Emi1 regulates the anaphase-promoting complex by a different mechanism than Mad2 proteins

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The anaphase-promoting complex/cyclosome (APC) ubiquitin ligase is activated by Cdc20 and Cdh1 and inhibited by Mad2 and the spindle assembly checkpoint complex, Mad2B, and the early mitotic inhibitor Emi1. Mad2 inhibits APC^{Cdc20}, whereas Mad2B preferentially inhibits APC^{Cdh1}. We have examined the mechanism of APC inhibition by Emi1 and find that unlike Mad2 proteins, Emi1 binds and inhibits both APC^{Cdh1} and APC^{Cdc20}. Also unlike Mad2, Emi1 stabilizes cyclin A in the embryo and requires zinc for its APC inhibitory activity. We find that Emi1 binds the substrate-binding region of Cdc20 and prevents substrate binding to the APC, illustrating a novel mechanism of APC inhibition.

[*Key Words*: Emi1; Cdc20; Cdh1; APC; Mad2; mitosis]

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The anaphase-promoting complex/cyclosome (APC) is a ubiquitin ligase that controls mitotic progression by ubiquitylating key mitotic regulators, including the anaphase inhibitor securin and the mitotic cyclins A and B, targeting them for destruction by the 26S proteasome (for review, see Page and Hieter 1999; Zachariae and Nasmyth 1999). The APC is present throughout the cell cycle, but selective binding of the activator proteins Cdc20 or Cdh1 results in a peak of APC^{Cdc20} activity in mitosis and APC^{Cdh1} activity in late mitosis and G_1 (Sigrist and Lehner 1997; Visintin et al. 1997; Fang et al. 1998b; Kramer et al. 1998, 2000; Lorca et al. 1998; Prinz et al. 1998; Zachariae et al. 1998).

Cdc20 and Cdh1 target for ubiquitylation proteins containing a destruction box motif (D-box; Glotzer et al. 1991). Cdh1 also recognizes proteins with a KEN-box motif (Pfleger and Kirschner 2000). APC substrates were recently found to bind and be recruited directly to the APC by Cdc20/Cdh1 in a D-box- and KEN-box-dependent manner (Burton and Solomon 2001; Hilioti et al. 2001; Pfleger et al. 2001a).

APC substrate destruction is temporally regulated: cyclin A in prometaphase, securin at metaphase-anaphase, and the mitotic polo-like kinase upon mitotic exit (Cohen-Fix et al. 1996; Shirayama et al. 1998; den Elzen and Pines 2001; Geley et al. 2001). Tight regulation of APC activity ensures the sequential destruction of APC substrates and the correct timing of mitotic events. We recently identified the APC inhibitor Emi1, which binds Cdc20 to inhibit premature APC activation in mitosis (Reimann et al. 2001). In *Xenopus* embryos, Emi1 is required for cyclin B accumulation and mitotic entry and Emi1 destruction is required for mitotic exit.

APC^{Cdc20} activity is also regulated by the spindle assembly checkpoint (SC), a pathway that delays sister chromatid separation until chromosome alignment at metaphase (for review, see Shah and Cleveland 2000). The SC protein Mad2 acts at unattached kinetochores in prometaphase to inhibit the APC until chromosome alignment, and is activated following spindle damage. Mad2 binds and inhibits Cdc20 in vitro (Fang et al. 1998a; Hwang et al. 1998; Kallio et al. 1998; Kim et al. 1998). BubR1, another SC component, also forms a complex with Cdc20 and inhibits APC activation by Cdc20 in vitro (Sudakin et al. 2001; Tang et al. 2001). The Mad2-like protein Mad2B was recently identified as an APC^{Cdh1} inhibitor in vitro and in vivo (Chen and Fang 2001; Pfleger et al. 2001b). Mad2 and Mad2B have been proposed to inhibit APC activity by inhibiting substrate release from APC^{Cdc20} and APC^{Cdh1}, respectively (Pfleger et al. 2001b).

To understand how Emi1 regulates APC activity, we investigated its APC inhibitory activity in several different assays. We find that Emi1 inhibits Cdh1–APC as well as Cdc20–APC activation, acting more broadly than either Mad2 or Mad2B. Unlike Mad2 or Mad2B, Emi1 can inhibit APC already activated by Cdc20 or Cdh1. Emi1 binds the Cdc20 N terminus in the substrate-binding region, and directly inhibits substrate binding to

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Cdc20, potentially explaining its mechanism of APC inhibition.

Results

Emi1 binds Cdh1 and inhibits APC^{Cdh1} activity

Studies of the likely *Drosophila* homolog of Emi1, Regulator of cyclin A (Rca1), show that Rca1 overexpression in G_1 cells stabilizes cyclin A (Dong et al. 1997). Cdh1 activates the APC to ubiquitylate cyclin A and other G_1 substrates (for review, see Zachariae and Nasmyth 1999). Because Emi1 binds and inhibits Cdc20, we considered whether Emi1 also inhibits the related protein Cdh1. Baculovirus-expressed Emi1 and Cdh1 coimmunoprecipitated from insect cell lysate and ^{35}S-labeled Cdh1 precipitated with GST–Emi1 protein (Fig. 1A). Human Emi1 and Cdh1 also form a complex in vivo (J. Hsu, J. Reimann, C. Sorensen, J. Lukas, and P. Jackson, in prep.).

