Protein Phosphatase 2A and Separase Form a Complex Regulated by Separase Autocleavage*[S]

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The onset of anaphase is triggered by the activation of a site-specific protease called separase. Separase cleaves the chromosomal cohesins holding the duplicated sister chromatids together, allowing sisters to simultaneously separate and segregate to opposite ends of the cell before division. Activated separase cleaves not only cohesin, but also itself; however, the biological significance of separase self-cleavage has remained elusive. Before anaphase, separase is inhibited by at least two mechanisms. The first involves the binding of securin, whereas the second requires the phosphorylation-dependent binding of cyclin-dependent kinase 1 (Cdk1)/cyclin B1. Because securin and Cdk1/cyclin B1 interact with separase in a mutually exclusive manner, the degradation of both these inhibitors plays an important role in activating separase at anaphase. Here we identify a new separase interacting partner, a specific subtype of the heterotrimeric protein phosphatase 2A (PP2A). PP2A associates with separase through the B′ (B56) regulatory subunit and does so independently of securin and cyclin B1 binding. The association of PP2A with separase requires a 55-amino acid domain closely juxtaposed to separase autocleavage sites. Strikingly, mutation of these cleavage sites increases PP2A binding, suggesting that separase cleavage disrupts the interaction of PP2A with separase. Furthermore, expression of a non-cleavable separase, but not a non-cleavable mutant that cannot bind PP2A, causes a premature loss of centromeric cohesion. Together these observations provide a new mechanistic insight into a physiological function for separase self-cleavage.

Each time a cell divides it must first faithfully duplicate its chromosomes and then accurately distribute the sister chromatids between the two daughter cells. After their generation during S phase, sister chromatids are held together through the action of the multisubunit chromosomal cohesion complex (1, 2). During mitosis, cohesin is dissolved, and sister chromatids are segregated toward opposite ends of the cell. In all eukaryotes studied chromosome segregation is initiated by the activation of a site-specific protease known as separase (3, 4). Separase cleaves the cohesins linking each duplicated chromatid pair, allowing sisters to separate simultaneously at anaphase. Because the separation of sister chromatids is an irreversible event, it is clearly vital that separase activation is tightly regulated; activation before the correct attachment of all chromosomes on the microtubule spindle risks chromosome missegregation and the production of aneuploid daughter cells (5).

Curiously, upon activation, vertebrate separase cleaves not only cohesin but also itself. Cleavage occurs at three closely spaced sites in between separase conserved C-terminal catalytic domain and its large N-terminal portion (6, 7). Once cleaved, the N- and C-terminal fragments of separase remain associated and are catalytically active. However, separase cleavage is not required for separase activation *in vitro*. Thus, despite the conservation of separase cleavage sites in vertebrates, the cellular function of this regulation has remained largely mysterious. Recently, human HCT-116 cells were generated in which all but one of the six endogenous separase cleavage sites were mutated (8). These cells delayed entry into mitosis and exhibited chromosome alignment defects. However, how separase cleavage acts to promote mitotic entry and progression through mitosis remains unclear.

For most of the cell cycle, separase is bound by its chaperone securin, which inhibits separase proteolytic activity (9, 10). Once all chromosomes have bioriented, securin is targeted for proteasomal degradation by the anaphase promoting complex/cyclosome, thereby liberating and activating separase (11–16). Surprisingly, however, securin is not an essential gene in mice, and human cells lacking securin execute mitosis normally (17–19). This strongly suggests securin-independent mechanisms are able to inhibit separase. Indeed, a second regulatory mechanism has been described in vertebrates and is mediated by the Cdk1/cyclin B1 protein kinase (20–22). Cdk1 phosphorylates separase on serine 1126 during mitosis (20), thereby promoting the stable binding of Cdk1/cyclin B1 to separase via the cyclin B1 subunit (21, 22). Importantly, securin and Cdk1/cyclin B1 bind separase in a mutually exclusive manner (21, 22), suggesting that these two mechanisms may play redundant roles in inhibiting separase during mitosis. Thus, at least in vertebrates, the anaphase promoting complex/cyclosome triggers separase activation through the concomitant destruction of securin and cyclin B1.

We recently investigated the role Ser-1126 phosphorylation and cyclin B1 binding plays in inhibiting separase in human

cells. By stably expressing a non-phosphorylatable S1126A (S:A)[4] mutant of separase above endogenous securin levels, we were able to create a situation in which cyclin B1-mediated inhibition of separase becomes essential for accurate chromosome segregation (22). Nevertheless, despite overriding both securin- and cyclin B1-mediated inhibition of separase, cells expressing non-phosphorylatable separase still aligned most of their chromosomes at metaphase, with premature sister chromatid disjunction occurring just 5 min before control cells. Because human cells lacking cohesin fail to align their chromosomes at all (23), it appears that cohesion is still maintained during early mitosis in separase S:A cells. This suggests that mechanisms other than securin- and cyclin B1-mediated inhibition of separase are able to protect cohesin from cleavage. Consistent with this notion, securin$^{-/-}$ mouse embryonic stem cells expressing a non-phosphorylatable separase allele are viable and appear to divide normally (24).

If a novel mechanism is able to regulate cohesin cleavage, then it is possible that this pathway acts directly upon separase to regulate its proteolytic activity. To investigate the possibility that a protein other than securin and cyclin B1 is able to regulate separase activation, we set out to identify novel separase-associated proteins. Here, we show that separase binds protein phosphatase 2A (PP2A) via a direct interaction with PP2A regulatory B subunit. We define a 55-amino acid domain required for the association of PP2A and show that separase autocleavage disrupts PP2A binding. Together, these observations provide new insight into a physiological function of separase self-cleavage.

EXPERIMENTAL PROCEDURES

Molecular Cloning and DNA Manipulations—All separase constructs except for ZZ-Tev-Separase (20) were cloned into pcDNA5/FRT/TO/Myc as previously described (22). The S1126A, C2029A, LAG, and Δ12 mutants were as described (22). A non-cleavable mutant (E1483R, R1486E, E1503R, R1506E, E1532R, R1535E (6)) was generated by three rounds of site-directed mutagenesis (QuikChange, Stratagene). Separase fragments encoding amino acids 325–2120, 1278–1841, 1278–1556, and 1557–1841 were generated by PCR amplification. To create separase Δ55, an N-terminal fragment (encoding amino acids 1–1418) was amplified and cloned as an XhoI-AgeI fragment. The C-terminal portion (amino acids 1474–2120) was then amplified and cloned as an AgeI-NotI fragment. PP2A_A, PP2A_Bα, PP2A_B′α, and PP2A_C open reading frames were amplified by reverse transcriptase-PCR and cloned into pcDNA5/FRT/TO/Myc and pcDNA3/Myc (25) for transfection and *in vitro* transcription/translations respectively. To express securin, cyclin B1, and PP2A_B′α as C-terminal His-FLAG-tagged fusions, the open reading frames were cloned into pCS2 His$_6$-FLAG-His$_6$-FLAG. Plasmids encoding 4HA PP2A_B′ α, β, γ1, γ3, δ, and ε isoforms were obtained from the Addgene plasmid repository. All constructs were confirmed by DNA sequencing.

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[4] The abbreviations used are: Cdk, cyclin-dependent kinase; PP2A, protein phosphatase 2A; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; S:A, S1126A; C:A, C2029A; GST, glutathione *S*-transferase; WT, wild type; HA, hemagglutinin; Tet, tetracycline; MI, mitotic index; FRT, Flp recombinase target.

