The EMBO Journal vol.10 no.13 pp.4311–4320, 1991

Both cyclin AΔ60 and BΔ97 are stable and arrest cells in M-phase, but only cyclin BΔ97 turns on cyclin destruction

Francis C.Luca, Ellen K.Shibuya, Cord E.Dohrmann and Joan V.Ruderman

Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, MA 02115, USA

Communicated by T.Hunt

Previous work has established that destruction of cyclin B is necessary for exit from mitosis and entry into the next interphase. Sea urchin cyclin B lacking an N-terminal domain is stable, permanently activates cdc2 kinase, resulting in mitotic arrest, and permanently activates the destruction pathway acting on full length cyclin B. Here we have compared the properties of clam cyclins A and B lacking related N-terminal domains. Both cyclin AΔ60 and BΔ97 bind to cdc2 kinase, keep it hyperactivated and block the completion of mitosis. By adding purified Δcyclin proteins to a cell-free system at different cell cycle times, we find that when the cell-free system reaches the cyclin destruction point in the presence of either AΔ60 or BΔ97, the cyclin destruction pathway acting on full length cyclins fails to be turned off. However, the two cyclins differ dramatically in their ability to turn on cyclin destruction. When added to emetine-arrested interphase lysates devoid of endogenous cyclins, only cyclin BΔ97 activates the cyclin destruction system; cyclin AΔ60 does not. This functional difference between the two cyclin types, the first to be described, provides strong support for the idea that the two cyclins have different roles in the cell cycle and suggests that one specialized role of the cyclin B–cdc2 complex is to activate the cyclin destruction pathway and drive cells into interphase of the next cell cycle.

Key words: cdc2 kinase/cell cycle/fertilization/mitosis/proteolysis

Introduction

Progress through the eukaryotic cell cycle is controlled in part by the periodic accumulation and destruction of the cyclins, proteins that act as positive regulators of the protein kinase $p34^{cdc2}$. Newly synthesized cyclins bind to inactive monomeric $p34^{cdc2}$, promoting a series of post-translational modifications of the kinase complex that lead to its activation (Draetta *et al.*, 1988, 1989; Morla *et al.*, 1989; Dunphy and Newport, 1989; Gould and Nurse, 1989; Murray and Kirschner, 1989; Pondaven *et al.*, 1990; Solomon *et al.*, 1990; Kumagai and Dunphy, 1991; Parker *et al.*, 1991). In higher eukaryotes, two types of cyclins have been well characterized, cyclin A and cyclin B. Cyclin B, the best understood, targets $p34^{cdc2}$ for its mitotic functions. Cyclin B is highest in late M-phase cells (cyclin A is low or absent) and it is the major cyclin present in highly purified preparations of M-phase promoting factor (Swenson *et al.*, 1986; Westendorf *et al.*, 1989; Minshull *et al.*, 1989, 1991; Draetta *et al.*, 1989; Labbé *et al.*, 1989; Meijer *et al.*, 1989; Gautier *et al.*, 1990; Hunt *et al.*, 1992). In yeast, a related cyclin encoded by cdc13 is required for initiation of mitosis (Booher and Beach, 1987, 1988; Hagan *et al.*, 1988). The role of cyclin A, which rises earlier in the cell cycle, is not well understood. The observation that cyclin A mutants in *Drosophila* arrest in G_2 argues that cyclin A, like cyclin B, is required for mitosis (Lehner and O'Farrell, 1990). The effects of cyclin A and B are virtually indistinguishable in all assays reported so far, including activation of meiotic maturation, induction of cdc2 kinase activity towards histone H1, and T antigen-dependent SV40 DNA replication (Swenson *et al.*, 1986; Pines and Hunt, 1987; Westendorf *et al.*, 1989; Draetta *et al.*, 1989; Minshull *et al.*, 1990; D'Urso *et al.*, 1990; Roy *et al.*, 1991). In microtubule dynamic instability assays, however, cyclin A promotes the formation of centrosome-nucleated microtubule whose steady state lengths are considerably longer than those nucleated in the presence of cyclin B, suggesting that cyclin A plays an important and perhaps specialized role during prophase (Karsenti *et al.*, 1992).

Just as the rise in cyclin B is essential for cells to enter mitosis, its destruction is required for cells to exit mitosis. When mitotic exit is delayed by colchicine in clam embryos, cyclin A drops on schedule but cyclin B stays high for several hours and then gradually disappears; temporally, the loss of cyclin B correlates with entry into the next interphase (Minshull *et al.*, 1989; Hunt *et al.*, 1992). In frog eggs and egg lysates, sea urchin cyclin B lacking the first 90 amino acids keeps $p34^{cdc2}$ hyperactivated, maintains chromosomes in a condensed state and prevents cytokinesis (Murray *et al.*, 1989). Similarly, in yeast, a mutation that removes the N-terminus of a B-type cyclin confers a cell cycle arrest in mitosis (Ghiara *et al.*, 1991).

While cyclin A and B are degraded using similar pathways, their destruction is regulated individually, with cyclin A disappearing ahead of cyclin B. Destruction of both cyclins is ATP-dependent, sensitive to sulfhydryl reagents and appears to involve a ubiquitin-dependent system, with a ladder of higher molecular weight forms of cyclin appearing just at the onset of destruction phase (Luca and Ruderman, 1989; Glotzer *et al.*, 1991; Hershko *et al.*, 1991; Ruderman *et al.*, 1991). Advancement to the cyclin destruction point requires accumulation of cyclin beyond a threshold level, activation of cdc2 kinase and entry into M-phase (Luca and Ruderman, 1989; Murray and Kirschner, 1989; Hunt *et al.*, 1992). Most importantly, addition of cyclin B alone to *Xenopus* egg lysates can drive a full cycle of cdc2 kinase activation, entry into M-phase, cyclin destruction and kinase inactivation (Murray and Kirschner, 1989). Furthermore, the addition of cdc2–cyclin B complexes to an interphase

© Oxford University Press

lysate leads to cyclin destruction, suggesting that one or more of the components of the cyclin destruction pathway are activated directly by $p34^{cdc2}$ kinase (Félix *et al.*, 1990).

A truncated cyclin B lacking the first N-terminal 90 amino acids is stable and permanently activates both $p34^{cdc2}$ kinase and the destruction pathway acting on full length cyclin B (Murray *et al.*, 1989; Glotzer *et al.*, 1991). Fusion of the N terminus to a marker protein led to the correct, stage-specific destruction of the hybrid protein. This region contains a stretch of amino acids (RxxLxxIxN) conserved in all B-type cyclins; when the arginine is changed to cysteine, the resulting cyclin B was resistant to proteolysis (Glotzer *et al.*, 1991). Related regions were noted in cyclin A but were not tested, nor were the effects of truncated B on the destruction of cyclin A.

Here we show that a truncated clam cyclin A (AΔ60), like truncated clam cyclin B (BΔ97), is stable, permanently activates cdc2 kinase and arrests cells in M-phase. Extracts that pass through the cyclin destruction point in the presence of either AΔ60 or BΔ97 fail to switch off the cyclin destruction machinery and remain capable of destroying full length cyclins of both types for hours. However, cyclin A and cyclin B are not equivalent when it comes to turning on cyclin destruction: when added to emetine-arrested interphase lysates lacking endogenous cyclins, both AΔ60 and BΔ97 activate cdc2 kinase but only BΔ97 goes on to activate the cyclin destruction pathway. These results suggest that only cyclin B, and not cyclin A, is capable of driving a full cycle of cdc2 activation, entry into M-phase, cyclin destruction, cdc2 inactivation and exit into interphase of the next cell cycle.

Results

Production of truncated clam cyclin A and B proteins in bacteria

Clam cyclin A lacking the 60 N-terminal amino acids (AΔ60) and cyclin B lacking the 97 N-terminal amino acids (BΔ97) were expressed in *Escherichia coli* and purified as described in Materials and methods (Figure 1a). AΔ60 migrated more slowly than expected from its predicted molecular weight of 42 506 Daltons, whereas BΔ97 migrated in accordance with its predicted molecular weight of 39 446 Daltons.

Cell-free systems

We have previously described a cell-free system made from 12 000 *g* supernatants of synchronously dividing clam embryos which reproduces several aspects of the temporally regulated cyclin accumulation and destruction seen in intact cells (Luca and Ruderman, 1989). Most of the experiments presented here were done with 150 000 *g* supernatants, whose cyclin destruction properties are very similar. High-speed lysates, however, synthesize little or no protein and, thus, cannot proceed through a second cycle of cdc2 kinase activation without additional cyclin (see below). Furthermore, in M-phase cells, most cyclin A remains in the 12 000 *g* supernatant whereas a considerable portion of cyclin A pellets at 150 000 *g*. Cyclin B levels in the two types of lysates are comparable (not shown).

