Control of Programmed Cyclin Destruction in a Cell-free System

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Abstract. To ask what controls the periodic accumulation and destruction of the mitotic cyclins across the cell cycle, we have developed a cell-free system from clam embryos that reproduces several aspects of cyclin behavior. One or more rounds of cyclin proteolysis and resynthesis occur in vitro, and the destruction of the cyclins is highly specific. The onset, duration, and extent of cyclin destruction and the appropriately staggered disappearance of cyclin A and cyclin B are correctly regulated during the first cycle in the cell-free system. Just as in intact cells, lysates made from early interphase cells require further protein synthesis to reach the cyclin destruction point, and lysates made from later stages do not. Using the cell-free system we show that cyclin disappearance requires ATP and Mg^{2+}. By combining lysates from different cell cycle stages, we show that (*a*) interphase lysates do not contain a dominant inhibitor of cyclin destruction and (*b*) the timing of cyclin destruction is determined by the cell cycle stage of the cytoplasm rather than the cell cycle stage of the substrate cyclins themselves. Among a large variety of agents tested, only a few affect cyclin destruction. Tosyl-lysine chlormethyl ketone (TLCK, a protease inhibitor), 6-dimethylaminopurine (6-DMAP, a kinase inhibitor), certain sulfhydryl-blocking agents, $ZnCl_2$ and EDTA (but not EGTA) completely block cyclin destruction in vitro. Addition of 1 mM Ca^{2+} to the cell-free system has no effect on cyclin stability, but 5 mM Ca^{2+} leads to the rapid destruction of cyclins and a small number of other proteins.

In rapidly dividing embryos of marine invertebrates, newly synthesized cyclins accumulate steadily across the cell cycle and then abruptly disappear near the end of mitosis (reviewed by Swenson et al., 1989). Several different experimental approaches have established that the disappearance of cyclin near the end of each cycle is due to a burst of rapid and selective proteolysis (Evans et al., 1983; Swenson et al., 1986; Westendorf et al., 1989; Murray and Kirschner, 1989). In clam embryos, there are two prominently synthesized cyclins, cyclin A and cyclin B. While the levels of both oscillate across the cell cycle and both can act as M phase inducers, the two are distinguished by their amino acid sequences, their differential patterns of expression during oogenesis and meiosis, and their slightly offset kinetics of destruction (Swenson et al., 1986; Westendorf et al., 1989; Hunt, T., and J. Ruderman, unpublished). In normal meiotic and mitotic cycles, the proteolysis of cyclin A begins in late metaphase and is completed within ~5–6 min; the drop in cyclin B begins a few minutes after that of cyclin A and is similarly completed within a 5–6 min window. A and B cyclins have been identified in *Drosophila*, where they show dramatically different patterns of transcription in somatic and germ cell lineages (Lehner and O'Farrell, 1989; Whitfield et al., 1989), and in *Xenopus* and yeast, where they have been designated as A or B type on the basis of sequence homologies with clam cyclins (Reed et al., 1988; Solomon et al., 1988; Goebl and Byers, 1988; Nash et al., 1988; Hagan et al., 1988; Minshull et al., 1989).

A regulatory role for the cyclins was first inferred from the observation that cyclin levels oscillated in a way that could explain why newly made proteins are needed to enter M phase in each cell cycle (Evans et al., 1983). Several types of evidence now show that newly synthesized cyclins are required for the generation of active maturation or M phase-promoting factor (MPF)[1] and entry into M phase. First, when introduced into frog oocytes that are naturally arrested in meiotic prophase, cyclins induce entry into meiotic M phase (Swenson et al., 1986; Pines and Hunt, 1987; Westendorf et al., 1989). Conversely, depleting activated egg lysates of endogenous cyclin mRNAs prevents nuclei from entering mitotic M phase (Minshull et al., 1989). Second, newly synthesized cyclin joins with a preexisting component of active MPF (Draetta et al., 1989), a 34-kD protein kinase originally identified as the gene product of the fission yeast cdc2/budding yeast cdc28 gene (Gautier et al., 1988; Dunphy et al., 1988) and probably a small number of other proteins (Lohka et al., 1988; Westendorf et al., 1989) to generate a complex that resembles active MPF in several key ways. Whereas the relative level of $p34^{cdc2/28}$ protein kinase remains constant across the cell cycle, its activity changes markedly. When assayed for activity towards histone H1, a highly preferred substrate, both MPF kinase activity and $p34^{cdc2/28}$ ki-

1. *Abbreviations used in this paper*: MPF, M phase-promoting factor; NEM, *n*-ethymaleimide; pHMB, *p*-hydroxymercuribenzoic acid; 6-DMAP, 6-dimethylaminopurine; TLCK, tosyl-lysine chloromethyl ketone.

nase activity are low in interphase, rise markedly at M phase, and drop abruptly just around the time when the cyclins are destroyed (Sano, 1985; Meijer et al., 1987; Draetta and Beach, 1988; Arion et al., 1988; Draetta et al., 1989; Labbé et al., 1989; Murray and Kirschner, 1989). Third, in yeast there is compelling genetic and biochemical evidence for a physical association between cyclins and $p34^{cdc2/28}$ that is required for cells to enter mitosis (Reed et al., 1988; Booher and Beach, 1988; Solomon et al., 1988; Goebl and Byers, 1988; Nash et al., 1988; Booher et al., 1989).