Cell Culture, Drug Treatment, and Transient Transfections—TA-HeLa cells and Flip-In™ TRex™-293 cells were cultured under conditions described previously (26, 27). Stable isogenic cell lines expressing separase and PP2A transgenes were generated using FRT/Flp-mediated recombination as previously described (27). Briefly, pcDNA5 plasmids were co-transfected with a plasmid encoding the Flp recombinase (pOG44) into Flp-In™ TRex™-293 cells. After selection for hygromycin resistance, colonies were pooled and expanded, and transgene expression was induced with 1 μg/ml tetracycline. Flip-In™ TRex™-293 cells were used for all experiments unless stated otherwise. To enrich for mitotic cells, cultures were treated for 18 h with 0.2 μg/ml nocodazole. To arrest cells at G$_1$/S, cells were treated with 2 mM thymidine for 20 h. Transient transfections were performed using the calcium phosphate method according to the manufacturer's instructions (ProFection®, Promega).

Antibodies, Immunoblotting, and Immunoprecipitation—For immunoblot analysis, soluble cell proteins or immune complexes were resuspended in sample buffer, separated by SDS-PAGE, blotted onto nitrocellulose membranes (Bio-Rad), and probed with the following antibodies: 4A6 (mouse α-Myc, Upstate, 1:1,000); mouse α-cyclin B1 (Upstate, 1:2,000); DCS-280 (mouse α-securin, Abcam, 1:250); mouse α-separase (Abcam, 1:1,000); mouse α-N-terminal separase (20); rabbit α-P-S1126 (ref (22), 1:100); TAT-1 (mouse α-Tubulin, 1:10,000); rabbit α-PP2A_A (Cell Signaling, 1:1,000); mouse α-PP2A_C (BD Transduction Laboratories, 1:5,000); mouse α-PP2A_C (Upstate, 1:1,000), sheep α-GST (1:500); mouse α-FLAG (Sigma, 1:1,000). For immunoprecipitation, α-Myc, α-cyclin B1, α-separase, α-FLAG, or mouse IgG (Sigma) were coupled to protein G-Sepharose (Amersham Biosciences) at a final concentration of 0.2 μg/ml. Cells were harvested and lysed in 1× lysis buffer (0.1% (v/v) Triton X-100, 100 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 50 mM NaF, 1 μM okadaic acid, 1 μg/ml antipain, aprotinin, and pepstatin, and 5 μg/ml bestatin, chymostatin, and leupeptin) for 30 min on ice. Lysates were cleared by centrifugation at 13,000 rpm for 30 min at 4 °C, precleared with protein G-Sepharose for 1 h at 4 °C, then incubated for a further 2 h at 4 °C with the appropriate antibody coupled beads. After five washes in lysis buffer, immune complexes were immunoblotted or eluted with a synthetic Myc peptide (Sigma).

Large Scale Immunoprecipitations and Mass Spectrometry—Large scale immunoprecipitations were performed as above with the following modifications. ~2.5 × 10^8 cells were harvested and lysed on ice for 30 min in 1 ml of 2× lysis buffer (0.2% (v/v) Triton X-100, 200 mM NaCl, 20 mM Tris (pH 7.4), 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 50 mM NaF, 1 μM okadaic acid, 1 μg/ml antipain, aprotinin, and pepstatin, and 5 μg/ml bestatin, chymostatin, and leupeptin). Lysates were gently vortexed then centrifuged at 50,000 rpm for 30 min at 4 °C. Supernatants were precleared with protein G-Sepharose then incubated with 25 μl of anti-Myc coupled protein G beads (~5 μg of antibody). After 5 washes at 4 °C for 15 min, bound proteins were separated by SDS-PAGE and visualized by

silver staining. Protein bands were excised, digested with trypsin, and subject to analysis by MALDI-TOF mass spectrometry.

Tandem Affinity Purification—293T cells were co-transfected with ZZ-Tev$_4$-separase and securin or securin-His$_6$FLAGHis$_6$FLAG expression vectors using a calcium phosphate-based method and subsequently synchronized as described (28). 48 h after transfection the nocodazole-arrested cells were resuspended in lysis buffer (0.1% (v/v) Triton X-100, 100 mM NaCl, 5 mM MgCl$_2$, 20 mM Tris (pH 7.7), 1 mM EGTA, 5% (v/v) glycerol, 20 mM β-glycerophosphate, 10 mM NaF) supplemented with complete protease inhibitor mixture (plus EDTA, Roche Applied Science), Dounce-homogenized, and left on ice for 10 min. After ultracentrifugation for 45 min at 35,000 rpm, the supernatant was mixed with IgG-Sepharose™ 6 Fast Flow beads (Amersham Biosciences). The suspension was rotated overnight at 4 °C before the antibody-beads were pelleted and washed with lysis buffer. ZZ-Tev$_4$-tagged separase complexes were subsequently eluted with ⅕ bead volume His-Tev-protease for 1 h at 18 °C. As a negative control, ⅕ bead volume His-Tev-protease was added during the incubation procedure. His-Tev eluates were expanded to 1 ml in lysis buffer and rotated for 4 h at 4 °C together with 50 μl of α-FLAG M2-agarose beads (Sigma). Antibody-beads were pelleted and washed with lysis buffer. Proteins were eluted from antibody-beads with SDS and analyzed by immunoblotting.

Cell Biology—DNA content and mitotic index measurements were performed using flow cytometry as described (25). Briefly, cells were fixed in 70% ethanol, stained with the MPM-2 antibody (Upstate) followed by a fluorescein isothiocyanate-conjugated donkey α-mouse antibody, then stained with propidium iodide. All DNA content profiles were measured 24 h postinduction. Mitotic timings were determined by phase contrast time-lapse microscopy as described (22). Imaging began 4 h after tetracycline induction, and at least 50 cells were analyzed over the subsequent 16 h. Mitotic timings are presented as box-and-whisker plots, where the boxes represent the interquartile range, and the whiskers show the entire range. Metaphase spreads were carried out 8 h after transgene induction as described (27). At least 50 spreads were counted in three independent experiments; values in *bar graphs* represent the mean ± S.E.

Protein Purification and in Vitro Binding Assays—cDNA fragments were PCR-amplified and cloned into pGEX-4T-3 (Amersham Biosciences). GST fusion proteins were expressed in BL21 *Escherichia coli* cells by induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside at 37 °C for 2 h. Bacteria were lysed with ice-cold bacterial lysis buffer (0.1% (v/v) Triton X-100, 100 mM NaCl, 50 mM Tris (pH 7.5), 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg/ml antipain, aprotinin, and pepstatin and 5 μg/ml bestatin, chymostatin, and leupeptin), sonicated, and centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant was incubated at 4 °C overnight with glutathione-Sepharose (Amersham Biosciences). After 5 washes with lysis buffer, recombinant proteins were eluted in lysis buffer containing 6 μg/ml glutathione then dialyzed in phosphate buffered saline. *In vitro* transcription/translations were performed using the T7 RNA polymerase TNT® Quick Coupled system (Promega) according to the manufac-turer's instructions. Reaction mixtures were diluted in lysis buffer and incubated for 2 h at 4 °C with a recombinant GST fusion protein immobilized on glutathione-Sepharose. Beads were washed five times in lysis buffer, and bound proteins detected by immunoblotting.

Phosphatase Assays—Cells expressing Myc-tagged separase or PP2A subunits were lysed in lysis buffer lacking phosphatase inhibitors, and immunoprecipitations were performed using α-Myc antibody-coupled beads. Immune complexes were equilibrated with phosphatase wash buffer (0.1% (v/v) Triton X-100 (pH 7.4), 100 mM NaCl, 10 mM Tris, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) then washed once with assay buffer (50 mM Tris (pH 7.0), 100 μM CaCl$_2$). Half of the immune complex was immunoblotted, whereas the rest was assayed for phosphatase activity using a malachite green phosphatase assay (Upstate) according to the manufacturer's instructions. Where indicated, okadaic acid (Calbiochem) was added at a final concentration of 1 μM. For each assay, a control immunoprecipitation was performed from cells in which transgene expression had not been induced. The background reading obtained from this control (−Tet) reaction was subtracted from the value obtained in the induced (+Tet) immunoprecipitate. Experiments were repeated three times, and values were plotted as bar graphs of the mean ± S.E. Values were normalized to the average maximum phosphatase activity observed in the experiment.