Two kinds of high-speed lysates were used in this work. The first was made from cells arrested in interphase and lacking endogenous cyclins. To prepare these, the protein synthesis inhibitor emetine was added to cells in early

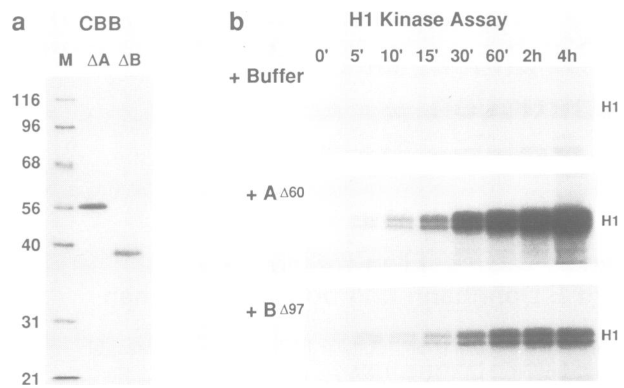

Fig. 1. Bacterially expressed clam cyclin AΔ60 and BΔ97 can each induce histone H1 kinase activity when added to interphase lysates lacking endogenous cyclins. (**a**) Purified cyclin AΔ60 (ΔA) and BΔ97 (ΔB) were produced in *E.coli* as described in the text. 1.5 μg of each purified protein and the indicated molecular weight markers (M) were electrophoresed on a 15% polyacrylamide gel and stained with Coomassie brilliant blue (CBB). (**b**) A 150 000 *g* supernatant lacking endogenous cyclins was prepared from two-cell embryos arrested in interphase as described in the text. Portions of the lysate were incubated with buffer, 875 nM cyclin AΔ60 or 1.6 μM BΔ97 and incubated at 18°C. At the indicated times, aliquots were taken and assayed for H1 kinase activity. Autoradiograms (equal exposures) of the polyacrylamide gels containing the ^{32}P-labeled histone H1 from each reaction are shown.

M-phase. These cells continued through mitosis, destroying their cyclins on schedule, and arrested in the next interphase; a lysate was then made. The second type was made from cells moving through the cycle, typically from cells taken at mid-interphase or early M-phase. These lysates contained endogenous cyclins and, when incubated at 18°C, advanced through the cyclin destruction point (see below).

Purified AΔ60 or BΔ97 cyclins bind and activate a homolog of $p34^{cdc2}$ kinase

Lysates made from emetine-arrested interphase cells lacking endogenous cyclins had very low histone H1 kinase activity (Figure 1b, top panel). When AΔ60 or BΔ97 was added, H1 kinase activity appeared after a lag of 5–10 min and continued to rise for several hours (Figure 1b, middle and bottom panels). In agreement with others (Solomon *et al.*, 1990; Roy *et al.*, 1991), dose-response experiments with both the A and B type cyclins from clam indicated that the time when H1 kinase activity first appeared was independent of the amount of input cyclin protein (not shown).

Seven different AΔ60 and four different BΔ97 preparations were tested. At the same final concentrations, AΔ60 consistently induced higher kinase activities than did BΔ97. Given the difficulties encountered in keeping BΔ97 protein soluble during purification (see Materials and methods), we suspect that its lower specific activity was due to problems in obtaining fully renatured BΔ97. It is, however, possible that this difference reflects different roles that cyclin A and B may play in activating $p34^{cdc2}$-related kinases or in conferring selective substrate specificities.

To establish that the H1 kinase activity induced by Δcyclins was due to their association with clam $p34^{cdc2}$ or a closely related kinase, the Δcyclin-activated lysates were subjected to binding with Sepharose beads coupled to $p13^{suc1}$, a protein that avidly binds cdc2 and related kinases, and co-purifies kinase-associated cyclins (Brizuela *et al.*,

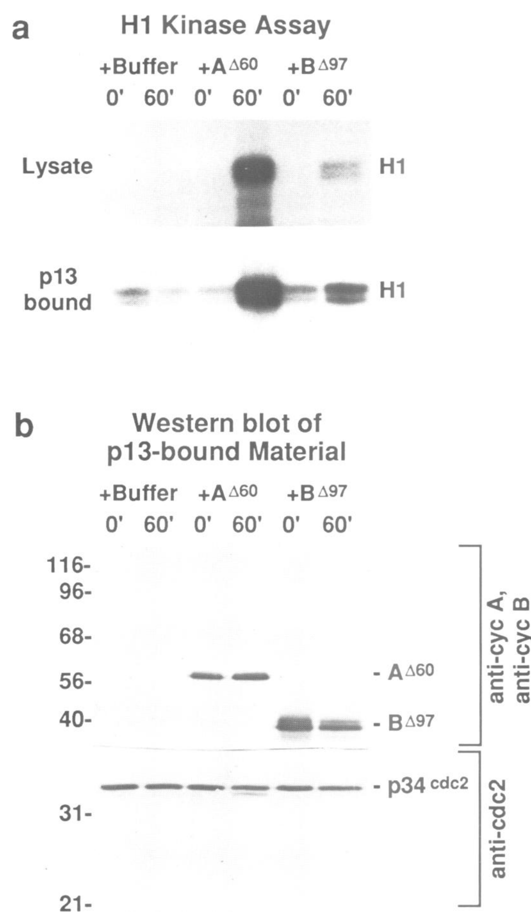

Fig. 2. Cyclin AΔ60, BΔ97 and the H1 kinase activity induced by these truncated cyclins are bound by $p13^{suc1}$ beads. Aliquots of the emetine-arrested interphase 150 000 *g* supernatant characterized in Figure 1 were mixed with buffer, 875 nM purified cyclin AΔ60 or 1.6 μM BΔ97 and incubated for 0 or 60 min after shifting to 18°C. A portion of each incubation was subjected to binding to $p13^{suc1}$ beads as described in the text. (**a**) Histone H1 kinase activity was assayed in the lysates (upper panel) and directly on $p13^{suc1}$-bound material (low panel). (**b**) Material bound to $p13^{suc1}$ beads was eluted with SDS gel sample buffer, electrophoresed on a 15% polyacrylamide gel, blotted to nitrocellulose and reacted with a mixture of affinity-purified cyclin A and cyclin B antibodies (upper panel) or cdc2 antibodies (lower panel). The positions of cyclin A, cyclin BΔ97 and $p34^{cdc2}$ are indicated.