Next, we tested whether Emi1 inhibits APC^{Cdh1} activity in *Xenopus* egg extracts. Radiolabeled in vitro translated (IVT) cyclin B and securin are stable in interphase extracts, where the APC is inactive (Fig. 1B). Addition of IVT Cdh1 to these extracts activated the APC for cyclin B and securin destruction. Emi1 addition to these Cdh1-supplemented extracts stabilized cyclin B and securin (Fig. 1B). Emi1 also inhibited Cdh1 activation of APC immunopurified from interphase extracts in a dose-dependent manner (Fig. 1C). Mad2, which does not interact with Cdh1, did not (Fig. 1C), as described (Chen and Fang 2001; Pfleger et al. 2001b). As with Cdc20 (Reimann et al. 2001), the Emi1 C but not the N terminus is sufficient to block APC^{Cdh1} activation (data not shown). Human Emi1 also inhibits both Cdc20 and Cdh1–APC activation in vitro and in vivo, indicating a conserved APC regulatory role for Emi1 (J. Hsu, J. Reimann, C. Sorensen, J. Lukas, and P. Jackson, in prep.). Neither Emi1 nor Mad2 inhibited the ubiquitylation activity of the core APC enzymatic components APC2/APC11 (Fig. 1D; Gmachl et al. 2000), further suggesting that both inhibitors act through Cdc20 or Cdh1.

Emi1 alignment with homologs from other organisms (Reimann et al. 2001) highlighted a conserved N-terminal KEN sequence, typically found in APC^{Cdh1} substrates (Pfleger and Kirschner 2000). Emi1 is degraded in mitosis independent of the APC in the embryo (Reimann