Our recent work suggests that active MPF is generated when $p34^{cdc2/28}$ is complexed with rising levels of cyclin, and MPF activity is lost as a consequence of the proteolytic destruction of cyclins near the end of M phase (Draetta et al., 1989; Westendorf et al., 1989). The experiments of Murray and Kirschner (Murray and Kirschner, 1989; Murray et al., 1989) directly confirm this, although these authors argue that cyclins are catalytic activators of MPF rather than essential components. Because the periodic rise and fall in cyclin levels is controlled by brief, periodic bursts of highly selective proteolysis near the end of M phase, it is important to understand what regulates cyclin destruction. Is cyclin protease constitutively active throughout the cell cycle, with cyclin destruction being regulated by periodic changes in the accessibility of the substrate cyclins? Or, instead, is cyclin protease inactive across most of the cell cycle then briefly activated near the end of each M phase? Is there one cyclin protease or two, one for cyclin A and one for cyclin B? To answer some of these questions, we turned to the use of a cell-free system, an approach that has been very productive for studying the regulation of several other aspects of cell cycle control (reviewed by Lohka and Maller, 1987; see also Burke and Gerace, 1986; Dessev et al., 1989). We have prepared a cell-free system from clam embryos that reproduces several features of in vivo cyclin behavior, including the programmed rise and fall of the cyclins and the temporally offset destruction of cyclin A and B. We show that interphase cells do not contain a dominant inhibitor of cyclin destruction, and that the timing of cyclin destruction is determined by the cell cycle stage of the cytoplasm rather than the cell cycle stage of the substrate cyclins. Of the numerous protease inhibitors and other agents tested in the cell-free system, all equally affect, or fail to affect, the destruction of cyclin A and B. This result suggests that if there are A and B type cyclin proteases, they are remarkably similar.

Materials and Methods

Fertilization and Labeling of Clam Embryos

Full-grown *Spisula solidissima* oocytes and sperm were collected and fertilized as described by Westendorf et al. (1989). Oocytes were suspended at a known concentration, typically 20,000–40,000/ml in sterile sea water, fertilized, and cultured at 18°C in the presence of 1 μg/ml Hoechst 33342 (Calbiochem-Behring Corp., La Jolla, CA). After entry into meiosis I at 11–12 min after fertilization, embryos were concentrated to 200,000/ml and cultured further at 18°C. Progress through the meiotic and mitotic cell cycles was followed by monitoring the state of H33342-stained chromosomes in living cells by fluorescence microscopy. If embryos were to be radiolabeled, 25 μCi ^{35}S-labeled Trans label (1,000–1,200 Ci/mmol; ICN Radiochemicals, Irvine, CA) was added per ml of cell culture upon the completion of second meiosis, as marked by the appearance of second polar bodies.

Preparation of the Cell-free System

1×10^7 embryos at the stages indicated below were pelleted rapidly in a clinical centrifuge and rinsed two to three times in cold (0°C) calcium-free sea water and once in cold buffer T (300 mM glycine, 120 mM K-gluconate, 100 mM taurine, 100 mM Hepes, 40 mM NaCl, 2.5 mM $MgCl_2$, adjusted to pH 7.2 with KOH). After the third wash, all buffer was aspirated from the cell pellet and the cells were homogenized with a stainless steel Dounce homogenizer (Wheaton Instruments Division, Milville, NJ). The homogenate was centrifuged twice at 12,000 *g* for 15 min. This step was essential: cyclin destruction was always seen in 12,000 *g* supernatants but never in unfractionated homogenates. Aliquots of the final supernatant were frozen in liquid nitrogen and stored at −80°C. Each microliter of lysate represented $\sim 1.5 \times 10^4$ homogenized cells.

Early interphase lysates were made from cells collected ~5–10 min after the second polar bodies had formed (~60–65 min after fertilization); at this time pronuclear envelopes were not detectable by phase-contrast microscopy. Mid-interphase lysates were prepared from cells that had progressed further to form well demarcated pronuclear membranes that were easily seen by phase-contrast microscopy (~70–75 min after fertilization). To make lysates from cells arrested in interphase of the second mitotic cell cycle, 100 μM emetine (Sigma Chemical Co., St. Louis, MO; Lodish et al., 1971) was added to embryos at the onset of first mitosis. This concentration inhibits protein synthesis by >99%. Embryos passed through first mitosis on schedule and entered second interphase but never entered second mitosis. After control embryos had reached the four-cell stage, emetine-arrested, two-cell embryos were collected and homogenized.