RESULTS

Separase Associates with PP2A—To identify separase interacting proteins, we immunoprecipitated exogenous Myc-tagged separase from 293 cells and analyzed isolated proteins by SDS-PAGE followed by silver staining. Because numerous separase autocleavage products obscured potential binding partners (not shown), we used 293 cells expressing a catalytically inactive C2029A mutant of separase (separase C:A), thereby minimizing autocleavage. Importantly, the separase C:A transgene was under tetracycline control, allowing us to directly compare induced and uninduced samples processed with the same antibody. Silver stain analysis of separase C:A complexes revealed several bands not present in the uninduced control (Fig. 1*A*). These were excised and identified by mass spectrometry. In addition to separase itself and securin, two PP2A subunits were identified. PP2A is a serine/threonine phosphatase that predominantly exists as a heterotrimeric complex in the cell containing a structural subunit (PP2A_A), a catalytic subunit (PP2A_C), and a third regulatory subunit (PP2A_B) comprised of four subfamilies (B, B′, B″, and B‴) (29, 30). Our analysis identified PP2A_A and PP2A_B′, also known as B56. To determine whether the catalytic subunit was present, we isolated immune complexes from 293 cells expressing Myc-tagged wild type (WT) separase. Immunoblotting revealed the presence of PP2A_C (Fig. 1*B*), which together with the silver stain analysis demonstrates that separase binds the A, B′, and C subunits of PP2A, most likely in the form of a heterotrimeric holoenzyme.

The PP2A_B′ family is composed of several isoforms (α, β, γ, δ, ε) that are >80% identical in the conserved central core (31). To determine which PP2A_B′ isoforms interact with separase,
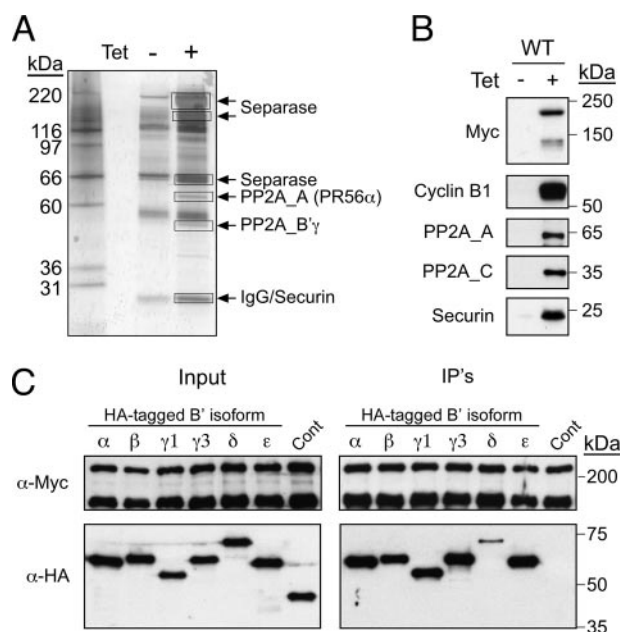
FIGURE 1. **Separase forms a complex with PP2A.** *A*, α-Myc immunoprecipitates from lysates of cells expressing Myc-tagged separase C:A. Co-purified proteins were separated by SDS-PAGE and silver-stained. The *boxed bands* were excised and identified by MALDI-TOF mass spectrometry. *B*, α-Myc immunoprecipitate from lysates of cells expressing Myc-tagged WT separase. Immune complexes were separated by SDS-PAGE and immunoblotted as indicated. *C*, α-Myc immunoprecipitates (*IP*) from lysates of cells co-expressing Myc-separase and various HA-tagged PP2A_B′ isoforms or a HA-tagged SLP2 control protein (*Cont*). Immune complexes were separated by SDS-PAGE and immunoblotted as indicated.

FIGURE 2. **Separase interacts with PP2A with during interphase.** *A*, cells expressing Myc-tagged PP2A_B′ were synchronized with thymidine (*Thym*) or nocodazole (*Noc*), and WT separase was purified from cell lysates by α-Myc immunoprecipitation. Immunoprecipitates (*IP*) were blotted with the antibodies indicated. *MI* represents the percent mitotic index of the population. *B*, lysates from nocodazole-arrested HeLa cells were immunoprecipitated with α-separase, α-cyclin B1 or α-Myc (*Control*) beads. Immunoprecipitates were separated by SDS-PAGE and immunoblotted as indicated. *C*, cells expressing Myc-tagged WT separase were synchronized with thymidine or nocodazole, and WT separase was purified from cell lysates by α-Myc immunoprecipitation. Immune complexes were separated by SDS-PAGE and blotted with the antibodies indicated. *MI* represents the percent mitotic index of the population.

HA-tagged B′ α, β, γ1, γ3, δ, and ε were transfected into 293 cells stably expressing Myc-tagged WT separase, and Myc immune complexes were isolated. Separase interacted with all five PP2A_B′ isoforms but failed to associate with a HA-tagged control protein (Fig. 1*C*). Thus, similar to the situation recently reported for SgoI (32, 34), separase can associate with any PP2A_B′ isoform. In all subsequent experiments the PP2A_B′α isoform was used.

To confirm the interaction between PP2A and separase, we asked whether PP2A could co-immunoprecipitate separase. Analysis of Myc-PP2A_B′α immune complexes revealed the presence of both PP2A_A and PP2A_C (Fig. 2*A*). Furthermore, phosphatase activity was associated with the immunoprecipitate (data not shown), indicating formation of an active complex. Importantly, endogenous separase was specifically detected in the α-Myc immune complex (Fig. 2*A*), demonstrating that endogenous separase associates with exogenous PP2A_B′α. Finally, to establish whether PP2A and separase interact at endogenous levels, separase was immunopurified from mitotic HeLa cells. PP2A_A and PP2A_C were specifically present in the separase immunoprecipitate but not in the control (Fig. 2*B*). Together, these observations indicate that separase interacts with heterotrimeric PP2A and that this interaction can be recapitulated by expressing either exogenous PP2A_B′ or exogenous separase.

Separase Binds PP2A during Interphase—Separase associates with securin in interphase but only binds cyclin B1 during mitosis after serine 1126 phosphorylation (20–22). To determine when PP2A binds separase, we immunopurified Myc-tagged
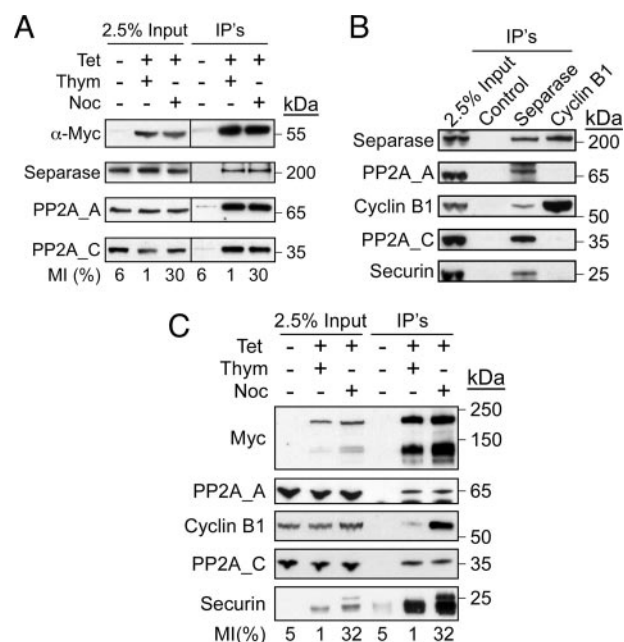
WT separase from G_1/S and mitotically enriched populations of cells. Similar amounts of PP2A_A and PP2A_C associated with separase in both G_1/S- and mitotic-enriched populations, suggesting that like securin, PP2A binds separase during interphase. Consistently, similar levels of endogenous separase bound Myc-tagged PP2A_B′ in G_1/S and mitotic populations (Fig. 2*A*).