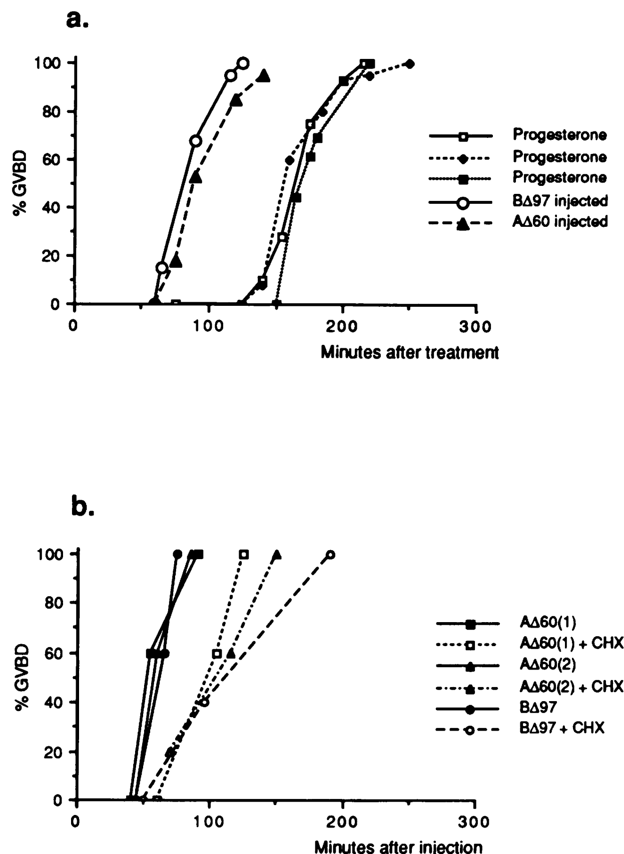

Fig. 3. Microinjection of cyclin AΔ60 and cyclin BΔ97 into *Xenopus* oocytes induces the resumption of meiotic maturation, even in the absence of protein synthesis. *Xenopus* oocytes were treated with progesterone or microinjected into cyclin AΔ60 or cyclin BΔ97. Entry into M-phase (GVBD) was monitored by the appearance of a white spot at the animal pole of the oocyte, and later confirmed by fixation and dissection of samples. (**a**) Time course of GVBD induced by progesterone or microinjection with AΔ60 or BΔ97. Recipient oocytes from the same batch of oocytes were used. 50 nl AΔ60 (0.15 mg/ml) or BΔ97 (0.25 mg/ml) was injected into each oocyte, giving final concentrations of 194 mM and 344 mM, respectively. (**b**) Oocytes were cultured in MBS (control) or MBS containing 10 μg/ml cycloheximide (+ CHX) for 1 h prior to microinjection. After injection, recipients pretreated with cycloheximide were cultured continuously in the presence of cycloheximide for the duration of the experiment. Similarly shaped symbols indicate that the same preparation of protein and same batch of recipient oocytes were used. The concentration of Δcyclin injected into each oocyte was: AΔ60 (prep. 1), 0.15 mg/ml (final concentration 194 nM); AΔ60 (prep. 2), 0.061 mg/ml (final concentration 78 nM); BΔ97, 0.25 mg/ml (final concentration 344 nM).

1987; Draetta and Beach, 1988; Draetta *et al.*, 1989). In the absence of cyclins, the $p13^{suc1}$-bound material showed a low but detectable H1 kinase signal (Figure 2a). The difference in H1 kinase activity of lysates compared with that of $p13^{suc1}$-bound material almost certainly reflects differences in the final specific activities of the [^{32}P]ATP in the kinase assays. The H1 kinase activities induced by the addition of AΔ60 and BΔ97 each bound efficiently to $p13^{suc1}$ beads (Figure 2a), with only a small fraction of the kinase activity remaining in the unbound material (not shown).

To monitor cyclins and clam cdc2, $p13^{suc1}$-bound samples were eluted and analyzed on immunoblots. The top half of the blot was reacted with a mix of affinity-purified antibodies against cyclins A and B, and the bottom half with affinity-purified antibodies against bacterially expressed full length *Schizosaccharomyces pombe* cdc2 protein (Figure 2b). Interphase lysates lacking endogenous cyclins contained a protein of ~34 kDa that bound to $p13^{suc1}$ beads and reacted with cdc2 antibodies. We refer to this protein as $p34^{cdc2}$. Virtually all of the $p34^{cdc2}$ had been bound by $p13^{suc1}$ beads; no other cross-reacting bands were obvious (not shown). Similar amounts of $p34^{cdc2}$ were bound to the beads in the presence or absence of the truncated cyclins, indicating that $p34^{cdc2}$ binding to $p13^{suc1}$ is not dependent on cyclin. $p13^{suc1}$-bound $p34^{cdc2}$ from interphase lysates and 4°C lysates to which cyclins had been added (the 0 min time point of this experiment) contained a single band of $p34^{cdc2}$ (Figure 2b); the same was true for the $p34^{cdc2}$ in the whole lysate (not shown). By 60 min after the addition of AΔ60, a more rapidly migrating form of $p34^{cdc2}$ had formed. This probably represents the activated form of the kinase (Morla

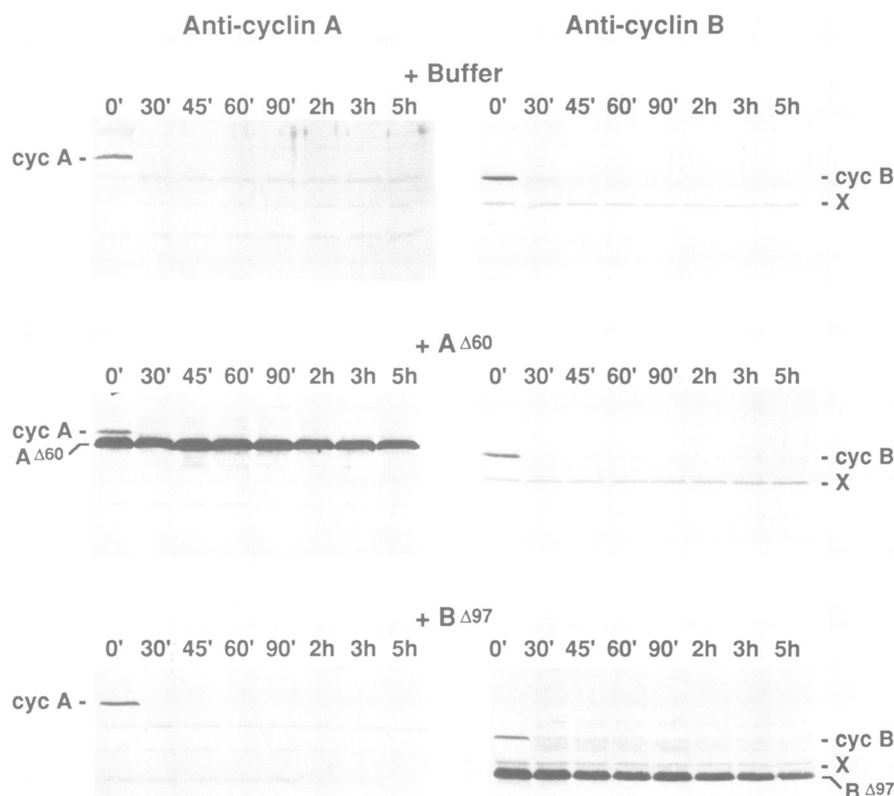

Fig. 4. Cyclin AΔ60 and cyclin BΔ97 are stable and do not inhibit the destruction of endogenous full length cyclins of either type. 150 000 *g* supernatants from embryos taken at late interphase were incubated with buffer (top panels), cyclin AΔ60 (middle panels) or BΔ97 (bottom panels). Δcyclins were added to ~300 nM, the amount needed to get maximal H1 kinase activation. At the indicated times after starting the incubation at 18°C, aliquots were taken, electrophoresed on a 15% polyacrylamide gel, blotted to nitrocellulose and probed with polyclonal serum raised against cyclin A (left panels) or cyclin B (right panels). The positions of endogenous cyclins A and B, and the Δcyclins are indicated. X indicates a non-cyclin cross-reacting band seen in all blots probed with this particular whole anti-cyclin B serum.

et al., 1989; Pondaven *et al.*, 1990; Solomon *et al.*, 1990; Kumagai and Dunphy, 1991). BΔ97 also induced the appearance of $p13^{suc1}$-associated H1 kinase activity and the formation of the lower $p34^{cdc2}$ band, although the latter was not as prominent as that seen with AΔ60.

Both AΔ60 and BΔ97 were bound by $p13^{suc1}$ beads (Figure 2b). Interestingly, similar amounts of Δcyclins from the 0 and 60 min time points were bound by $p13^{suc1}$ beads. Since the 0 min samples were kept at 4°C from the time of cyclin addition to the end of the $p13^{suc1}$ bead binding procedure (~70 min), this result indicates that cyclins bind efficiently to $p34^{cdc2}$ at 4°C.