In Vitro Cyclin Destruction Assay

[^{35}S]Methionine-labeled lysates were thawed on ice, diluted 1:1 with buffer T, and incubated at 18°C. At various intervals after the start of the incubation in vitro (typically 2, 5, 10, 20, 30, and 60 min; 2, and 5 h), 5-μl aliquots were taken, added to 95 μl SDS sample buffer (Laemmli, 1970) containing 5% β-mercaptoethanol, and boiled for 2–5 min. Samples were electrophoresed on 15% polyacrylamide gels containing SDS (Anderson et al., 1973), processed for autoradiography and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for 8–24 h.

Protease Inhibitors

Stocks of PMSF (Sigma Chemical Co.), calpain inhibitor I, calpain inhibitor II and E64 (Boehringer Mannheim Biochemicals, Indianapolis, IN) were dissolved in DMSO and used immediately. E64d, a gift from Dr. K. Imahori (Mitsubishi-Kasai Institute, Tokyo), was dissolved in DMSO. Stocks of tosyl-lysine chloromethyl ketone (TLCK; Sigma Chemical Co.), leupeptin and chymostatin (Sigma Chemical Co.) were dissolved in distilled water and stored at −20°C for up to 1 mo. Aprotinin (Sigma Chemical Co.) and soybean trypsin inhibitor (SBTI; Sigma Chemical Co.) were dissolved in water and stored at −20°C for not more than 6 mo. Hemin (Sigma Chemical Co.) was dissolved in buffer T and stored at −20°C. 6-dimethylaminopurine (6-DMAP, Sigma), α-2-macroglobulin and (4-amidinophenyl) methanesulfonyl fluoride (APMSF) (both from Boehringer Mannheim Biochemicals) were made fresh in either distilled water or buffer T. Stocks of *p*-hydroxymercuribenzoic acid (pHMB, Sigma Chemical Co.), *n*-ethylmaleimide (NEM; Sigma Chemical Co.), iodoacetamide (Sigma Chemical Co.), and dithiothreitol (DTT; Boehringer Mannheim Biochemicals) were dissolved in buffer T just before use. Stocks of $CaCl_2$, $CuCl_2$, LiCl, $ZnCl_2$, NaF, EDTA, and EGTA were made in buffer T and stored at 4°C. Colcemid and calcium ionophore A23187 (both from Sigma Chemical Co.) were dissolved in DMSO and stored in the dark at −20°C.

ATP-regenerating and ATP-depletion Systems

When indicated, lysates contained an ATP-regenerating system consisting of 50 μg/ml creatine kinase and 10 mM creatine phosphate both from (Sigma Chemical Co.). ATP (Sigma Chemical Co.) was dissolved in buffer T, pH 7.2, before adding to the lysates. ATPγ-S (Boehringer Mannheim Biochemicals) was dissolved in buffer T and then added immediately to an equal volume of lysate. To deplete the endogenous ATP, hexokinase (Sigma Chemical Co.) and glucose were added to final concentrations of 100 U/ml and 10 mM, respectively.

Dilute Lysates as Substrates for the Cyclin Destruction Assay

[^{35}S]Methionine-labeled lysates were diluted eightfold with buffer T, usually containing a final concentration of 200 μM emetine, so that the ratio of lysate volume/total volume was 0.125. 1 vol of dilute, radiolabeled lysate

was then added to one volume of an unlabeled concentrated test lysate. This brought the ratio of radiolabeled lysate/total volume to 0.063 and that of the test lysate to the standard ratio of 0.50. At each time point, 5-μl aliquots were taken, added to 45 μl SDS sample buffer and analyzed by gel electrophoresis followed by autoradiography. When incubated with buffer alone in the ratio of 0.063 lysate/total volume, none of the lysates showed any detectable cyclin proteolysis over the 5-h period assayed.

Immunoprecipitation

At each time point across the cyclin destruction assay, 10 μl of the concentrated [^{35}S]methionine-labeled lysate (representing ~150,000 cells) was diluted 50-fold in buffer T. This was incubated for at least 4 h at 4°C with 5–10 μl preimmune or immune rabbit polyclonal sera containing antibodies directed against about three quarters of the length of cyclin A or cyclin B proteins (Westendorf et al., 1989). Immune complexes were then precipitated by adding 50 μl of a 1:1 slurry of protein A-Sepharose CL4B beads (Pharmacia Fine Chemicals, Piscataway, NJ) and buffer, and incubated for another 4 h at 4°C. After the final incubation, beads were washed three times in buffer T containing 0.2% Tween 20 and three times in TBS (50 mM Tris, 150 mM NaCl, pH 7.4). Immune complexes were eluted by boiling in SDS sample buffer containing 5% β-mercaptoethanol for 2–5 min, and analyzed by gel electrophoresis followed by autoradiography.