Separase Associates with a Specific Subtype of PP2A—The B subunits of PP2A are typically responsible for substrate targeting and/or subcellular localization (29, 30). Therefore, we asked whether separase interacts directly with the regulatory B subunit. To test this, a fragment encoding the regulatory region of separase, which contains the PP2A binding domain (separase[RR], amino acids 1278–1556, see below), was expressed as a GST fusion in bacteria. Purified GST-separase[RR] was then incubated with *in vitro* translated Myc-tagged PP2A subunits. Although GST-separase[RR] did not bind PP2A_A or PP2A_C, it did interact with PP2A_B′α (Fig. 3*A*). This interaction was specific for GST-separase[RR] as neither GST nor GST-Mad2 bound (Fig. 3*B*). Thus, separase[RR] can directly associate with PP2A_B′α, consistent with the notion that the regulatory B′ subunit does indeed target PP2A to separase.

In these *in vitro* binding reactions, GST-separase[RR] could bind PP2A_B′α but not PP2A_Bα (Fig. 3*B*), suggesting that separase interacts with PP2A complexes containing B′ subunits rather than B subunits. To test this we isolated α-Myc immune complexes from cells expressing Myc-tagged-Bα or -B′α. As
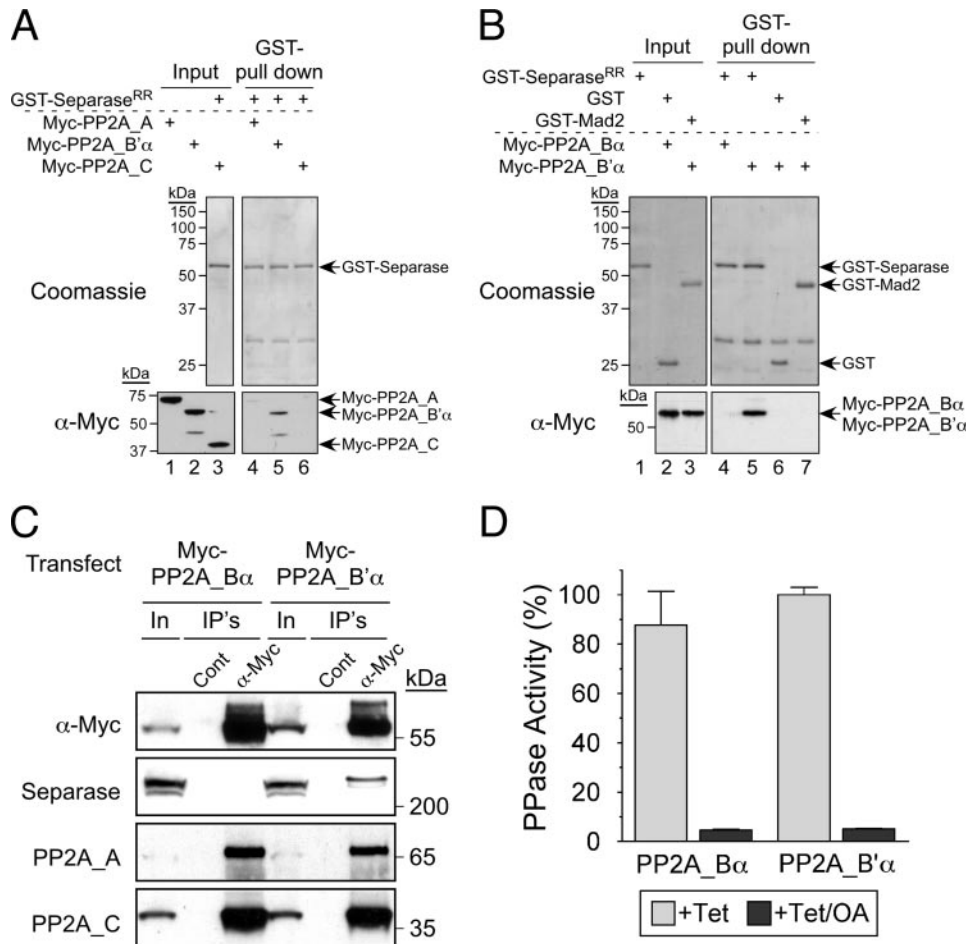
FIGURE 3. **Separase binds a specific subtype of PP2A.** *A*, recombinant GST-separase[RR] (amino acids 1278–1556) was pre-bound to glutathione-Sepharose and incubated with *in vitro* translated Myc-tagged PP2A subunits as indicated. After washing, bound PP2A subunits were separated by SDS-PAGE and detected by α-Myc immunoblotting. Coomassie Blue-stained gel demonstrates equal amounts of GST fusion protein were added to each binding reaction. *B*, equimolar amounts of recombinant GST, GST-Mad2, or GST-separase[RR] were pre-bound to glutathione-Sepharose and processed as in *A*. *C* and *D*, lysates from cells expressing Myc-tagged PP2A_B or PP2A_B' were immunoprecipitated with α-Myc or IgG (*cont*) beads. Immune complexes were divided and inputs (*In*) or immunoprecipitates (*IP's*) immunoblotted as indicated (*C*) or assayed for phosphatase (*PPase*) activity in the presence or absence of okadaic acid (*OA*) (D). Values are plotted relative to the average phosphatase activity of PP2A_B' immunoprecipitates.
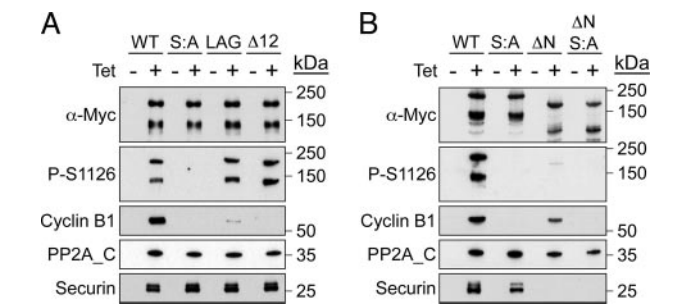

FIGURE 4. **The association of PP2A with separase does not require securin or cyclin B1 binding.** *A* and *B*, lysates from nocodazole-arrested cells expressing the indicated Myc-tagged separase transgenes were immunoprecipitated with α-Myc beads. Immune complexes were resolved by SDS-PAGE and immunoblotted as indicated. Note that the S:A, LAG, and Δ12 separase mutants are deficient in binding cyclin B1, whereas ΔN separase lacks the first 325 amino acids and does not bind securin. *P-S1126*, phosphorylated Ser-1126.

expected, both Myc-Bα and Myc-B'α bound PP2A_A and _C (Fig. 3*C*), and both were associated with phosphatase activity (Fig. 3*D*), consistent with their incorporation in an enzymati-

cally active complex. Significantly, separase associated with B'α, but not Bα, confirming that separase interacts specifically with PP2A enzymes containing the B' regulatory subunit.

The Association of PP2A with Separase Does Not Require Securin or Cyclin B1 Binding—Although separase binds PP2A in interphase, it only binds cyclin B1 in mitosis (20–22). This suggests that binding of separase to PP2A does not require cyclin B1 binding. Furthermore, separase appears to bind PP2A directly (Fig. 3*A*), suggesting that the PP2A interaction is also independent of securin binding. To test this, we first asked whether cyclin B1 binding was required for the interaction of PP2A by immunopurifying separase mutants that do not bind cyclin B1. For this experiment we used three separase mutants that we have previously shown are unable to interact with cyclin B1, namely separase Δ12, LAG, and S:A (20–22). Separase Δ12 and LAG contain mutations in the cyclin B1 binding domain of separase, whereas separase S:A contains a mutation that prevents the phosphorylation of Ser-1126, which is required for cyclin B1 binding. Significantly, all three of these mutants bound PP2A_C (Fig. 4*A*), demonstrating that the association of PP2A with separase does not require cyclin B1 binding.