Both AΔ60 and BΔ97 can induce M-phase in vivo

The appearance of H1 kinase activity has been used as a marker for activated $p34^{cdc2}$ and is often taken to indicate entry into M-phase. To test whether the purified Δcyclins actually do stimulate intact cells to enter M-phase, we injected them into frog oocytes, which are naturally arrested in prophase at the G_2-M border of meiosis. Like starfish (Pondaven *et al.*, 1990) and clam oocytes (Westendorf *et al.*, 1989; Ruderman *et al.*, 1992), they contain inactive cdc2 kinase, some of which is assembled with cyclin B into a complex of pre-MPF (Gautier and Maller, 1991; Kobayashi *et al.*, 1991). Injection of mRNAs encoding full length cyclins causes resumption of meiosis (Swenson

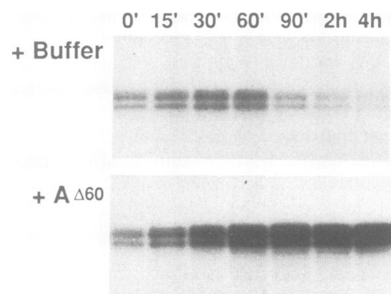

Fig. 5. The addition of cyclin AΔ60 to mid-interphase cytoplasm persistently hyperactivates histone H1 kinase activity. A 150 000 *g* lysate was prepared from cells collected in mid-interphase and incubated with buffer or 290 nM cyclin AΔ60. Aliquots taken at the indicated times after the start of the incubation were assayed for histone H1 kinase activity.

et al., 1986; Pines and Hunt, 1987; Westendorf *et al.*, 1989). It is not at all clear how exogenously provided cyclins do this, but one possibility is that they bind to free cdc2 and the resulting complex somehow escapes the inhibitory modifications that keep the endogenous complex inactive (Solomon *et al.*, 1990; Kumagai and Dunphy, 1991; E.Shibuya and J.Ruderman, in preparation). Both AΔ60 and BΔ97 stimulated resumption of meiosis, with each cyclin inducing germinal vesicle breakdown (GVBD) more rapidly

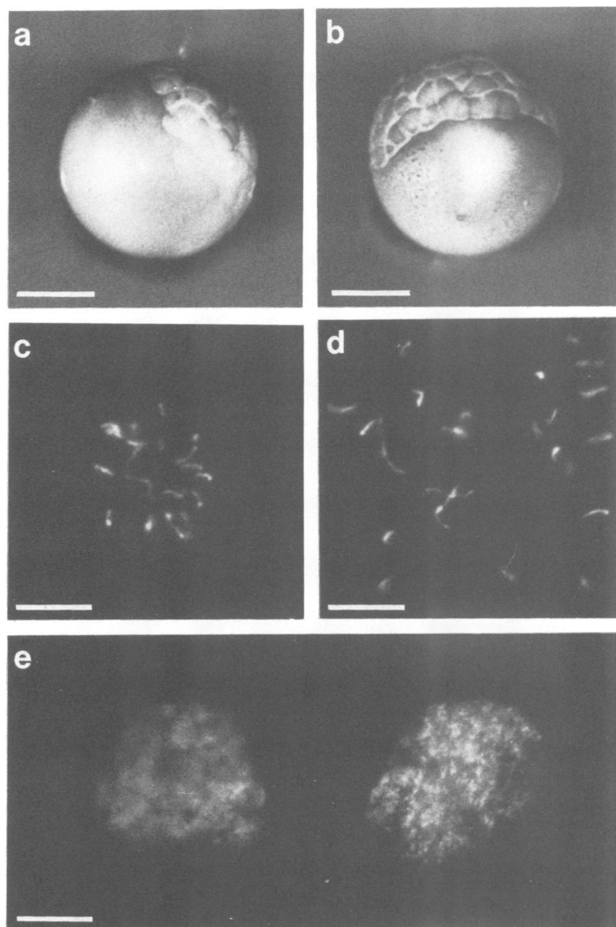

Fig. 6. Cyclin AΔ60 and cyclin BΔ97 into cleaving *Xenopus* embryos can each block the completion of mitosis and cytokinesis. Single blastomeres of cleaving two-cell embryos were injected with 30 nl of AΔ60, 0.15 mg/ml (**a**) or BΔ97, 0.25 mg/ml (**b**). Recipients were cultured for 4–6 h before fixation and cytology. The Hoechst-stained chromosomes in the blastomeres were arrested by AΔ60 (**c**) or BΔ97 (**d**) were visualized as described in Materials and methods. The appearance of decondensed, interphase nuclei in cleaving cells from the uninjected half-blastulae are shown in (**e**). Bars in (a) and (b) = 0.5 mm. Bars in (c), (d) and (e) = 5.0 μm.

than progesterone (Figure 3a). AΔ60-injected oocytes contained condensed chromosomes that had migrated to the animal pole (not shown). These results indicate that the bacterially-produced Δcyclins are active *in vivo* and can promote full resumption of meiosis.

A number of purified proteins, including the regulatory subunit of cAMP-dependent protein kinase (Maller and Krebs, 1980), PKI (Maller and Krebs, 1980) and ras (Birchmeier *et al.*, 1985) can induce GVBD. When protein synthesis is blocked, however, only preparations containing MPF are capable of inducing GVBD (Wasserman and Masui, 1985; Drury and Schorderet-Slatkine, 1975; Lohka *et al.*, 1988; Schroetke and Maller, cited in Allende *et al.*, 1988). As shown in Figure 3b, injection of either Δcyclin alone is sufficient to induce GVBD in 100% of cycloheximide-treated oocytes. It should be noted, however, that the rate of GVBD was slightly retarded when protein synthesis was blocked. Thus, of the purified proteins tested so far, only cyclin can induce GVBD in the absence of protein synthesis.

AΔ60 and BΔ97 are stable and do not inhibit destruction of endogenous cyclins

As outlined above, the N termini of B-type cyclins contain sequences necessary for their programmed destruction. Here, we have directly tested the role of a related region in cyclin A, which contains the candidate A-type destruction motif RAALGVITN (Glotzer *et al.*, 1991). A lysate of late interphase one-cell embryos was supplemented with buffer, AΔ60 or BΔ97. Cyclin levels remaining at various times after the start of the incubation were analyzed on blots (Figure 4). When only buffer was added, the endogenous full length cyclin A and cyclin B were destroyed within 30 min (top panel), just as would have occurred in intact cells. When AΔ60 was added, both endogenous cyclins were destroyed on schedule but AΔ60 itself was stable for at least 5 h, the duration of the experiment (middle panel). Similar results were seen for BΔ97 (bottom panel). The stability of the Δcyclins cannot be explained by incomplete renaturation, for example, since they are highly active as cdc2 kinase/ M-phase inducers (Figures 1–3). Nor can their stability be attributed to their overwhelming the destruction machinery, since full length cyclins are destroyed perfectly well in their presence (Figure 4; see also Figure 7 below) and 10-fold lower amounts of Δcyclins are equally stable (not shown). Thus, our results confirm the importance of the conserved N-terminal sequences in cyclin B and establish that the N terminus of cyclin A also carries a destruction determinant.

Both AΔ60 and BΔ97 can block the completion of mitosis and cell division

In all eukaryotes examined so far, $p34^{cdc2}$ histone H1 kinase activity rises near the beginning of mitosis and drops abruptly near the end, close to the time of destruction of cyclin B and the onset of anaphase. Intact cells containing truncated B-type cyclins arrest in M-phase and fail to divide (Murray and Kirschner, 1989; Ghiara *et al.*, 1991), indicating that the loss of cyclin B is necessary for cells to complete one cell cycle and enter the next. Here we have asked whether truncated cyclin A has the same effects. In lysates from mid-interphase clam embryos incubated without additional cyclin, H1 kinase activity rose and fell (Figure 5, top panel). The kinetics of this cycle were much slower than in intact cells or in lysates prepared from late interphase, presumably reflecting the finding that lysates or cells with lower cyclin levels take longer to activate cdc2 and enter M-phase (Murray *et al.*, 1989; Hunt *et al.*, 1992). When AΔ60 was added at the start of the incubation, H1 kinase activity rose well beyond control levels, suggesting the presence of free, activatable cdc2 kinase; this activity stayed high for >4 h (Figure 5, bottom panel). Thus, just as with cyclin B, failure to destroy cyclin A leads to persistent H1 kinase activation.

To compare the effects of AΔ60 and BΔ97 in living cells, each was injected into one cell of a two-cell frog embryo. Both AΔ60 and BΔ97 were potent inhibitors of cell division (Figure 6a and b). 100% of the recipients in each set arrested without undergoing any further cleavage, whereas all of the buffer-injected cells continued to divide normally (10–20 embryos/set in each of three experiments). All the Δcyclin-arrested cells contained highly condensed chromosomes (Figure 6c and d), in contrast to the normally cleaving cells which often displayed decondensed, interphase nuclei (Figure 6e). In the absence of information about the mitotic spindle,