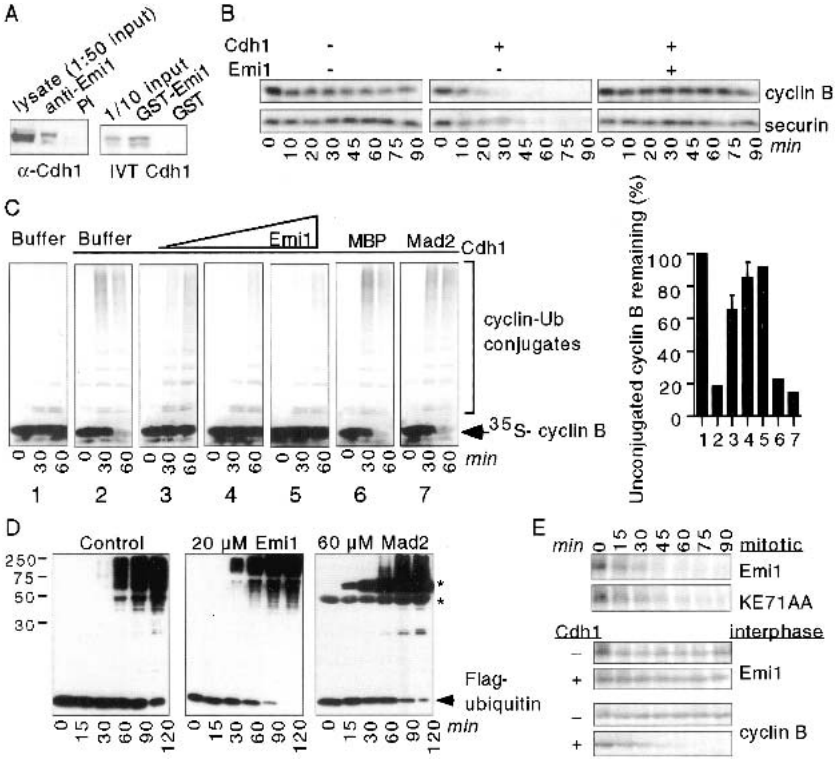

Figure 1. Emi1 binds Cdh1 and inhibits APC^{Cdh1} activity. (*A*) Emi1 interacts with Cdh1. SF9 cells were coinfected with baculoviruses expressing Emi1 and Cdh1, lysed, and lysates precipitated with preimmune (PI) or anti-Emi1 antisera and analyzed for Cdh1 by immunoblotting (*left*). GST–Emi1 or GST was incubated with ^{35}S-labeled in vitro translated (IVT) Cdh1, bound to glutathione agarose, and analyzed by SDS-PAGE and autoradiography (*right*). (We typically observe ~20–30% of input ^{35}S-labeled Cdh1 precipitating with GST–Emi1.) (*B*) Emi1 inhibits APC^{Cdh1} activity in *Xenopus* egg extracts. ^{35}S-labeled IVT N terminus *X*cyclin B or *X*securin was incubated in *Xenopus* interphase extracts treated with buffer, buffer + IVT Cdh1, or IVT Cdh1 plus MBP–Emi1 (1 μM). Aliquots were removed at the indicated times and analyzed by SDS-PAGE and autoradiography. (*C*) Emi1, but not Mad2, inhibits APC^{Cdh1}-dependent activation in vitro. IVT Cdh1 (panels *2*–*7*) or rabbit reticulocyte lysate (*1*) was incubated with buffer (*1* and *2*), MBP–Emi1 (*3*, 1 μM; *4*, 3 μM; *5*, 10 μM), 10 μM MBP (*6*), or 80 μM GST–Mad2 (*7*). APC immunopurified from interphase egg extracts was then incubated with the Cdh1/protein mixtures. The ability of treated APC to ubiquitylate ^{35}S-labeled N-terminal cyclin B fragment was assayed. The percentage of cyclin B remaining unconjugated to ubiquitin after 60 min was quantitated on a PhosphorImager (graph). (*D*) Neither Emi1 nor Mad2 inhibits the substrate-independent reaction of the APC. Baculovirus-expressed and purified APC2/APC11 was incubated with 20 μM MBP (control), 20 μM MBP–Emi1, or 60 μM GST–Mad2 in the presence of E1, E2, ATP, and Flag-tagged ubiquitin. Aliquots were taken at the indicated times and analyzed for the formation of polyubiquitin chains by immunoblotting with αFlag antibodies. (*, GST–Mad2; αFlag antibody cross-reacts with GST, which is ubiquitylated in this assay.) (*E*) Emi1 destruction is not mediated by APC^{Cdh1}. ^{35}S-labeled IVT wild-type Emi1, KE71AA mutant (substitution of K 71 and E 72 with alanines), or N terminus cyclin B fragment was added to mitotic extracts, interphase extracts, or Cdh1-supplemented interphase extracts. Aliquots were removed at the indicated times and analyzed for substrate destruction by SDS-PAGE and autoradiography.

et al. 2001), but Cdh1 is not present in *Xenopus* embryos (Lorca et al. 1998). To test whether Emi1 is an APC^{Cdh1} substrate, we assayed the stability of ^{35}S-labeled Emi1 in Cdh1-supplemented interphase extracts. Cdh1 addition to extracts destabilized cyclin B but not Emi1 (Fig. 1E). Additionally, a KEN box mutant (KE71AA) did not stabilize Emi1 in mitotic extracts (Fig. 1E), and Emi1 was not ubiquitylated by APC^{Cdh1} in vitro (data not shown). Thus, Emi1 does not appear to be an APC^{Cdc20} or APC^{Cdh1} substrate, but rather a Cdh1/Cdc20 regulator.

Emi1 but not Mad2 stabilizes cyclin A in Xenopus *eggs*

APC-dependent cyclin A destruction in prometaphase is not inhibited by the SC (Hunt et al. 1992; den Elzen and Pines 2001; Geley et al. 2001). In contrast, Emi1 prevents cyclin A destruction in *Xenopus* eggs (Fig. 2A; Reimann et al. 2001), whereas addition of GST–Mad2 to cycling extracts prevented cyclin B but not cyclin A destruction (Fig. 2B). Thus, unlike Emi1, Mad2 is not competent to stabilize cyclin A in either somatic or embryonic cells.

Cyclin A is a key APC^{Cdh1} target in G_1 (Lukas et al. 1999; Sørensen et al. 2001), so we tested Emi1 inhibition of APC^{Cdh1}-mediated cyclin A ubiquitylation. Emi1 blocked APC^{Cdh1} ubiquitylation of cyclin A in a dose-dependent manner (Fig. 2D). Human Emi1 also inhibits APC^{Cdh1}-mediated cyclin A ubiquitylation in vitro and in vivo (J. Hsu, J. Reimann, C. Sorensen, J. Lukas, and P. Jackson, in prep.), indicating conservation of Emi1's ability to regulate cyclin A stability.

Emi1 interacts with and inhibits Cdc20/Cdh1 already bound to the APC

Fractionation experiments show separate Emi1–Cdc20 and APC^{Cdc20} complexes in eggs (Reimann et al. 2001). However, exogenously added Emi1 can inhibit the APC in mitotic egg extracts, where the APC is already activated by Cdc20. One possibility is that Cdc20–APC binding is dynamic, and exogenous Emi1 sequesters Cdc20 as it dissociates from the APC. An alternative explanation is that exogenous Emi1 binds and inhibits Cdc20 already associated with the APC. Emi1 may fail to bind APC^{Cdc20} in mitotic extracts because Emi1 is already degraded before Cdc20 is fully bound to the APC (Reimann et al. 2001). We found that ^{35}S-labeled Emi1 precipitated with APC prebound to either IVT Cdc20 or Cdh1, but not to APC preincubated with reticulocyte lysate or with control beads; Emi1 reproducibly bound APC^{Cdh1} more strongly than it did APC^{Cd20} (Fig. 3A). Emi1 did not prevent Cdc20 or Cdh1 binding to the APC in vitro; when IVT ^{35}S-labeled Cdc20 or Cdh1 was incubated with APC beads, a similar amount of either protein was recovered in the presence or absence of Emi1 (Fig. 3B; data not shown).