Using a similar approach we next set out to establish whether securin binding was required for the separase-PP2A interaction. Securin contacts separase in both the N and C termini, and a distinct securin binding site has not yet been defined (6, 35, 36). Nevertheless, the first 325 amino acids of an N-terminal separase fragment are required for securin binding in a yeast two-hybrid screen (37). Therefore, we created a cell line expressing a Myc-tagged separase mutant missing the first 325 amino acids (separase ΔN). Although this deletion completely abolished the ability of separase to bind securin, PP2A_C did associate with separase ΔN (Fig. 4*B*). Furthermore, separase ΔN S:A, which binds neither securin nor cyclin B1, also bound PP2A_C.

Separase Forms a Trimeric Complex with Securin and PP2A—Having established that PP2A can associate with separase mutants which cannot bind cyclin B1 and securin, we next asked whether PP2A binding prevented the binding of cyclin B1 and securin. Mutually exclusive interactions are not unprecedented; separase interacts with either cyclin B1 or securin but
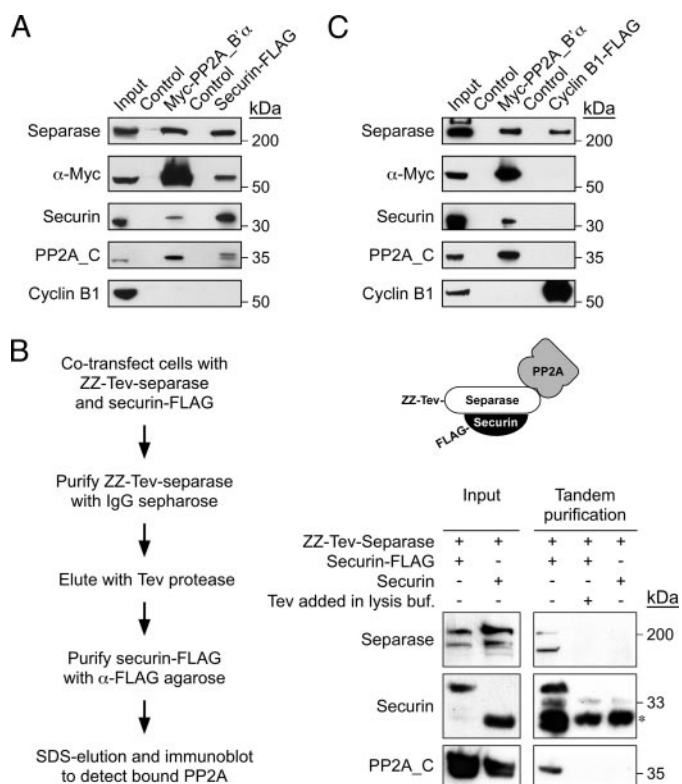
FIGURE 5. **Separase forms a heterotrimeric complex with securin and PP2A.** *A,* lysates from cells co-expressing Myc-PP2A_B′ and securin-FLAG were immunoprecipitated with α-Myc, α-FLAG, or IgG (control) beads. Immunoprecipitates were separated by SDS-PAGE and immunoblotted as indicated. *B,* cells co-expressing ZZ-Tev-separase and securin-FLAG or securin were synchronized with nocodazole. A tandem affinity purification was performed first using IgG-Sepharose with subsequent Tev elution. Released proteins were then purified in a second step over anti-FLAG beads, and bound proteins were detected by immunoblotting. The *asterisk* (*) corresponds to the IgG light chain and securin. *C,* lysates from cells co-expressing Myc-PP2A_B′ and cyclin B1-FLAG were immunoprecipitated and immunoblotted as in *A.*

FIGURE 6. **Mapping the PP2A binding determinants in separase.** *A,* schematic representation of human separase showing the ARM-repeat domain, the active and inactive caspase-like domains, the cyclin B1 binding site, the autocleavage sites (*AC*), and the PP2A binding site. *B,* schematic representation of Myc-tagged separase fragments used in *C. C,* lysates from cells stably expressing Myc-tagged separase fragments were immunoprecipitated with α-Myc beads. Immune complexes were resolved by SDS-PAGE and immunoblotted as indicated. *D–E,* lysates from cells stably expressing Myc-tagged WT or Δ55 separase were immunoprecipitated with α-Myc beads. Immune complexes were divided and either immunoblotted as indicated (*D*) or assayed for phosphatase activity in the presence or absence of okadaic acid (*OA*) (*E*). Values are plotted relative to the average phosphatase activity of WT separase immunoprecipitates. Note that separase Δ55 lacks 55 amino acids required for PP2A binding.

not both (21, 22). Therefore, we co-expressed in cells Myc-PP2A_B′ with either securin-FLAG or cyclin B1-FLAG. We then separately isolated α-Myc and α-FLAG immune complexes. Importantly, PP2A_B′ co-precipitated both separase and securin, whereas securin co-purified both separase and PP2A_B′ (Fig. 5*A*), suggesting separase, securin, and PP2A may form a heterotrimeric complex. To formally demonstrate the existence of such a complex, we carried out a tandem affinity purification from cells co-expressing ZZ-Tev-separase and securin-FLAG (Fig. 5*B*). First, ZZ-Tev-separase was isolated with IgG-Sepharose, and purified proteins were released by cleavage of the tag with Tev protease. Securin-FLAG was then purified from the resulting eluate using anti-FLAG-agarose, and bound proteins were detected by immunoblotting. Although PP2A was present in the tandem purified separase-securin complex (Fig. 5*B*), it did not bind non-specifically to either IgG-Sepharose or anti-FLAG-agarose. Thus, a separase-securin-PP2A trimeric complex does exist.

Importantly, neither Myc-tagged PP2A_B′α nor endogenous PP2A_C co-precipitated with cyclin B1-FLAG, although separase was present (Fig. 5*C*). To rule out the possibility of tag interference, endogenous cyclin B1 was immunopurified from mitotic HeLa cells. Although separase was present in the cyclin
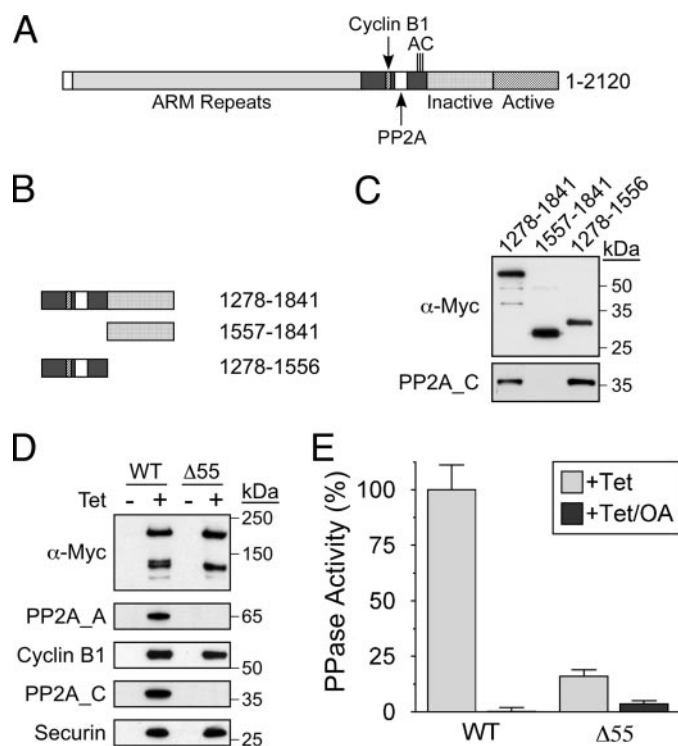
B1 immune complex, PP2A_A and PP2A_C were not detected (Fig. 2*B*), indicating that binding of PP2A and Cdk1/cyclin B1 to separase is mutually exclusive.