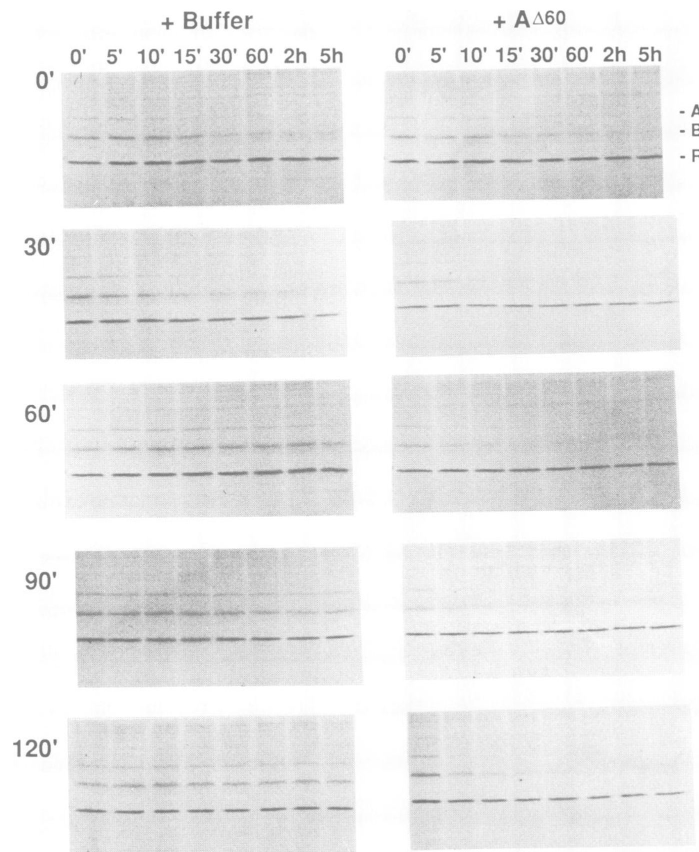

Fig. 7. Cyclin AΔ60 keeps the cyclin destruction pathway permanently activated. A 150 000 *g* supernatant from mitotic M-phase embryos was incubated with buffer or with 290 nM cyclin AΔ60 for 0, 30, 60, 90 or 120 min before adding [^{35}S]methionine-labeled substrate proteins from a radiolabeled M-phase 12 000 *g* lysate, as described in the text. Aliquots were taken at the indicated times after substrate addition and analyzed by SDS–PAGE followed by autoradiography. The positions of cyclin A, cyclin B and ribonucleotide reductase (R) are indicated.

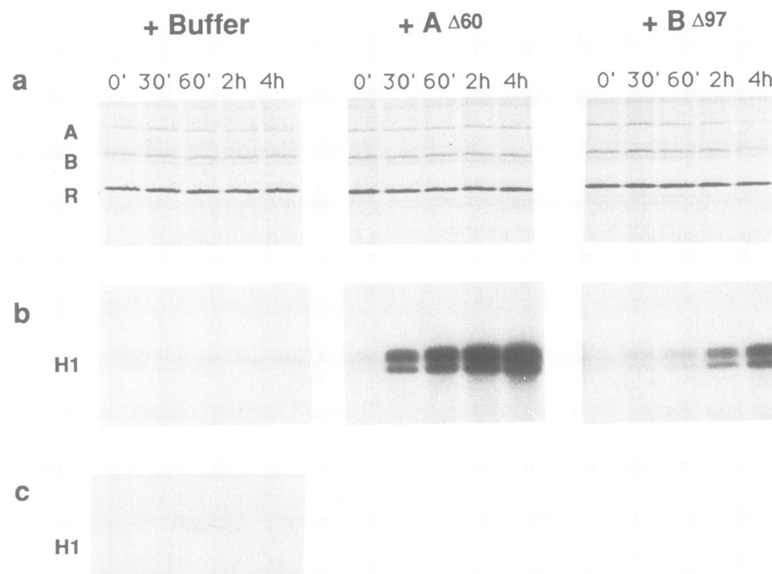

Fig. 8. Cyclin BΔ97 switches on cyclin destruction but AΔ60 does not. A 150 000 *g* supernatant from mitotic M-phase embryos was incubated at 18°C for 4 h, put on ice and [^{35}S]methionine-labeled substrate proteins were added. After adding buffer, 1 μm AΔ60 or 1.5 μM BΔ97, the reactions were moved to 18°C and incubated for the indicated times. (**a**) Cyclin destruction activity, assayed by monitoring the fate of the [^{35}S]methionine-labeled cyclin, was monitored by SDS–PAGE followed by autoradiography. (**b**) Histone H1 kinase activity of each aliquots was assayed. (**c**) Histone H1 activity was assayed from aliquots of the same lysate plus buffer incubated in the absence of added ^{35}S-labeled M-phase substrate proteins. The positions of cyclin A, cyclin B and ribonucleotide reductase (RR) are indicated. All autoradiograms were exposed for the same amount of time.

the exact stage of the M-phase arrest could not be assigned. Many of the Δcyclin-arrested embryos contain two separate groups of condensed chromosomes, but whether these two groups arose naturally or from shearing is not known.

Both AΔ60 and BΔ97 prevent cyclin destruction from being turned off

In normally dividing embryonic cells and lysates derived from them, cyclin destruction is a brief and transient process (Minshull *et al.*, 1989; Hunt *et al.*, 1992). In contrast, when sea urchin cyclin BΔ90 is added to interphase frog egg lysates, it permanently activates both cdc2 and the cyclin destruction pathway (Murray *et al.*, 1989; Glotzer *et al.*, 1991). Here, we have investigated the properties of truncated clam A- and B-type cyclins in the homologous cell-free system.

In these experiments, we took advantage of previous work showing that dilute radiolabeled lysates, which themselves cannot progress to the cyclin destruction point, can be used as test substrates (Luca and Ruderman, 1989). When labeled substrate proteins prepared from one cell cycle stage were added to concentrated unlabeled lysates from the same stage, or a different stage, the substrate cyclins are destroyed with the timing appropriate to the concentrated 'host' lysate. Other labeled proteins remain stable for several hours. Here, a concentrated unlabeled high-speed lysate from early M-phase cells was prepared and kept on ice; emetine plus buffer, AΔ60 or BΔ97 was added, and the incubation was started by shifting the mix to 18°C. At various times thereafter, a small amount of labeled substrate proteins was added and the fate of the labeled proteins followed. As shown on the left side of Figure 7, when labeled substrates were added to early M-phase lysate at time 0, the cyclins were subsequently destroyed within 15–30 min, with cyclin A falling in advance of cyclin B. When the host lysate was incubated for 30 min and substrates then added, cyclins were destroyed after a 10–15 min lag, suggesting that the host lysate went into cyclin destruction mode some time between 15 and 30 min after the start of the incubation and stayed in destruction mode longer than the intact cells would have. After 60, 90 and 120 min of incubation, the lysate no longer destroyed input substrate cyclins. These results show that, just as *in vivo*, the cell-free system advanced to the cyclin destruction point, turned on the destruction pathway and then turned it off, albeit after a longer interval than *in vivo*.

When AΔ60 was added to the M-phase lysate at 0 min, the labeled cyclins were destroyed in all cases (Figure 7, right panels). The kinetics of destruction varied, however. Cyclins added early in incubation, at 0 and 30 min, were not destroyed until the time at which the control lysate had moved on to the cyclin destruction point. However, cyclins added after that point, namely at 60, 90 or 120 min (or 4 h, not shown), were destroyed more quickly, with the bulk of the labeled cyclins disappearing within 5 min. Significantly, both cyclin A and cyclin B were destroyed and cyclin A always disappeared in advance of cyclin B. Similar results were seen with BΔ97 (not shown). Thus, when the cell-free system reaches the cyclin destruction point in the presence of stable, truncated cyclin of either type, the cyclin destruction pathway fails to be switched off and that pathway continues to act on both cyclin types for several hours.

Similar results were seen with low-speed supernatants, with these lysates passing through the lysate destruction point more rapidly than high-speed lysates (not shown). This suggests that low-speed lysates might be more suitable for studies focusing on the inactivation of cyclin destruction.

Only cyclin BΔ97 can switch on the cyclin destruction pathway; AΔ60 cannot

We next asked whether AΔ60 or BΔ97 could reactivate cyclin destruction in high-speed lysates that had passed the cyclin destruction point, switched off cyclin destruction and arrested in interphase (Figure 8). To do this, an M-phase lysate was incubated with emetine for 4 h at 18°C to take it well past the cyclin destruction point. Radiolabeled substrate proteins were then added; these were stable over the next 4 h indicating that, as expected, the lysate had passed the cyclin destruction point. When labeled substrate proteins and AΔ60 were added together at the end of the first incubation, high levels of H1 kinase activity were induced but cyclin destruction was not turned on. Thus cyclin A-activated cdc2 kinase is not by itself sufficient, even at very high levels, to turn on the cyclin destruction pathway. In striking contrast, however, cyclin BΔ97 was able both to activate both cdc2 kinase, albeit to only modest levels, and to turn on the cyclin destruction pathway. The long lag between the addition of BΔ97 and the appearance of cyclin destruction activity most likely reflects the relatively long time required to generate high cdc2 kinase activity. These results suggest that the two cyclins, while qualitatively indistinguishable in all other assays reported so far, are indeed functionally different and that it is cyclin B which is responsible for activating the cyclin destruction pathway, an event that allows entry into interphase of the next cell cycle.