We next tested whether Emi1 could inhibit immunopurified APC already activated by Cdc20/Cdh1. Emi1 addition to preformed APC^{Cdh1} complexes inhibited cyclin B ubiquitylation to a similar extent as when Cdh1 was preincubated with Emi1 (Fig. 3C). Preincubation of the APC with Emi1 reduced activation by Cdh1 somewhat, consistent with the small amount of Emi1 that associates with the APC in our binding assays (Fig. 2A). We obtained similar results with APC^{Cdc20} (data not shown), indicating that Emi1 can inhibit the APC with either Cdc20 or Cdh1 already bound.

Emi1 inhibits substrate binding to Cdc20

The N-terminal 158 residues of Cdc20 are sufficient for binding to Emi1 (Reimann et al. 2001). This Cdc20 fragment contains both a Mad2-binding region (MBR, residues 118–158) and a substrate-binding region (SBR, residues 1– 118) (Luo et al. 2000; Pfleger et al. 2001a; Zhang and Lees 2001). We tested Emi1 binding to these domains, and found that Emi1 specifically bound the Cdc20 SBR, and not the MBR (Fig. 3E). Both Cdc20 bind-

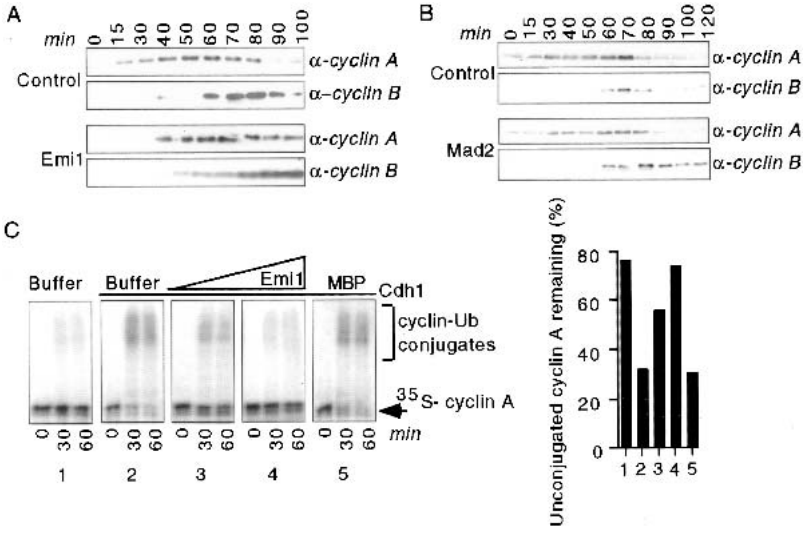

Figure 2. Emi1 but not Mad2 inhibits cyclin A destruction in *Xenopus* eggs. (*A*,*B*) Emi1 prevents cyclin A and B destruction in egg extracts whereas Mad2 only stabilizes cyclin B. Activated *Xenopus* cycling egg extracts were incubated with buffer alone, MBP–Emi1, or GST–Mad2. Aliquots were removed at the indicated times and assayed for *Xenopus* cyclins A and B by immunoblotting. (*C*) Emi1 inhibits APC^{Cdh1}-mediated cyclin A ubiquitylation in vitro. IVT Cdh1 (panels *2–5*) or rabbit reticulocyte lysate (*1*) was incubated with buffer (*1* and *2*), MBP–Emi1 (*3*, 3 μM; *4*, 6 μM), or 6 μM MBP (*5*). APC immunopurified from interphase egg extracts was incubated with the Cdh1/protein mixtures. The ability of treated APC to ubiquitylate ^{35}S-labeled cyclin A was assayed. The percentage of cyclin A remaining unconjugated to ubiquitin after 60 min was quantitated on a PhosphorImager (graph).