In Vitro Transcription and Translation

SP6-cyclin A and cyclin B mRNAs were transcribed in vitro as described previously (Swenson et al., 1986; Westendorf et al., 1989). Total RNA was isolated from emetine-arrested two-cell embryo lysates as described by Rosenthal et al. (1980). RNAs were translated in a rabbit reticulocyte lysate (Promega Biotec, Madison, WI) in the presence of [^{35}S]methionine (175 Ci/mM final concentration from a 1,444 Ci/mM stock; Amersham Corp., Arlington Heights, IL).

Mixed Lysate Experiments: Cyclins Made in Reticulocyte Lysate as Substrates in Clam Embryo Lysates

Various amounts of reticulocyte lysate containing SP6-cyclin mRNA translation products (ranging from one-half to one-sixty-fourth the total volume) were added to previously characterized [^{35}S]methionine-labeled or -unlabeled M phase lysates in the presence of 100 μM emetine and incubated at 18°C. Aliquots were taken at stated intervals and analyzed as above. Added reticulocyte lysate, with or without cyclin translation products, did not affect the destruction of endogenous cyclins contained within the clam lysates.

Results

Timing of the First Mitotic Cell Cycle

In *Spisula*, full-grown oocytes are arrested at the G2/M border of meiosis I. Upon fertilization, the embryos go through the two meiotic divisions and then begin a series of rapid mitotic divisions (Fig. 1). Embryos from an individual batch of oocytes goes through the early cell cycles with considerable synchrony. For different batches, the exact times vary by a few minutes. When cultured at 18°C, embryos generally pass through the metaphase-anaphase transition of meiosis II and put out the second polar body at 55–60 min after fertilization. They enter interphase of the first mitotic cell cycle at 60–65 min, as marked by chromosome decondensation and formation of distinct pronuclear envelopes. Entry into first mitotic M phase, signaled by chromosome condensation and nuclear envelope breakdown, occurs ~82 min. By ~90 min, the chromosomes become fully aligned on the metaphase plate; anaphase begins shortly thereafter, ~92 minutes, and is followed by cytokinesis at 95 min. The destruction of cyclin A begins several minutes before the onset of anaphase and is completed within 5-6 min. Destruction of cyclin B begins a few minutes later. The drop in cyclin B immediately precedes and parallels the time course of the metaphase-anaphase transition. Cyclin A completely disappears at the end of each mitosis, but cyclin B levels drop by only 85–90% (Swenson et al., 1986; Westendorf et al., 1989; Hunt, T., and J. Ruderman, manuscript in preparation).

Cyclin Destruction Occurs In Vitro

Lysates were prepared and frozen from embryos cultured at 18°C, labeled with [^{35}S]methionine during interphase of the first mitotic cycle and collected at the onset of first mitosis (82–84 min after fertilization). To follow the fate of the labeled cyclins in vitro, an aliquot was thawed, diluted with one volume of buffer, and incubated at 18°C. Samples were taken at various times after the start of the incubation, typically 2, 5, 10, 20, 30, and 60 min and 2 and 5 h, and analyzed by SDS gel electrophoresis followed by autoradiography.

In the experiment shown in Fig. 2, the protein synthesis inhibitor emetine was added to the lysate at the start of the incubation (t = 0 min) so that the destruction of prelabeled cyclins would not be masked by the appearance of newly synthesized ones. Most proteins, such as ribonucleotide reductase (RR), were stable in vitro for at least 5 h. In contrast, cyclin levels remained high for several minutes and then dropped precipitously, with the disappearance of cyclin A preceding that of cyclin B. Indeed, in almost all lysates prepared from early M phase cells, the destruction of cyclin A began 5–10 min after the start of the in vitro incubation, a little later than would have occurred in the intact embryo. Once initiated, most cyclin A disappeared within a period of 5 min and virtually all of it was gone by the next time point. Thus, for cyclin A, the onset, duration, and extent of destruction in vitro closely resembled that seen in vivo. The behavior of cyclin B was also reproduced in vitro, but less well. In the lysate shown in Fig. 2, the drop in cyclin B began ~10 min and had ceased by the next time point 10 min later. These times are slightly delayed compared with the times routinely seen with intact cells. These sorts of delays and extensions in cyclin B proteolysis were typical of most lysates. Curiously, the details of cyclin B destruction, whereas identical in all aliquots of an individual lysate, varied among different lysates. In general, the onset of cyclin B destruction was delayed by several minutes, the duration was longer and the final extent of destruction was less.