Discussion

Cyclins A and B display several differences in their behavior during the cell cycle, but they have been virtually indistinguishable in all assays reported so far. Both cyclins can bind and activate the H1 activity of cdc2 (Draetta *et al.*, 1989; Parker *et al.*, 1991; Roy *et al.*, 1991), both cyclin–cdc2 complexes phosphorylate the same sites on various histones (Minshull *et al.*, 1990), both can induce oocytes to enter M-phase and resume meiosis (Swenson *et al.*, 1986; Pines and Hunt, 1987; Westendorf *et al.*, 1989), both can stimulate the centrosome-mediated nucleation of microtubules, although with quantitatively different results (Karsenti *et al.*, 1992) and both can induce T antigen-dependent SV40 DNA replication in extracts of G_1 somatic cells (D'Urso *et al.*, 1990). And, as we show here, both truncated cyclins lacking an N-terminal region essential for cell cycle stage-specific proteolysis are stable, both permanently activate cdc2 kinase, both prevent intact embryonic cells from completing mitosis and both prevent the cyclin destruction pathway from being switched off.

In striking contrast, only truncated cyclin B was capable of turning on the cyclin destruction pathway. When truncated cyclin A was provided to emetine-arrested interphase lysates lacking endogenous cyclins, it promptly activated the H1 kinase activity of cdc2 but failed to turn on the cyclin destruction pathway. This conclusion differs from that of Roy *et al.* (1991) who found that the addition of baculovirus-infected cell lysates overexpressing clam cyclin A to an interphase-arrested frog embryo lysate stimulated one oscillation of H1 kinase activity. In those experiments,

however, it is important to note that a complex mix of total insect cell proteins plus cyclin A was added to extracts prepared from mid-interphase frog embryo cells; furthermore, these frog extracts contained substantial amounts of A- and B-type cyclins, including $\sim 10^9$ molecules/cell of frog cyclin B1 (Minshull *et al.*, 1990; Kobayashi *et al.*, 1991). By contrast, in the experiments reported here, we added purified cyclin AΔ60 protein to interphase lysates that were totally devoid of endogenous cyclins. Under these conditions, cyclin A clearly activates cdc2 kinase but fails to activate the cyclin destruction system.

Our results support the conclusion that, while cyclins A and B can induce many apparently identical responses, they are indeed functionally distinct, with only cyclin B having the capacity to induce cyclin destruction. They further support the idea that cyclin B–cdc2 is responsible for activating the cyclin destruction pathway, providing a negative feedback loop that leads to the inactivation of cdc2 and exit from mitosis into the interphase of the next cell cycle (Félix *et al.*, 1990). In this model, the appearance of cyclin B-activated cdc2 kinase at the beginning of mitosis would phosphorylate a collection of M-phase-specific substrates, including one or more proteins involved in the cyclin destruction pathway. In some way, appropriate time lags for the destruction of cyclins A and B would be built into the system. Since both cyclins appear to be degraded by ubiquitin-mediated proteolysis (Glotzer *et al.*, 1991; Hershko *et al.*, 1991; Ruderman *et al.*, 1992), cyclin-specific ubiquitin ligases would be good candidates for such regulation. The recent findings that okadaic acid, a potent inhibitor of type 1 and 2A phosphatases, also keeps the cyclin destruction pathway activated in lysates of both frog (Lorca *et al.*, 1991) and clam embryos (A.Hershko, personal communication) suggest the existence of one or more okadaic acid-sensitive phosphatases that negatively regulate cyclin destruction and whose activity might also be controlled by cyclin B–cdc2.

Finally, our conclusion that cyclin A–cdc2 cannot, on its own, switch on the cyclin destruction pathway might even make sense. In all organisms examined so far, the oscillations of cyclins A and B are offset, with cyclin A and cyclin A–cdc2 kinase activity rising in advance of cyclin B (e.g. Evans *et al.*, 1983; Westendorf *et al.*, 1989; Pines and Hunter, 1989; Minshull *et al.*, 1990). If cyclin A–cdc2 by itself could turn on the cyclin destruction pathway, this would be expected to happen early in the cell cycle and cells would never be able to accumulate enough cyclin B.

One of the most striking features of both truncated cyclins is their effect on the persistence of cyclin destruction. As shown first for ΔB by Glotzer *et al.* (1991), and here for ΔA, the presence of Δcyclin causes the cyclin destruction pathway to remain active for hours, instead of the few minutes it normally occupies. With purified cyclins and responsive cell-free systems, it should now be possible to investigate this property further, as well as the individual roles of cyclin A and cyclin B, in considerably more detail.

Materials and methods

Construction of AΔ60 and BΔ97 cyclin expression vectors

The cyclin A cDNA pAXH(+) (Swenson *et al.*, 1986) was digested with *Hin*dIII which cuts at nucleotide (nt) 1539 in the 3′ non-coding region, blunt-ended with DNA polymerase Klenow fragment (New England BioLabs) and ligated to phosphorylated *Eco*RI linkers (NEB #1020). Subsequent digestion with *Eco*RI yielded a fragment extending from nt 326 to 1596 which encodes cyclin A from Glu61 through the C-terminal Leu422. This fragment was purified by electrophoresis in low melting point agarose (SeaPlaque, FMC) followed by phenol extraction (Sambrook *et al.*, 1989) and inserted into the *Eco*RI site of pET5a expression vector (Studier *et al.*, 1990). The predicted amino acid sequence of the new construct, pFLA1, includes the first 11 amino acids of T7 gene 10 protein, Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly, followed by Arg-Gly-Ser, which is specified by the linker region, and cyclin A Glu61–Leu422 to yield a 42 506 Dalton protein. The cyclin B construct pJW401 (Westendorf *et al.*, 1989) was cut with *Eco*RI to yield the fragment nt 379–2762 which encodes cyclin B from His97 to the C-terminal Val429 and continues to the end of the poly(A) tail. The fragment was inserted into the *Eco*RI site of pET5c (Studier *et al.*, 1990). The predicted amino acid sequence of this construct, pFLB1, includes the first 11 amino acids of T7 gene 10 protein, followed by Arg-Ile-Arg-Ile which is specified by the linker region, and His98 to Val429 from cyclin B to yield a 39 446 Dalton protein. pFLA1 and pFLB1 were transfected into BL21(DE3) (Hanahan, 1983).

Purification of recombinant Δcyclins

500 ml of NCZYM media + 100 μg/ml ampicillin (Sigma) was inoculated with 1 ml of an overnight culture of BL21(DE3) harboring pFLA1 or pFLB1 and incubated with shaking at 37°C until it reached an OD_{595} of 0.5. 1 mM IPTG (Sigma) was added and Δcyclin synthesis was induced for 2–4 h (Sambrook *et al.*, 1989). Δcyclins were isolated from the inclusion body fraction as described by Sambrook *et al.* (1989) as modified by Marston (1987). Cell pellets were resuspended in 3 ml lysis buffer/g cells (50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 8) containing 0.25 mM PMSF and treated with 0.3 mg/ml lysozyme (Sigma) for 20 min at 4°C. The lysate was incubated at 37°C with deoxycholate (4 mg/g cells) until it became viscous; at this point, DNA was degraded with 10 μg/ml DNase I (Sigma). After centrifugation at 12 000 *g*, the pellet was resuspended in 9 vol of lysis buffer containing 0.5% Triton X-100, 0.25 mM PMSF and 10 mM EDTA, incubated for 5 min on ice and centrifuged as before. The pellet was then washed in several volumes of 5 M urea + 0.1 M Tris, pH 8.5, dissolved in freshly made 8 M urea sample buffer (40 mM Tris, pH 6.8, 8 M urea, 3% SDS, 10% glycerol, 5% mercaptoethanol and bromophenol blue) and boiled for 10 min. Δcyclins were electrophoresed through 3 mm thick 15% polyacrylamide gels (Anderson *et al.*, 1973), and visualized on the gel by incubating in ice-cold 0.25 M KCl for 10 min (Westendorf *et al.*, 1989). Δcyclin-containing regions were excised, placed in dialysis tubing (Spectropor MWCO 6-8,000; Spectrum) containing ~20 ml elution buffer (0.5% SDS, 2 mM EDTA, 40 mM Tris–acetate, pH 8.5), and electroeluted with 40 mA constant current for 24–48 h. The eluted protein was dialyzed in buffer lacking SDS and the pH was gradually lowered by 0.2 pH unit decrements down to a pH of 7.2–7.5, a process that was needed to maintain solubility. Δcyclins were concentrated several-fold by dialysis against pH 7.5 buffer containing 20% PEG 20 000. Some samples were dialyzed in 0.5 × buffer T (Luca and Ruderman, 1989), pH 7.2. Protein concentrations were determined using a BioRad assay kit (Bradford, 1976) or by comparison with known amounts of BSA on polyacrylamide gels.