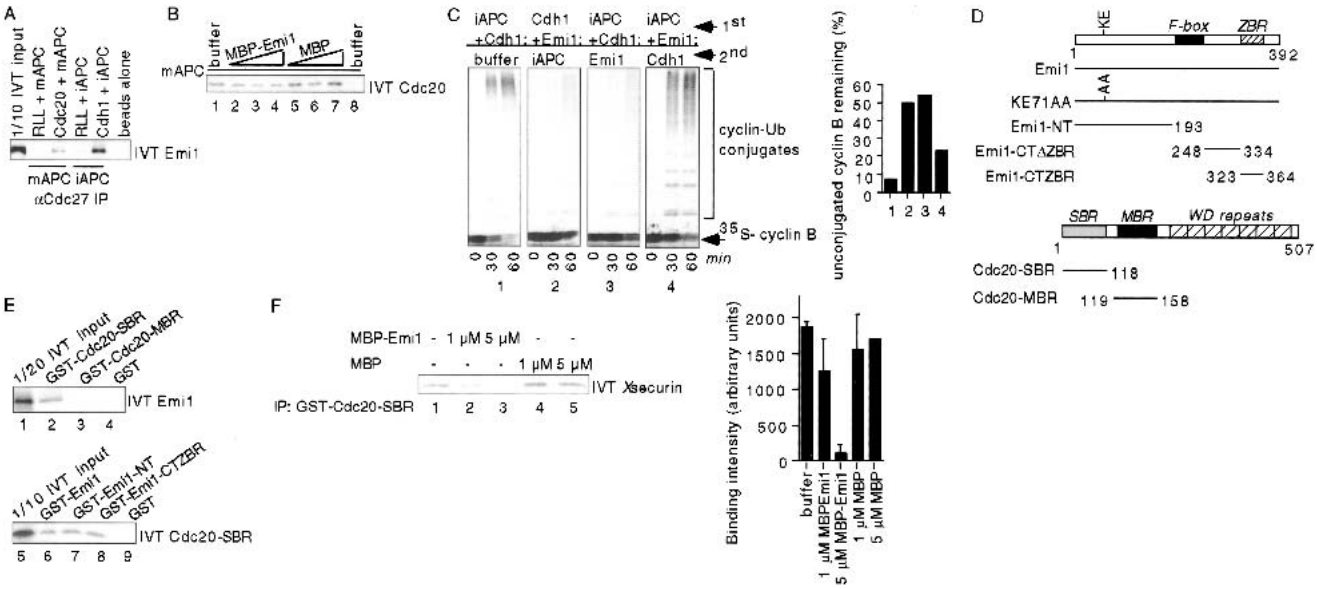

Figure 3. Emi1 inhibits substrate binding to the APC. (*A*) Emi1 can bind Cdc20 and Cdh1 already associated with the APC. Mitotic (m), interphase (i), or no APC was immobilized on αCdc27 beads, bound to rabbit reticulocyte lysate, IVT Cdc20, or IVT Cdh1, and then incubated with ^{35}S-labeled IVT Emi1. Bound Emi1 was analyzed by SDS/PAGE and autoradiography. (*B*) Emi1 does not prevent Cdc20 binding to the APC in vitro. ^{35}S-labeled IVT Cdc20 was preincubated with buffer (lanes *1* and *8*), MBP–Emi1 (*2*, 1 μM; *3*, 3 μM; *4*, 6 μM) or MBP (*5*, 1 μM; *6*, 3 μM; *7*, 6 μM) and then incubated with mitotic APC (*1–7*) or no APC (*8*) on αCdc27 beads. Bound Cdc20 was analyzed as in *A*. (*C*) Emi1 inhibits previously activated APC. Interphase APC (iAPC) complex immobilized on αCdc27 beads was first incubated with IVT Cdh1 (panels *1* and *3*), buffer (*2*), or 10 μM MBP–Emi1 (*4*). Beads were then washed and incubated with buffer (*1*), IVT Cdh1 + 10 μM MBP–Emi1 (*2*), 10 μM MBP–Emi1 (*3*), or IVT Cdh1 (*4*). APC beads were washed and then assayed for cyclin B ubiquitylation activity. The percentage of cyclin B remaining unconjugated to ubiquitin after 60 min was quantitated on a PhosphorImager (graph). (*D*) Schematics of Emi1 and Cdc20 constructs used in this study. Emi1 F-box (residues 196–245), Emi1 zinc-binding region (ZBR, residues 323–364), Cdc20 substrate-binding region (SBR, residues 1–118), Cdc20 Mad2-binding region (MBR, residues 119–158), and Cdc20 WD-repeats (residues 181–480) are indicated. (*E*) Emi1 binds the substrate-binding region (SBR) of Cdc20. GST fusion proteins (lane *2*, GST–Cdc20–SBR; *3*, GST–Cdc20–MBR; *4* and *9*, GST; *6*, GST–Emi1; *7*, GST–Emi1–NT; *8*, GST–Emi1–CTZBR) were incubated with ^{35}S-labeled IVT Emi1 or Cdc20–SBR, bound to glutathione agarose, and analyzed as in *A*. (*F*) Emi1 can inhibit substrate binding to Cdc20. GST–Cdc20–SBR (1 μM) was prebound to glutathione agarose and then incubated with buffer (lane *1*), MBP–Emi1 (lanes *2*,*3*) or MBP (lanes *4*,*5*). ^{35}S-labeled IVT *X*securin was then added and the amount of securin bound to Cdc20 was analyzed as in *A* and was quantitated on a PhosphorImager (graph).

ing domains of Emi1 (the Emi1 N terminus and zinc-binding region; Reimann et al. 2001) interact specifically with the Cdc20 SBR (Fig. 3E).

Because both Emi1 and substrates bind the Cdc20 SBR, we assayed the ability of Emi1 to inhibit substrate binding to Cdc20. MBP–Emi1 addition strongly reduced ^{35}S-labeled securin from binding to the Cdc20 SBR in a dose-dependent manner (Fig. 3F). Using this substrate-binding assay, we find that Emi1 proteolytically cleaved and purified from MBP and a purified his-tagged Emi1 (which both inhibit APC activity similarly) also inhibit substrate–Cdc20 binding (data not shown). GST–Mad2 did not inhibit substrate binding to Cdc20 in our assay, consistent with earlier results (Pfleger et al. 2001b). Emi1 also blocks substrate binding to Cdh1 in vitro (data not shown), providing further evidence of the role of Emi1 as a general substrate inhibitor of APC activity.