No obvious intermediates of cyclin destruction were seen during the in vitro destruction assay. This indicates that, whatever pathway is used to destroy the cyclins, it operates rapidly and efficiently in the cell-free system. Most importantly, even after multiple rounds of freezing and thawing, lysates retained the ability to selectively destroy the cyclins on schedule. This feature allowed us to check the basic characteristics on an individual lysate, and then go on to assay other aliquots of the same lysate under a variety of experimental conditions.

In Most Lysates, Cyclin Destruction Was Followed by at Least One Additional Round of Cyclin Synthesis and Destruction

Most lysates displayed at least one full round of cyclin destruction and reappearance in vitro (Fig. 3 *a*), and some went on to destroy the cyclins a second time although with considerably slower kinetics (not shown). The reappearance of

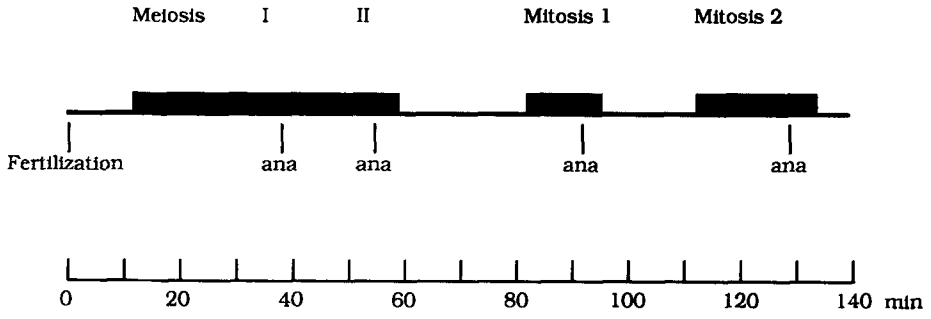

Figure 1. Timing of the first four cell cycles in *Spisula* embryos. Full-grown *Spisula* oocytes are naturally arrested at the G2/M border of meiosis I with an intact nuclear envelope and a 4C set of partially condensed chromosomes. Fertilization activates the resumption of meiosis and leads to the mitotic cleavage divisions. When cultured at 18°C, fertilized oocytes enter M phase of meiosis I at 11–12 min, marked by nuclear envelope breakdown and an increase in chromosome condensation. The maternal chromosomes go through anaphase of the two meiotic divisions, I and II, at 40 and 55 min, respectively. After anaphase II, the remaining haploid set of maternal chromosomes and the haploid sperm nucleus decondense fully and form two haploid pronuclei; this occurs around 60 minutes and marks the transition into interphase of the first mitotic cleavage cycle. Embryos enter M phase of mitosis 1 around 82 min (marked by nuclear envelope breakdown, chromosome condensation), begin to exit M phase of mitosis 1 around 92 minutes (marked by the onset of anaphase 1) and pass through the second mitotic cycle as indicated. Thin line, interphase; heavy line, M phase; ana, anaphase.

labeled cyclins in vitro was surprising at first, as we had made no attempt to optimize lysates for protein synthesis. Blocking protein synthesis in M phase lysates did not in any way affect the timing or extent of cyclin destruction but did prevent the reappearance of cyclin (Fig. 3 *b*), just as in vivo (Hunt, T., and J. Ruderman, unpublished). These results indicate that the cyclin proteases and other components responsible for cyclin destruction are present in early M phase cells at levels sufficient to ensure rapid and complete degradation in vitro. Furthermore, the protease activity is remarkably specific: by the end of a 5-h incubation at 18°C, and in the absence of any exogenous protease inhibitors, only the cyclins were targets for destruction. Even after 24 h the bulk of the proteins remained intact (not shown).

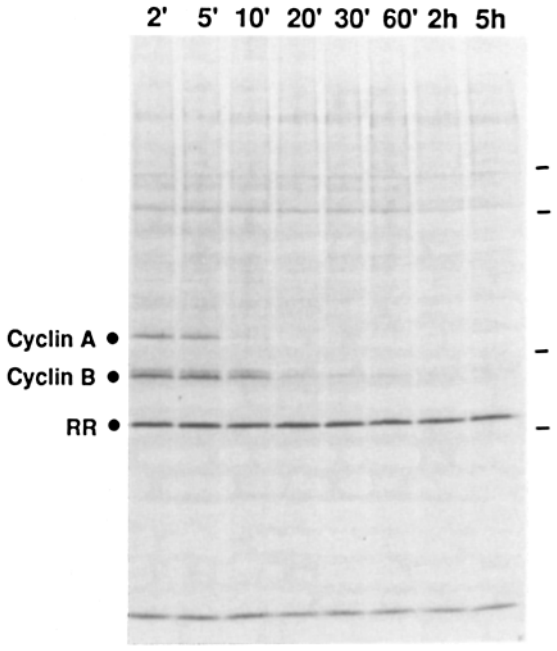

Figure 2. Cyclin destruction in vitro. Embryos were labeled with [^{35}S]methionine during the first mitotic cell cycle, and a lysate was prepared from early M phase cells and incubated at 18°C as described in the text. After the start of the incubation in vitro (t = 0 min), samples were taken at the indicated times and analysed by gel electrophoresis followed by autoradiography. The positions of cyclin A, cyclin B, and ribonucleotide reductase (*RR*) are indicated on the left. The positions of molecular weight markers are indicated by dashes on the right, from top to bottom: 116, 94, 56, and 40 kD.