Preparation of cell-free systems from clam embryos

Spisula solidissima oocytes were cultured as decribed by Luca and Ruderman (1989). Progress through the meiotic and mitotic cell cycles was monitored by adding 1 μg/ml Hoechst 33342 (Calbiochem) to the cultures and examining aliquots of living cells by fluorescence microscopy. Homogenates were prepared from cell pellets, generally 1–3 ml, at selected cell cycle stages and centrifuged twice at 12 000 *g* for 15 min (Luca and Ruderman, 1989), or once at 150 000 *g* for 45 min, at 4°C using a TL100.1 rotor in a Beckman TLA100 ultracentrifuge. Aliquots of the supernatants were frozen in liquid nitrogen and stored at −80°C. M-phase lysates containing [^{35}S]methionine-labeled cyclins were made as described previously (Luca and Ruderman, 1989). Unless indicated otherwise, M-phase lysates were prepared from cells that had just entered first mitosis, as marked by the appearance of threadlike chromosomes. Mid- to late-phase interphase lysates were made from cells that had completed meiosis and gone on to form well defined nuclei containing decondensed chromosomes. To generate embryos arrested in interphase of the second mitotic cell cycle, 100 μM emetine (Sigma) was added to a suspension of embryos which had entered first mitosis; the embryos went on to complete first mitosis and arrested in interphase of the next cell cycle with decondensed chromosomes and well defined nuclei that were easily observed by bright-field microscopy.

In vitro cyclin destruction assays

Lysates were thawed on ice and mixed with an equal volume of buffer T (300 mM glycine, 120 mM potassium gluconate, 100 mM taurine, 40 mM NaCl, 2.5 mM $MgCl_2$, 100 mM HEPES, adjusted to pH 7.2 with KOH),

40 mM Tris−acetate, pH 7.2, or Δcyclin protein in the appropriate buffer. In the presence of Δcyclins, although not in their absence, cyclin destruction activity was dependent on providing an ATP regenerating system (50 μg/ml creatine kinase, 10 mM creatine phosphate). This requirement presumably reflects the depletion of ATP by the high constitutive H1 kinase activity induced by stable Δcyclins. After shifting the mix to 18°C to start the incubation, 5 μl aliquots were taken at the indicated times, mixed with 95 μl SDS sample buffer (Laemmli, 1970) containing 5% β-mercaptoethanol, boiled for 2−5 min, electrophoresed on 15% polyacrylamide gels (Anderson *et al.*, 1973) and analyzed by autoradiography or Western blotting.

When dilute [^{35}S]methionine-labeled M-phase low-speed lysates were used as the source of radiolabeled substrate cyclins (Luca and Ruderman, 1989), incubation mixes contained 5% [^{35}S]methionine-labeled M-phase lysate, 50% unlabeled lysate and 45% buffer or buffer containing Δcyclin. 100 μM emetine was always included to prevent further protein synthesis. After shifting the mix to 18°C, aliquots were taken at the indicated times and analyzed by electrophoresis followed by autoradiography against Kodak XAR-5 film for 12−48 h.

For Western blotting, proteins were transferred from 15% polyacrylamide gels to nitrocellulose (BA85, Schleicher & Schuell) using a Hoefer transfer apparatus set at 45 V for 3 h (Towbin *et al.*, 1979; Westendorf *et al.*, 1989; Draetta *et al.*, 1989) and reacted with rabbit antibodies specific for cyclin A, cyclin B or cdc2 (see below), followed by anti-rabbit IgGs conjugated to alkaline phosphatase (Promega). Binding of the secondary antibodies was visualized according to the manufacturer's protocol. Cyclin B antiserum and affinity purified antibodies were obtained as described in Westendorf *et al.* (1989). Cyclin A antibodies were obtained as described by Draetta *et al.* (1989) and affinity purified (Harlow and Lane, 1988) on a matrix of Sepharose CL4B coupled to the AΔ60 protein. cdc2 antibodies were generated against gel-purified recombinant *S.pombe* cdc2 protein (Draetta *et al.*, 1987) and affinity purified on Sepharose CL4B beads coupled to the cdc2 protein.

H1 kinase assays

H1 kinase assays were carried out with a protocol modified from Dunphy and Newport (1989). 5 μl aliquots were taken from reaction mixes at the indicated times and either processed immediately or frozen in liquid N_2 and used later. Aliquots were diluted 10-fold in buffer T, pH 7.2 (final concentration of lysate = 50%) which halts further progression of the lysates (Luca and Ruderman, 1989). One volume of this was added to one volume of 2 × kinase mix [0.2 mg/ml H1 (Boehringer), 10 μM cAMP dependent kinase inhibitor (Sigma), 500 μCi/ml [^{32}P]ATP (New England Nuclear Corp., 3000 Ci/mmol), 28 mM $MgCl_2$, 14 mM EGTA, 56 mM HEPES−KOH, pH 7.2]. Mixes were prepared on ice and then shifted to room temperature for a 10 min incubation. Reactions were stopped by the addition of one volume of 2 × SDS sample buffer, electrophoresed through 15% polyacrylamide gels and processed for autoradiography. No detectable differences in H1 kinase activity were seen between frozen and fresh samples.

p13^{suc1} binding of p34^{cdc2}−cyclin complexes

55 μl aliquots of lysate mix were diluted with an equal volume of stop buffer (50 mM HEPES−KOH, pH 7.2, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 1 mM ammonium molybdate) and incubated with an equal volume of packed, preswelled protein A−Sepharose beads (Sigma) for 30 min at 4°C. 50 μl of the supernatant was incubated with an equal volume of Sepharose beads coupled to p13^{suc1} (Brizuela *et al.*, 1987) for 30 min at 4°C. The beads received two 10 min washes with 0.5% Tween 20, 1 M NaCl, HEPES−KOH, pH 7.2, and two 10 min washes with stop buffer + 0.5% Tween 20, all at 4°C. The beads were rinsed with stop buffer lacking detergent and then resuspended in 50 μl stop buffer. 5 μl of the bead slurry was mixed with 5 μl of 2 × kinase mix and assayed for H1 kinase activity as above. The remainder of the pelleted beads were mixed with one volume of 2 × SDS sample buffer and boiled for 5 min. 5 μl of the eluate was analyzed by Western blotting.

Frog oocyte maturation assays, in vitro fertilization and microinjection

Xenopus laevis (Xenopus I, Ann Arbor, Michigan) females were primed with 50 IU pregnant mares' serum (PMS, Sigma). Two to 3 days later, ovarian pieces were excised and placed in s-Modified Barth's Solution (MBS, Gurdon and Wickens, 1983) plus 25 mg/l penicillin G (Na), 50 mg/l streptomycin sulfate and 100 mg/l sulfathiazole (Sigma). Oocytes were manually defolliculated and kept in MBS at 19−21°C in agar-lined Petri dishes. Uninjected oocytes were induced to mature by incubation with 5 μg/ml progesterone (Sigma). Oocytes were injected with 50 nl of recombinant cyclin. In some cases, oocytes were incubated in MBS with 10 μg/ml cycloheximide (Sigma) for 1 h before microinjection and continued in this medium afterwards. GVBD was scored initially by the appearance of a white spot at the animal pole, and verified by fixation in 10% trichloroacetic acid followed by dissection. *In vitro* fertilization, microinjection and embryo incubations were performed exactly as previously described (Shibuya and Masui, 1988). One blastomere of a cleaving, two-cell embryo was microinjected with 30 nl of sample. Microinjected embryos were incubated at 19−21°C for 4−6 h before fixation.

Cytology

To determine chromosome morphology, embryos were fixed in 63% ethanol, 30% distilled H_2O, 7% glacial acetic acid overnight at 4°C. After decanting the fixative, embryos were rinsed twice for 1 h each in H_2O, then stained in 1 μg/ml Hoechst 33342 for several hours to overnight. After dissection, a portion of the stained embryo was placed on a slide and immersed in a drop of 10% acetic acid. After a few minutes, the softened tissue was covered with a coverslip and squashed. The samples were observed using fluorescent optics.