PP2A Interacts with the Regulatory Region of Separase—To further understand how PP2A interacts with separase, we set out to define the separase PP2A binding site (Fig. 6*A*). First, we expressed several Myc-tagged separase fragments in human cells and assayed their ability to co-immunoprecipitate PP2A subunits. This analysis identified a fragment spanning amino acids 1278–1841 capable of co-immunoprecipitating PP2A_C as efficiently as WT separase (not shown). To further define the binding site, this fragment was subdivided into two fragments encoding amino acids 1278–1556 and 1557–1841 (Fig. 6*B*). Significantly, the 1278–1556 fragment retained the ability to co-immunoprecipitate PP2A_C (Fig. 6*C*), demonstrating that the PP2A binding site resides within amino acids 1278–1556 of separase. Interestingly, in addition to the PP2A binding site, the 1278–1556 region also contains the cyclin B1 binding site and the autocleavage sites (Fig. 6*A*), indicating this is an important regulatory region of separase. Note that this fragment corresponds to separase[RR] used in the *in vitro* binding studies described above (Fig. 3, *A* and *B*).

Having defined a 279-amino acid region as being sufficient for PP2A binding, we set out to create deletions in full-length separase that would abolish PP2A binding. Remarkably, deleting amino acids 1419–1473 (separase Δ55) reduced PP2A binding to below the limit of detection, whereas the binding of securin and cyclin B1 was unaffected (Fig. 6D). Taken together with the *in vitro* binding studies (Fig. 2A) plus the observation that PP2A binding does not require separase ability to bind cyclin B1 or securin (Fig. 4, A and B), this confirms that PP2A interacts with separase directly rather than indirectly via an association with securin or cyclin B1.

Separase Interacts with Enzymatically Active PP2A—Having identified a separase mutant that could bind securin and cyclin B1 but not PP2A, we asked whether the PP2A associated with separase was enzymatically active. Myc-tagged WT and separase Δ55 were immunoprecipitated from cells, and the immune complexes were assayed for their ability to dephosphorylate a synthetic peptide using a malachite green phosphatase assay. Importantly, active phosphatase co-purified with WT separase isolated from cells, and moreover, this activity was sensitive to the phosphatase inhibitor okadaic acid (Fig. 6E). However, the phosphatase activity associated with separase Δ55 was reduced by ~80%, indicating that the activity associated with WT separase is largely accounted for by the co-purifying PP2A. Although we have not compared the activity of separase-bound PP2A with free PP2A, this observation suggests that the PP2A bound to separase is indeed catalytically active.

PP2A Binding Is Not Required to Inhibit Separase in Vivo—To determine the functional significance of the separase-PP2A interaction, we asked whether PP2A binding is required to activate or inhibit the protease in cells. Recently, we have shown that overexpression of separase S:A in human cells alleviates cyclin B1-mediated inhibition of separase, resulting in a premature loss of sister chromatid cohesion that can be readily assayed by flow cytometry, time-lapse microscopy, and chromosome spreads (22). By contrast, expression of WT separase at a similar level has little effect. Thus, we predicted that if PP2A binding was required to inhibit separase, expression of separase Δ55 would cause a premature loss of sister chromatid cohesion, thereby inducing phenotypes similar to those observed after expression of separase S:A. We, therefore, expressed separase Δ55 in cells at levels similar to WT separase (supplemental Fig. S1A). Surprisingly, however, induction of separase Δ55 had no obvious effect on cells, as judged by flow cytometry, time-lapse microscopy, or analysis of chromosome spreads (Fig. 7, A, B, and C and supplemental Table S1). This indicates that PP2A binding is unlikely to play a critical role in inhibiting separase in cells. We did, however, observe an increase in the level of serine 1126 phosphorylation on separase Δ55 compared with WT (supplemental Fig. S1B), suggesting that phosphorylated serine 1126 may be a substrate for PP2A.

To establish whether PP2A binding is required for separase ability to cleave cohesin, we took advantage of our previous observation that the premature loss of cohesion observed in separase S:A cells is dependent on separase catalytic activity (22). We, therefore, generated a tetracycline-inducible separase S:A Δ55 cell line (supplemental Fig. S1C) and tested whether preventing PP2A binding was able to abolish the catalytic activ-

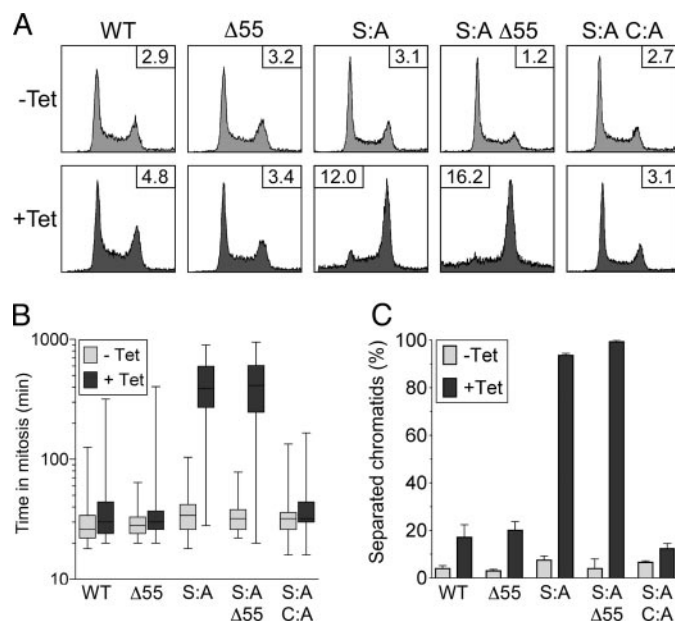FIGURE 7. **Preventing PP2A binding does not abolish separase catalytic activity *in vivo*.** *A*, DNA content profiles of cells 24 h after induction of separase transgenes. *Numbers* represent the mitotic index (%), as determined by MPM-2 staining. *B*, box and whisker plots showing the time spent in mitosis following induction of separase transgenes. Mitotic timings were determined by phase contrast time-lapse microscopy beginning 4 h post-induction. *C*, bar graph quantitating the number of metaphase spreads with separated chromatids 8 h post-induction of Myc-tagged separase transgenes. Note that separase Δ55 lacks 55 amino acids required for PP2A binding; separase S:A contains a S1126A mutation which prevents cyclin B1 binding and results in separase becoming active prematurely; separase S:A Δ55 lacks 55 amino acids required for PP2A binding and contains a S1126A mutation; separase S:A C:A harbors a mutation in the catalytic cysteine (C2029A) in combination with the S1126A mutation.

ity of separase S:A. Significantly, cells expressing separase S:A Δ55 phenocopied separase S:A cells (Fig. 7, A, B, and C and supplemental Table S1), thereby demonstrating that PP2A binding is not directly required for separase catalytic activity *in vivo*.

