finally, anaphase-promoting complex or cyclosome might not be required for female meiosis I, but separase clearly is (21, 39, 40).

The phosphorylation sites at Ser<sub>1126</sub> and Thr<sub>1346</sub> match the consensus of Cdk1 substrates, the degree of Ser<sub>1126</sub> phosphorylation closely correlates with Cdk1 activity in vivo, and Ser<sub>1126</sub> is efficiently phosphorylated by Cdk1 in vitro (22). All of this points to Cdk1 promoting its association with separase and, thus, its own inactivation. The crucial Cdk1 phosphorylation sites in separase might therefore serve as a sensor and regulator of Cdk1 activity. Applying this model to late meiosis I is particularly attractive, because here Cdk1 inactivation, despite being required for spindle disassembly, must not go to completion if relicensing of replication ought to be prevented. When first stripped of securin at the beginning of anaphase, separase would still be highly phosphorylated and efficiently inactivate Cdk1. As Cdk1 activity would drop, however, so would the phosphorylation status of separase, thereby limiting separase-dependent inactivation of the kinase. By liberating sequestered Cdk1 upon dephosphorylation, separase might even contribute to the reaccumulation of active kinase upon entry into meiosis II.

Acknowledgments—We thank A. Gutsmiedl, C. Schultheis, and especially A. Strasser for excellent technical assistance; J. Gorr for Ptx1 kinase; and J. Walther for anti-xCdk1 antibody. Regarding LIV-mediated cross-linking, we greatly appreciate the help and advice from Dr. S. A. Etchells as well as the gift of pSup-MjBpARS-6TRN from P. G. Schultz.

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