Acknowledgements

We are grateful to the staff of the Marine Biology Laboratory and to Joe De Giorgis, Woods Hole, for the collection and maintenance of clams. We thank Michael Glotzer, Andrew Murray, Mark Kirschner and Eric Karsenti for communicating their results prior to publication, Giulio Draetta and David Beach for the bacterial expression vectors encoding p34^{cdc2} and p13^{suc1}, Ellen LeMosy for generating cdc2 antibodies and Kim Gavin for purifying p13^{suc1}. F.C.L. was supported in part by the Genetics Program, Duke University; C.D. by an NIH Training Grant to the Program in Cell and Developmental Biology, Harvard Medical School; and E.K.S. by a Jane Coffin Childs postdoctoral fellowship.

References

Allende,C.C., Hinrichs,M.V., Santos,E. and Allende,J.E. (1988) *FEBS Lett.*, **234**, 426−430.

Anderson,C.W., Baum,P.R. and Gesteland,R.F. (1973) *J. Virol*, **12**, 241−252.

Birchmeier,C., Broek,D. and Wigler,M. (1985) *Cell*, **43**, 615−621.

Booher,R. and Beach,D. (1987) *EMBO J.*, **6**, 3441−3447.

Booher,R. and Beach,D. (1988) *EMBO J.*, **7**, 2321−2327.

Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248−254.

Brizuela,L., Draetta,G. and Beach,D. (1987) *EMBO J.*, **6**, 3507−3514.

Draetta,G. and Beach,D. (1988) *Cell*, **54**, 17−26.

Draetta,G., Brizuela,L., Potashkin,J. and Beach,D. (1987) *Cell*, **50**, 319−325.

Draetta,G., Piwnica-Worms,H., Morrison,D., Druker,B., Roberts,T. and Beach,D. (1988) *Nature*, **336**, 738−744.

Draetta,G., Luca,F., Westendorf,J., Brizuela,L,. Ruderman,J. and Beach,D. (1989) *Cell*, **56**, 829−838.

Drury,K.C. and Schorderet-Slatkine,S. (1975) *Cell*, **4**, 269−274.

Dunphy,W. and Newport,J. (1989) *Cell*, **58**, 181−191.

D'Urso,G., Marraccino,R.L., Marshak,D.R. and Roberts,J.M. (1990) *Science*, **250**, 768−791.

Evans,T., Rosenthal,E.T., Youngbloom,J., Distel,D. and Hunt,T. (1983) *Cell*, **33**, 389−396.

Felix,M.A., Labbé,J.C., Dorée,M., Hunt,T. and Karsenti,E. (1990) *Nature*, **346**, 379−392.

Gautier,J. and Maller,J.L. (1991) *EMBO J.*, **10**, 177−182.

Gautier,J., Minshull,J., Lohka,M., Hunt,T. and Maller,J.L. (1990) *Cell*, **60**, 487−494.

Ghiara,J.B., Richardson,H.E., Sugimoto,K., Henze,M., Lew,D.J., Wittenberg,C. and Reed,S.I. (1991) *Cell*, **65**, 163−174.

Glotzer,M., Murray,A.W. and Kirschner,M.W. (1991) *Nature*, **349**, 132−138.

Gould,K.L. and Nurse,P. (1989) *Nature*, **342**, 39−45.

Hagan,I., Hayles,J. and Nurse,P. (1988) *J. Cell Sci.*, **91**, 587−595.

Hanahan,D. (1983) *J. Mol. Biol.*, **166**, 557−572.

Harlow,E. and Lane,D. (1988) *Antibodies. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hershko,A., Ganoth,D., Pehrson,J., Palazzo,R.E. and Cohen,L.H. (1991) *J. Biol. Chem.*, **266**, 16376−16379.

Hunt,T., Luca,F.C. and Ruderman,J.V. (1992) *J. Cell Biol.*, in press.

Karsenti,E., Buendia,B., Clarker,P., Verde,F., Decommun,B. and Draetta,G. (1992) *Cold Spring Harbor Symp. Quant. Biol.*, in press.

Kobayashi,J., Minshull,J., Ford,C., Golsteyn,R., Poon,R. and Hunt,T. (1991) *J. Cell Biol.*, **114**, 755−765.

Kumagai,A. and Dunphy,W.G. (1991) *Cell*, **64**, 903–914.
Labbé,J.C., Picard,A., Peaucellier,G., Cavadore,J.C., Nurse,P. and Dorée,M. (1989) *Cell*, **57**, 253–263.
Laemmli,U.K. (1970) *Nature*, **277**, 680–685.
Lehner,C.F. and O'Farrell,P.H. (1990) *Cell*, **61**, 535–547.
Lohka,M.J., Hayes,M.K. and Maller,J.L. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 3009–3013.
Lorca,T., Fesquet,D., Zindy,F., Le Bouffant,F., Cerruti,M., Brechot,C., Bevauchelle,G. and Dorée,M. (1991) *Mol. Cell. Biol.*, **11**, 1171–1175.
Luca,F. and Ruderman,J.V. (1989) *J. Cell Biol.*, **109**, 1895–1909.
Maller,J.L. and Krebs,E.G. (1980) *Curr. Top. Cell Regul.*, **16**, 217–311.
Marston,F.A.O. (1987) In Glover,D.M. (ed.), *DNA Cloning: A Practical Approach.* IRL Press, Oxford. Vol. III, pp. 59–88.
Meijer,L., Arion,D., Golsteyn,R., Pines,J., Brizvela,L., Hunt,T. and Beach,D. (1989) *EMBO J.*, **8**, 2275–2282.
Minshull,J., Pines,J., Golsteyn,R., Standart,N., Mackie,S., Colman,A., Blow,J., Ruderman,J.V. and Hunt,T. (1989) *J. Cell Sci.*, Suppl. **12**, 77–97.
Minshull,J., Golsteyn,R., Hill,C. and Hunt,T. (1990) *EMBO J.*, **9**, 2865–2875.
Minshull,J., Murray,A., Colman,A. and Hunt,T. (1991) *J. Cell Biol.*, **114**, 767–772.
Morla,A., Draetta,G., Beach,D. and Wang,J.Y.J. (1989) *Cell*, **58**, 193–203.
Murray,A.W. and Kirschner,M.W. (1989) *Nature*, **339**, 275–280.
Murray,A.W., Solomon,M.J. and Kirschner,M.W. (1989) *Nature*, **339**, 280–286.
Parker,L., Atherton-Fessler,S., Lee,M.S., Ogg,S., Falk,J.L., Swenson,K.I. and Piwnica-Worms,H. (1991) *EMBO J.*, **10**, 1255–1263.
Pines,J. and Hunt,T. (1987) *EMBO J.*, **6**, 2987–2995.
Pines,J. and Hunter,T. (1989) *Cell*, **58**, 833–846.
Pondaven,P., Meijer,J. and Beach,D. (1990) *Gene Dev.*, **4**, 9–17.
Roy,L., Swenson,K.I., Walker,D.H., Gabrielli,B.G., Li,R.S., Piwnica-Worms,H. and Maller,J.L. (1991) *J. Cell Biol.*, **113**, 507–514.
Ruderman,J., Luca,F., Shibuya,E., Gavin,K., Boulton,R. and Cobb,M. (1992) *Cold Spring Harbor Symp. Quant. Biol.*, in press.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Shibuya,E.K. and Masui,Y. (1988) *Dev. Biol.*, **129**, 253–264.
Solomon,M.J., Glotzer,M., Lee,T., Philippe,M. and Kirschner,M. (1990) *Cell*,, **63**, 1013–1024
Studier,F.W., Rosenberg,A.H., Dunn,J.J. and Dubendorff,J.W. (1990) *Methods Enzymol.*, **185**, 60–89.
Swenson,K., Farrell,K.M. and Ruderman,J.V. (1986) *Cell*, **47**, 861–870.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl Acad. Sci. USA*, **76**, 4350–4354.
Wasserman,W.J. and Masui,Y. (1975) *J. Exp. Zool.*, **193**, 369–375.
Westendorf,J.M., Swenson,K.I. and Ruderman,J.V. (1989) *J. Cell Biol.*, **108**, 1431–1444.

Received on July 23, 1991; revised on September 18, 1991