Not all lysates were equally active in protein synthesis. Furthermore, multiple rounds of freezing and thawing resulted in progressive loss of protein synthesis activity. Because the major issue addressed here was the regulation of cyclin proteolysis, and ongoing protein synthesis was not needed for cyclin destruction (and, in fact, often partly obscured it), we did not attempt to optimize the lysates for protein synthetic activity.

Cyclin Destruction In Vitro Does Not Reveal Any Intermediates

Once cyclin destruction begins in vivo, it proceeds rapidly and without the appearance of any detectable intermediates (Evans et al., 1983; Swenson et al., 1986; Standart et al., 1987; Westendorf et al., 1989). Because the duration of cyclin destruction in vitro is slightly longer, it seemed possible that we might be able to visualize transient intermediates by immunoprecipitation. This approach would be particularly useful in detecting a diffuse ladder of higher molecular weight intermediates of the type expected for ATP-dependent, ubiquitin-mediated proteolysis (reviewed by Rechsteiner, 1987). No such intermediates of cyclin A were apparent in immune precipitates from the cell-free system (Fig. 4), even after long exposures. Immunoblots using the same polyclonal antiserum, which is directed against >75% of the length of cyclin A, confirmed this (not shown). Immune precipitates of cyclin B revealed a few faint, more rapidly migrating bands but no larger forms. However, these could represent products of a nonspecific, low level protease present in the antiserum.

These results show that intermediates of cyclin proteolysis are destroyed nearly as rapidly in the cell-free system as they are in the intact cell. They also suggest that the lag in the kinetics of cyclin destruction in vitro is due to deficiencies in activities that may mark the cyclins for destruction or to deficiencies in the activation of cyclin proteases.

Cyclin B occasionally splits into a tight doublet (compare Figs. 2, 4, 5, and 7 with Fig. 6). In light of other work (Stan-

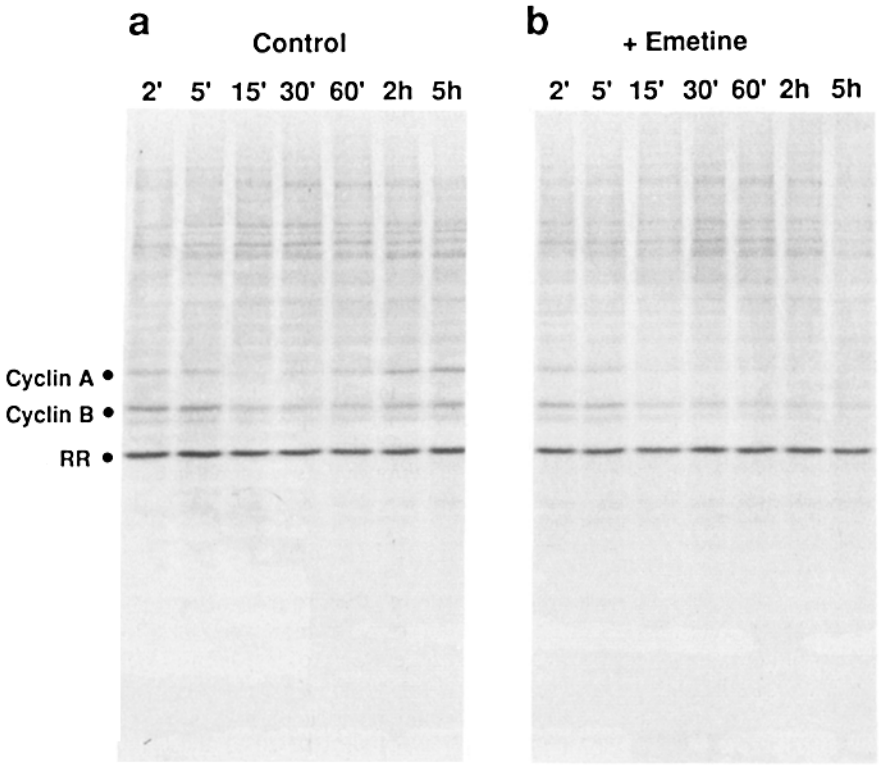

Figure 3. Cyclin destruction is followed by resynthesis in vitro. Embryos were labeled with [^{35}S]methionine during the first mitotic cell cycle and a lysate was prepared from early M phase cells and incubated in vitro as described in the text. Portions of the lysate were then incubated in (*a*) the absence or (*b*) presence of 100 μM emetine. Samples were taken at the indicated times after the start of the incubation (t = 0 min) and analyzed by gel electrophoresis followed by autoradiography. The positions of cyclin A, cyclin B, and ribonucleotide reductase (*RR*) are indicated on the left.