Zinc is required for Emi1 to inhibit APC activity

A highly conserved cluster of cysteines and histidine in Emi1, a likely zinc-binding region (ZBR), is required for inhibiting APC activity (Reimann et al. 2001). Here, we found that at high concentrations, the Emi1 ZBR fragment is sufficient to inhibit APC activity in vitro (Fig. 4A).

To formally test whether zinc is required for Emi1's inhibitory activity, we chelated zinc from the Emi1 protein with the zinc chelator TPEN. The ability of TPEN-treated MBP–Emi1 to inhibit APC activity was strongly reduced and was restored by zinc addition (Fig. 4B). We see similar loss of activity with DPTA, another zinc chelator, and with zinc chelation from the Emi1 C terminus fragment.

Discussion

The APC is regulated by multiple mechanisms, including phosphorylation and binding of the Cdc20/Cdh1 activators or the Mad2 and Emi1 inhibitor proteins. Cdc20–APC activation is regulated by Emi1, Mad2, and the SC proteins including BubR1; Cdh1 activation of the APC is regulated by Mad2B and as shown here by Emi1. The activity of Emi1 toward the APC is partially con-

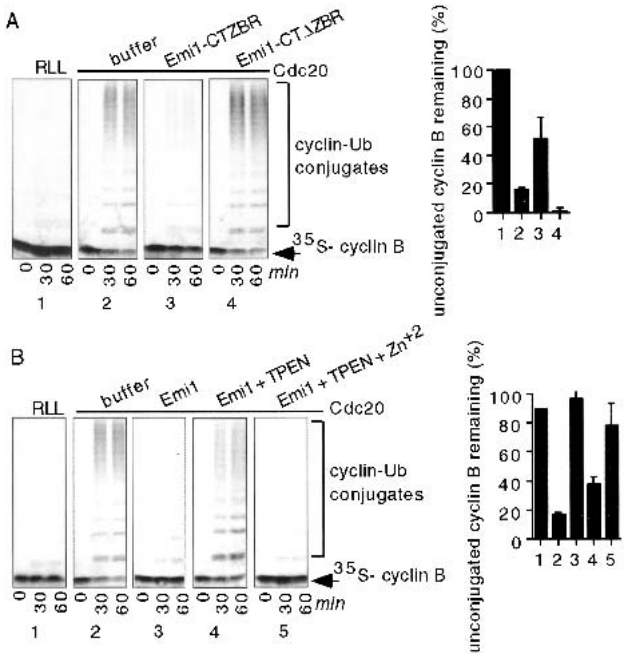

Figure 4. Zinc is required for the APC inhibitory activity of Emi1 in vitro. (*A*) The zinc-binding region (ZBR) of Emi1 is sufficient to inhibit the APC in vitro. IVT Cdc20 (panels *2–4*) or rabbit reticulocyte lysate (*1*) was preincubated with buffer (*1* and *2*), 80 μM GST–Emi1–CTZBR (*3*), or 80 μM GST–Emi1–CTΔZBR (*4*). APC immunopurified from mitotic egg extracts was incubated with the Cdc20/protein mixtures. The ability of treated APC to ubiquitylate ^{35}S-labeled N terminus cyclin B fragment was assayed. The percentage of cyclin B unconjugated to ubiquitin by 60 min was quantitated on a PhosphorImager (graph). (*B*) Zn^{2+} chelation strongly reduces the ability of Emi1 to inhibit the APC in vitro, and addition of $ZnCl_2$ rescues activity. MBP–Emi1 was incubated with TPEN and then dialyzed into XB^- or XB^- plus 50 μM $ZnCl_2$. IVT Cdc20 (panels *2–5*) or rabbit reticulocyte lysate (*1*) was incubated with buffer (*1* and *2*), untreated MBP–Emi1 (*3*), TPEN-treated MBP–Emi1 (*4*), or TPEN-treated MBP–Emi1 plus $ZnCl_2$ (*5*). APC was immunopurified and cyclin B ubiquitylation was analyzed as in *A*.