Separase Autocleavage Disrupts PP2A Binding—Interestingly, the 55-amino region required for PP2A binding lies just 13 amino acids upstream of the first separase autocleavage site (Fig. 6A). We, therefore, asked whether separase cleavage influences PP2A binding by creating a non-cleavable mutant (separase N/C) in which the three major autocleavage sites were mutated from EXXR to RXXE (6). We expressed separase N/C as a tetracycline-inducible Myc-tagged fusion protein in cells and examined the levels of co-purifying PP2A by immunoblot. Although both WT and separase N/C immunoprecipitated similar amounts of cyclin B1 and securin, increased levels of PP2A were associated with separase N/C (Fig. 8A). Furthermore, separase N/C immune complexes contained ~2.6-fold more phosphatase activity than WT separase immunoprecipitates (Fig. 8B). We next asked whether the increased PP2A binding to uncleaved separase was dependent upon the PP2A binding site that we had identified, as opposed to promoting binding at a new site. To this end we generated a line expressing a non-cleavable separase Δ55 mutant (separase Δ55 N/C). Importantly, the ability of separase Δ55 N/C to bind PP2A was largely abolished (Fig. 8A), and the phosphatase activity associated with the immune complex was reduced by ~7-fold (Fig. 8B). Thus, the
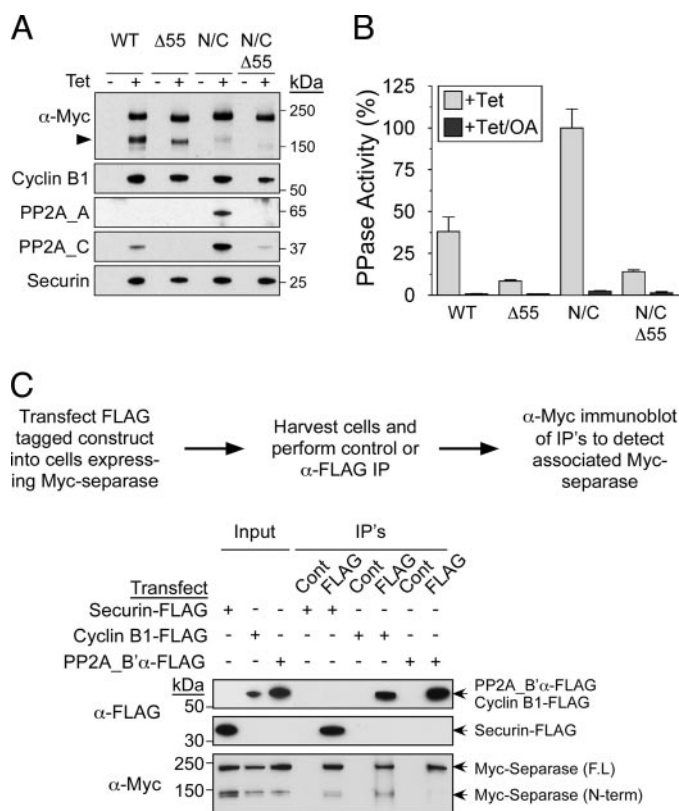
FIGURE 8. **Separase autocleavage disrupts PP2A binding.** *A–B*, lysates from cells stably expressing the indicated Myc-tagged separase transgenes were immunoprecipitated with α-Myc beads. Immune complexes were divided and either immunoblotted as indicated (*A*) or assayed for phosphatase (*PPase*) activity in the presence or absence of okadaic acid (*OA*) (*B*). The *arrow-head* in *A* marks the position of the separase N-terminal autocleavage products. Values in *B* are plotted relative to the average phosphatase activity of separase N/C immunoprecipitates. *C*, cells stably expressing Myc-tagged WT separase were transfected with FLAG-tagged securin, cyclin B1, or PP2A_B'. Cells were lysed, and immunoprecipitations (*IP*) were performed using α-FLAG or IgG (control) beads. Immune complexes were separated by SDS-PAGE and immunoblotted as indicated. Loading was normalized to ensure equal amounts of co-purifying WT separase. In contrast to cyclin B1 and securin, the majority of WT separase associated with PP2A is the uncleaved, full-length (*F.L*) form. Note, separase N/C contains mutations in the separase three main autocleavage sites, whereas separase N/C Δ55 is deficient in both autocleavage and PP2A binding.

increased association of PP2A with separase N/C is dependent upon the 55-amino acid region juxtaposed to the autocleavage sites. Note that trace amounts of PP2A_C are detectable in the separase Δ55 N/C immune complex, suggesting that the Δ55 deletion does not completely abolish PP2A binding (Fig. 8*A*). In addition, whereas the Δ55 deletion in GST-separase[RR] substantially reduces PP2A binding *in vitro*, it does not totally abolish it (supplemental Fig. S1, *D* and *E*).

One explanation for the increased association between PP2A and separase N/C is that separase cleavage disrupts or inhibits PP2A binding (see "Discussion"). This predicts that PP2A should associate with full-length but not cleaved separase. To test this we co-expressed in cells Myc-tagged WT separase along with either securin, cyclin B1, or PP2A_B', all as FLAG-tagged fusion proteins. Securin, cyclin B1, and PP2A_B' were then immunopurified via the FLAG tag, and bound separase was detected by immunoblotting for Myc. N-terminal separase cleavage products were readily detectable in the inputs of all
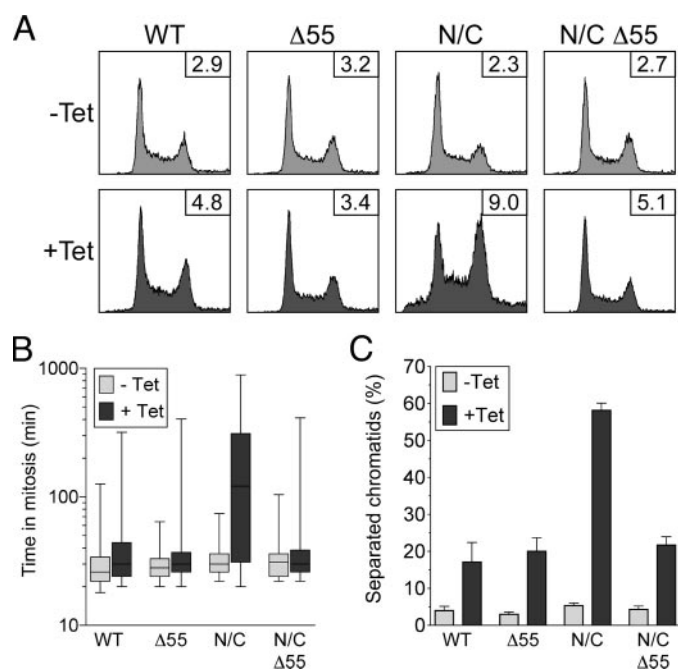

FIGURE 9. **The phenotype caused by expression of non-cleavable separase is rescued by preventing PP2A binding.** *A*, DNA content profiles of cells 24 h after induction of separase transgenes indicated. *Numbers* represent the mitotic index (%) as determined by MPM-2 staining. *B*, *box* and *whisker plots* showing the time spent in mitosis after induction of Myc-tagged separase transgenes. Mitotic timings were determined by phase contrast time-lapse microscopy beginning 4 h post-induction. *C*, *bar graph* quantitating the number of metaphase spreads with separated chromatids 8 h post-induction of Myc-tagged separase transgenes. Note that the data for WT and Δ55 separase are taken from Fig. 7 and are shown alongside merely for comparison.

three-cell lysates and when cyclin B1 and securin were immunoprecipitated (Fig. 8*C*). However, the separase present in the PP2A_B' immune complex was predominantly full-length separase, consistent with the notion that PP2A does not efficiently bind cleaved separase.