dart et al., 1987; Hunt et al., 1988; Draetta et al., 1988; Swenson et al., 1989; Westendorf et al., 1989), we suspect the upper band is phosphorylated cyclin B. However, neither band was preferentially proteolysed nor was there any obvious shift from one form to the other during the in vitro destruction assay.

Behavior of Interphase Lysates

Whereas M phase lysates took 5–15 min to reach the cyclin destruction point (Figs. 2, 3, and 7 *a*), mid-interphase lysates took ~10–20 min (see Fig. 7 *c*, for example) and early interphase lysates took even longer, ~30–60 min (Fig. 5). This result shows that, whatever sets the timing for the switchover to cyclin destruction, it is reproduced fairly well in the cell-free system.

Spisula embryos, like others, require protein synthesis up to a particular point in early interphase, the "commitment point," (~65 minutes after fertilization) in order to proceed into and through the subsequent M phase (Hunt, T., and J. Ruderman, unpublished; reviewed by Swenson et al., 1989).

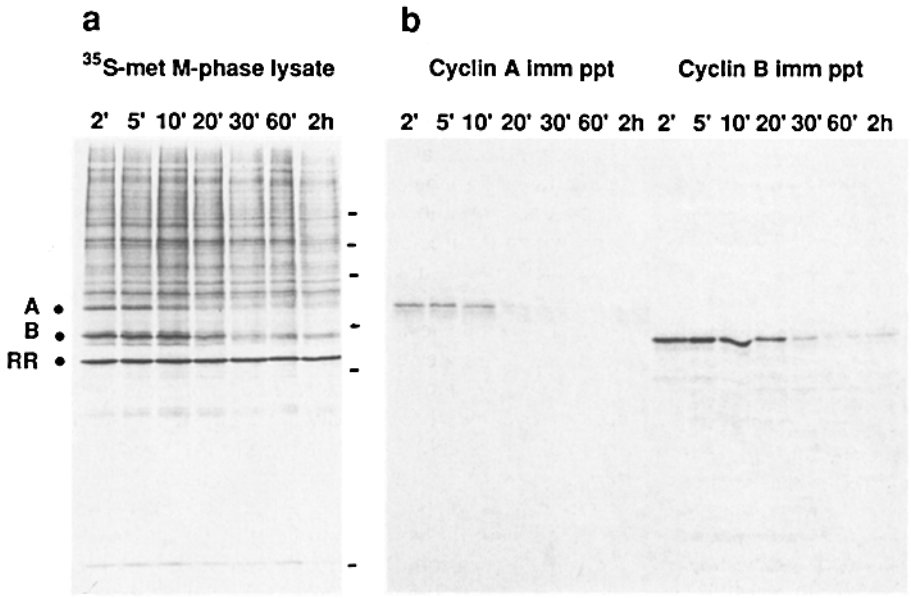

Figure 4. Immunoprecipitation of cyclins A and B from [^{35}S]methionine-labeled M-phase lysate. Embryos were labeled with ^{35}S-methionine during the first mitotic cell cycle, and a lysate was prepared from early M phase cells and incubated in vitro. Samples were taken at the indicated times after the start of the incubation (t = 0 min) and analyzed by gel electrophoresis followed by autoradiography. This lysate showed very little protein synthesis activity so emetime was not added to the incubation mix. (*a*) Total ^{35}S-labeled proteins. (*b*) ^{35}S-labeled proteins immunoprecipitated with polyclonal cyclin A or cyclin B antibodies. No ^{35}S-labeled proteins immunoprecipitated when preimmune sera were used (not shown). The positions of molecular weight markers are indicated by dashes on the right of *a*, from top to bottom: 116, 94, 68, 56, 40, and 21 kD.