trolled by its abundance in both the embryo (Reimann et al. 2001) and somatic cells, where Emi1 accumulates in late G_1 much like cyclin A (J. Hsu, J. Reimann, C. Sorensen, J. Lukas, and P. Jackson, in prep.). Previous studies suggested that in addition to the ability of cyclin A/Cdk2 to phosphorylate Cdh1 and inactivate the APC-^{Cdh1} in S phase (Lukas et al. 1999; Sørensen et al. 2001), an additional E2F target might block APC^{Cdh1} activity. Like cyclin A, Emi1 proves to be an E2F target and Emi1 inhibits Cdh1's ability to block cyclin A accumulation and S phase entry in vivo (J. Hsu, J. Reimann, C. Sorensen, J. Lukas, and P. Jackson, in prep.). The *Drosophila* Emi1 homolog Rca1 also blocks Cdh1 activity in flies (F. Sprenger, pers. comm.). As shown here, Emi1 binds Cdh1 to inhibit APC^{Cdh1} activity in vitro. These data strongly suggest that Emi1 inactivates the APC^{Cdh1} complex to promote cyclin A accumulation at the G_1–S transition. Although it is unclear whether Emi1 continues to inhibit Cdh1 during S phase, as cells approach G_2 Emi1 is available to inhibit Cdc20 as it is expressed, thereby promoting cyclin B accumulation and mitotic entry (Reimann et al. 2001).

APC regulation by the Mad2 proteins is complex, involving additional factors. Mad2/Mad2B inhibit in vitro APC activation by Cdc20/Cdh1, but neither can inhibit preactivated APC complexes in vitro, despite being able to form ternary complexes with activator and APC (Fang et al. 1998a; Kallio et al. 1998; Chen and Fang 2001; Pfleger et al. 2001b; Sudakin et al. 2001; Tang et al. 2001). However, both Mad2 proteins can inhibit activated APC in *Xenopus* egg extracts and in vivo, suggesting that cellular factors activate Mad2 proteins to inhibit APC activity. Notably, APC inhibition by the SC requires Mad1 and BubR1 in vivo (Hwang et al. 1998; Jin et al. 1998; Chen et al. 1999; Sudakin et al. 2001; Tang et al. 2001). Although Mad2 and BubR1 are present and biochemically competent to inhibit the APC throughout the cell cycle, the APC is only sensitive when phosphorylated in mitosis and when spindle tension and/or microtubule attachment at kinetochores is lost (Abrieu et al. 2001; Skoufias et al. 2001; Sudakin et al. 2001).

In contrast, Emi1 can bind and inhibit activation of APC prebound to Cdc20 or Cdh1 in vitro and in vivo. The ability of Emi1 to inhibit an already activated APC would be a necessary feature for Emi1 to inactivate Cdh1 already bound to the APC at the G_1–S transition. Thus, Emi1's APC inhibitory activity is likely controlled by Emi1 protein levels and its ability to bind Cdc20/Cdh1, and not at the level of APC phosphorylation.

APC-dependent cyclin A ubiquitylation is not inhibited by the SC or by Mad2, but is inhibited by Emi1 (den Elzen and Pines 2001; Geley et al. 2001; our present results). Consistently, Emi1 itself is destroyed in mitosis slightly before cyclin A levels drop, and Emi1 is not stabilized by SC activation (J. Hsu, J. Reimann, C. Sorensen, J. Lukas, and P. Jackson, in prep.). Moreover, Emi1 is not present in the purified Mad2 and BubR1-containing APC inhibitory complex (J. Hsu, V. Sudakin, T. Yen, P. Jackson, unpubl.). Thus, Emi1 activity is distinct from and independent of Mad2/BubR1.

Both the N and C termini of Emi1 bind the Cdc20 SBR. Mad2 binds just C-terminal to the SBR, and neither Mad2 nor Mad2B prevent Cdc20/Cdh1 substrate binding. Instead, they appear to inhibit substrate release from Cdc20/Cdh1 (Pfleger et al. 2001b), suggesting that Mad2 proteins prevent APC substrate turnover. Emi1 binding to the SBR directly inhibits substrate binding to Cdc20 in vitro. Thus, Emi1 appears to prevent substrate ubiquitylation by inhibiting substrate binding, although additional mechanisms may function in vivo.

Both the ZBR (Reimann et al. 2001) and, as we show here, zinc itself are required for the APC inhibitory activity of Emi1. Whether zinc fulfills a structural role, facilitates binding interactions, or has another role, such as a catalytic function, is unclear. We did find that zinc chelation did not appear to affect Emi1–Cdc20 binding in vitro (J.D.R. Reimann, B. Gardner, and P.K. Jackson, unpubl.).

The identification of other Cdc20/Cdh1-like proteins in various species (e.g., Cooper et al. 2000; Chu et al.

2001; Wan et al. 2001) suggests additional pathways of APC regulation. An attractive model is that Cdc20, Cdh1, and their homologs regulate the timing of APC activity by regulated binding of specific substrates. The ability of these APC adapters to bind and activate substrate ubiquitylation by the APC might in turn be restricted by a range of inhibitory proteins like Emi1 and the Mad2 proteins.

Materials and methods

Recombinant protein and construct preparation

Full-length, Emi1–NT, and Emi1–CTΔZBR constructs were described (Reimann et al. 2001). Emi1–CTZBR (amino acids 323–364) was cloned into pGEX6P1. Cdc20–SBR (amino acids 1–118) and Cdc20–MBR (amino acids 119–158) were cloned into pGEX6P1 and $pCS2^+$ vectors. The Emi1 KE71AA site-directed mutant was cloned into $pCS2^+$–5mt and verified by sequencing.