Overexpression of Non-cleavable Separase Triggers Premature Sister Chromatid Disjunction by Recruiting PP2A—To probe the functional relationship between separase cleavage and PP2A binding, we examined the cell cycle consequences of expressing Myc-tagged separase N/C and separase Δ55 N/C. In contrast to WT separase, induction of separase N/C had a profound effect on the cell cycle profile, with ~47% of cells accumulating DNA contents ≥4N 24 h post-induction (Fig. 9*A*) (note that the WT and Δ55 data in Fig. 9 are taken from Fig. 7 and are shown alongside merely for comparison). Time-lapse microscopy demonstrated that whereas uninduced cells spent an average of ~32 min in mitosis (Fig. 9*B*, supplemental Table S1), induction of separase N/C resulted in 54% of cells spending >100 min in mitosis (Fig. 9*B*). By contrast, expression of WT separase had only a marginal effect, with 12% of cells spending >100 min in mitosis (Fig. 9*B*, supplemental Table S1). To determine whether the prolonged mitotic delay was due to a premature loss of sister chromatid cohesion, we analyzed chromosome spreads. Significantly, whereas 83% of WT separase spreads contained chromosomes with paired sisters, 58% of separase N/C cells had completely separated their sister chromatids (Fig. 9*C*). Thus, similar to the phenotype induced by

overexpressing separase S:A (22), expression of separase N/C causes a premature loss of sister chromatid cohesion, thereby activating the spindle checkpoint that induces a prolonged mitotic arrest.

Remarkably, deleting the PP2A binding domain from separase N/C completely rescued the phenotype induced by separase N/C expression (Fig. 9, *A*, *B*, and *C*). Because preventing PP2A binding does not abrogate separase catalytic activity *in vivo* (see above), these data strongly suggest that the phenotypes caused by separase N/C are a result of the increased PP2A binding.

DISCUSSION

Here we describe the identification of PP2A as a novel separase interacting partner. Separase forms a complex with a subtype of enzymatically active heterotrimeric PP2A, and this interaction occurs at physiological concentrations in cells. Importantly, the binding of PP2A to separase requires a 55-amino acid domain right between the cyclin B1 binding site and separase autocleavage sites. Preventing self-cleavage of separase at these sites results in an increased association of PP2A with separase, and moreover, immunopurified PP2A preferentially binds full-length separase. We suggest, therefore, that separase cleavage may release bound PP2A. In addition, because PP2A binding does not appear to play a direct role in activating or inhibiting separase catalytic activity *in vivo*, separase cleavage may play a role in regulating the mitotic functions of specific PP2A isoforms.

Separase Associates with a Specific Subtype of PP2A—The specificity of phosphatase function arises from the ability of highly conserved catalytic subunits to associate with regulatory subunits. In the case of PP2A, a variable regulatory B subunit from one of four subfamilies (B, B′, B″, and B‴) associates with a conserved scaffolding (A) and catalytic (C) subunit to form a heterotrimeric holoenzyme. The B regulatory subunits are, therefore, responsible for modulating the substrate specificity, localization, and temporal functions of PP2A. Here, we show separase binds the A, C, and any of the five B′ isoforms of PP2A, suggesting that separase specifically associates with a subtype of PP2A containing B′ family members. In support of this proposal, the B′α but not Bα subunit of PP2A was found to immunoprecipitate endogenous separase from cells. Furthermore, GST-separase^{RR} interacts directly with the B′α but not Bα *in vitro*. The simplest explanation, therefore, is the B′ subunit acts to directly target the PP2A heterotrimer to separase.

PP2A is the third separase interacting partner identified. Because the two previously known separase interacting proteins, securin and cyclin B1, associate with separase in a mutually exclusive manner, we investigated whether PP2A was able to interact with separase bound to cyclin B1 or securin. Our tandem purification clearly demonstrates that a heterotrimeric securin-separase-PP2A complex exists. However, human securin has recently been reported to associate with heterotrimeric PP2A (38), raising the possibility that the PP2A may associate with separase through an indirect interaction with securin; our observations argue that this scenario is unlikely for several reasons. First, *in vitro*, the B′ subunit of PP2A associates with GST-separase^{RR} in the absence of securin, indicating a

direct interaction between separase and PP2A regulatory subunit. Second, securin was shown to interact with a trimeric PP2A holoenzyme containing the B regulatory subunit, as opposed to the B′ subunit. Third, mutations in separase that abolished the interaction of securin with separase did not eliminate PP2A binding. Finally, deletion of the PP2A binding site removed all detectable PP2A from the separase immunoprecipitate without affecting securin binding. Thus, taken together these observations strongly indicate that PP2A is unlikely to interact with separase via securin; the most parsimonious explanation is that both securin and PP2A directly interact with separase to form a heterotrimeric complex.

In contrast to securin, analysis of both endogenous and exogenous cyclin B1 immune complexes revealed that PP2A did not associate with separase bound to Cdk1/cyclin B1. Interestingly, the binding of PP2A to separase was found to require a 55-amino acid region located just 16 amino acids downstream of the cyclin B1 interaction site. Thus, an appealing model for the mutually exclusive nature of the binding of PP2A and Cdk1/cyclin B1 to separase is that Cdk1/cyclin B1 and PP2A compete for a similar binding site in separase, with the interaction of one complex physically excluding docking of the other.

PP2A Binding Is Regulated by Separase Self-cleavage—The close proximity of the 55 amino acids required for PP2A binding to the autocleavage sites prompted us to ask whether self-processing of separase regulates PP2A binding. Indeed, mutation of separase cleavage sites increased the amount of PP2A associated with separase, and furthermore, this increased association required the 55-amino acid PP2A binding domain. Together, this suggests separase self-cleavage antagonizes the binding of PP2A. Consistently, PP2A purified from cells associated almost exclusively with full-length as opposed to cleaved separase. One explanation for these observations is that the PP2A binding site comprises the self-cleavage sites and is, therefore, disrupted by separase cleavage. An alternatively explanation is that separase self-cleavage causes a conformational change that indirectly disrupts or inhibits the binding of PP2A. Because the cleavage of separase is autocatalyzed, separase activity may itself be responsible for regulating PP2A binding.

If separase cleavage releases PP2A, then a non-cleavable mutant of separase would stably bind PP2A, preventing the phosphatase from carrying out other cellular functions. Consistently, overexpression of non-cleavable separase induced a premature loss of sister chromatid cohesion and a prolonged mitotic arrest. This phenotype was completely reverted by deletion of the PP2A binding domain. One explanation, therefore, is that overexpression of non-cleavable separase sequesters PP2A and, thus, perturbs its function. Indeed, the PP2A_B′-containing holoenzyme localizes to the centromeres during mitosis, where it is required to localize SgoI and maintain centromeric cohesion (32–34). This raises the possibility that overexpressed non-cleavable separase binds and titrates PP2A_B′ away from the centromeres, thereby disrupting SgoI function and causing a premature loss of centromeric cohesion.

An alternative explanation for the loss of cohesion observed in separase N/C cells is that the increased association of PP2A with separase antagonizes Ser-1126 phosphorylation and cyclin

B1-mediated inhibition. Because separase N/C is expressed above the levels of endogenous securin (22), incomplete cyclin B1-mediated inhibition of separase N/C may account for the premature activation of separase N/C.

Recently, budding yeast separase has been shown to initiate mitotic exit by interacting with and down-regulating PP2A activity at anaphase (39). However, several differences indicate that separase-PP2A might serve a different function in vertebrates. First, budding yeast and human separase appear to interact with different PP2A isoforms, namely the B (Cdc55) and B′ regulatory subunits, respectively. Second, mouse embryonic fibroblasts lacking separase enter and exit mitosis without any obvious delay, suggesting separase does not play an essential role in mitotic exit in vertebrates (40, 41). Third, the interaction of PP2A with human separase is impeded by separase self-cleavage, which does not occur in yeast. Finally, although budding yeast separase counteracts PP2A activity only after securin degradation, we show that separase-securin-PP2A can form a trimeric complex and that PP2A associated with human separase is catalytically active.

Although the functional significance of the human separase-PP2A interaction remains to be determined, it is interesting to note that human cells in which endogenous separase has been made non-cleavable by targeted mutagenesis exhibit defects with entry into and progression through mitosis (8). In the future, it will be interesting to determine whether these phenotypes can be attributed to non-cleavable separase perturbing the regulation of PP2A_B′.

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