All Emi1 and Cdc20 variants produced as MBP or GST fusion proteins were purified by standard protocols. Cdh1 baculovirus protein was as described (Kramer et al. 2000).

Binding assays

In vitro GST–Emi1 and GST–Cdc20 binding assays First, 750 nM GST fusion protein was incubated with in vitro translated (IVT) ^{35}S-labeled proteins (TNT Promega) in RIPB (100 mM NaCl, 50 mM β-glycerophosphate, 5 mM EDTA, 0.1% Triton X-100, 1 mM DTT) (1 h at 4°C). Samples were spun (14,000 rpm for 10 min), supernatant incubated with glutathione agarose (40 min at 4°C), beads washed 4× in RIPB, and bound proteins analyzed by SDS-PAGE and autoradiography.

Baculovirus reconstitution was performed as described (Reimann et al. 2001).

APC binding assays APC was immunopurified from mitotic or interphase egg extracts on αCdc27 beads as described (Fang et al. 1998a) and incubated (room temperature for 1 h) with 10 µL IVT Cdc20, Cdh1 or rabbit reticulocyte lysate. Beads were washed 2× in XB^- (20 mM HEPES, 100 mM KCl), incubated with 4 µL ^{35}S-labeled IVT Emi1 diluted 1:38 in RIPB (4°C for 45 min), and washed 5× in Q-A buffer (20 mM HEPES, 500 mM KCl, 0.5% NP-40). Bound Emi1 was analyzed by SDS-PAGE and autoradiography. αCdc27 beads were subjected to identical binding and washing conditions. For testing the ability of Emi1 to inhibit Cdc20 APC binding, 2 µL of ^{35}S-labeled IVT Cdc20 was prebound to MBP–Emi1 or MBP before binding to APC beads.

Substrate binding competition assay GST–Cdc20–SBR (1 µM) prebound to glutathione agarose was incubated (4°C for 45 min) with 1 or 5 µM MBP–Emi1, his–Emi1, Emi1 with MBP removed, MBP, or BSA in NETN buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM DTT, 1 mM EDTA, 1% aprotinin). Four microliters of ^{35}S-labeled IVT securin was diluted 1:25 in NETN, then incubated with the above mixture (4°C for 45 min). Beads were washed 5× in NETN, and bound securin was analyzed by SDS-PAGE and autoradiography.

Zinc chelation experiments

MBP–Emi1 protein was incubated (4°C, 24 h) with two changes of XB^- plus 2 mM TPEN, then incubated (4°C for 3 h) in either 50 µM $ZnCl_2$ or XB^-, and dialyzed into XB^- (4°C for 18 h).

Degradation and ubiquitylation assays

Emi1 stability experiments in egg extracts ^{35}S-labeled IVT Emi1, KE71AA, or N terminus sea urchin cyclin B substrate (Glotzer et al. 1991) was incubated at 23°C in Δ90 mitotic extracts (Reimann et al. 2001), interphase extracts with IVT Cdh1 (1:20 volume), or interphase extracts with unprogrammed reticulocyte lysate. Aliquots were removed and analyzed by SDS-PAGE and autoradiography.

Effect of Emi1 and Mad2 on cyclin A and B stability Buffer, 1 µM MBP–Emi1, or 20 µM GST–Mad2 fusion protein was added to cycling extracts (Murray 1991). Aliquots were removed at the indicated times, and endogenous cyclin A and B levels were assayed by immunoblotting with αcyclin B2 or αcyclin A1 antibodies.

Effect of Emi1 on APC^{Cdh1} activity in extracts ^{35}S-labeled IVT Xl cyclin B1 (amino acids 2–97) fragment or securin was added (1:20 volume) to interphase extracts preincubated with either XB^- buffer, IVT Cdh1 (1:25 volume) plus XB^- buffer, or IVT Cdh1 (1:25 volume) plus 1 µM MBP–Emi1. Aliquots were removed and analyzed by SDS-PAGE and autoradiography.

APC2/APC11 substrate-independent ubiquitylation reaction Twenty µM MBP, 20 µM MBP–Emi1, or 60 µM GST–Mad2 was incubated at room temperature in ULAA buffer (50 mM Tris at pH 7.5, 5 mM $MgCl_2$, 2 mM NaF, 0.6 mM DTT) containing 1.5 ng/µL baculovirus expressed and purified APC2/APC11, 7.2 ng/µL Ubc5, 0.2 ng/µL E1 (Calbiochem), 2 mM ATP, 10 nM okadaic acid, and 3.2 ng/µL Flag-ubiquitin. Aliquots were removed at the indicated times and analyzed for polyubiquitin chains by immunoblotting with αFlag antibody (Sigma).

In vitro APC assays Mitotic or interphase extract aCdc27 immunoprecipitates were incubated (25°C for 1 h) with 10 µL IVT Cdc20 or Cdh1 preincubated (4°C for 30 min) with protein or buffer as indicated in the figure legend, washed in XB^-, and assayed for cyclin ubiquitylation as described (Fang et al. 1998b).

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