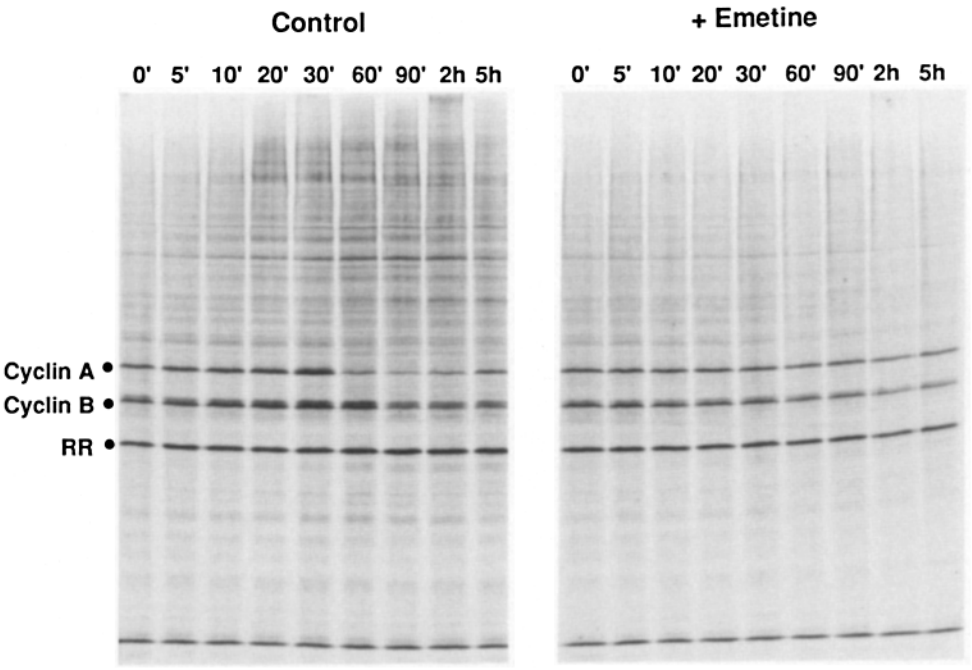

Figure 5. The cyclins present in early interphase lysates are not destroyed in vitro if protein synthesis is inhibited. Embryos were labeled with [^{35}S]methionine during the first mitotic cell cycle and a lysate was prepared from early interphase cells. Portions of the lysate were incubated in the absence (control) or presence (emetine) of 100 μM emetine. Samples were taken at the indicated times after the start of the incubation (t = 0 min) and analyzed by gel electrophoresis followed by autoradiography. The positions of cyclin A, cyclin B, and ribonucleotide reductase (*RR*) are indicated.

These protein synthesis requirements were completely reproduced in the cell-free system: lysates made from early interphase cells taken before the commitment point required further protein synthesis to reach the cyclin destruction point (Fig. 5), whereas those from mid interphase (Fig. 7) or M phase cells (Figs. 2 and 3) did not. Certain early-interphase lysates made around the time of the commitment point did not require protein synthesis to proceed to the cyclin destruction point, but took a very long time to reach that point (not shown). This delay suggests that once the threshold level of cyclin is reached, there is an additional rate limiting process required before various M phase events including cyclin destruction can be activated.

The Timing of Cyclin Destruction Is Set By the Cell Cycle Stage of the Lysate, Not the Cell Cycle Stage of the Cyclins

The kinetics of cyclin destruction in M phase lysates were indistinguishable in concentrated lysates and lysates diluted with one volume buffer (not shown). When lysates were diluted further, there were corresponding increases in the times taken to reach the onset of cyclin destruction (Fig. 6). At the highest dilution shown (6.3%), cyclins were stable for >5 h (Fig 6, *bottom*). Dilution of interphase lysates to 6.3% similarly blocked cyclin destruction (not shown).

To see whether the kinetics of destruction are determined by the cyclins themselves or by other components of the lysates, we asked if radiolabeled "substrate" cyclins within a dilute lysate made at one stage of the cell cycle would be recognized and destroyed by a concentrated "host" lysate made at a different stage of the cell cycle. When incubated alone, the radiolabeled cyclins in dilute M phase lysates (Fig. 7 *a*) or dilute interphase lysates (not shown) were stable. When added to a concentrated unlabeled M phase lysate, the radiolabeled M phase substrate cyclins were destroyed in <10 min and, as expected, their destruction did not require ongoing protein synthesis (Fig. 7 *b*). When added to a concentrated mid interphase lysate, radiolabeled M phase substrate cyclin A was stable for 10–20 min and then began to be destroyed, i.e., with typical midinterphase kinetics; cyclin B levels declined later (Fig. 7 *c*). This mixed lysate did not require ongoing protein synthesis to reach the onset of cyclin destruction (Fig. 7 *d*). When added to a concentrated early interphase lysate, the radiolabeled M phase cyclin substrates were stable for even longer. Cyclin A destruction began after 60 min; cyclin B destruction began even later and never reached completion during the 5 h assayed. Furthermore, when emetine was added to the dilute M phase-concentrated early interphase lysate mix at the beginning of the incubation, the M phase cyclin substrates failed to be destroyed.

In all cases examined, the ability of the labeled substrate cyclins to undergo destruction, and the timing of their destruction, was determined by the concentrated host lysate (summarized in Table I). Thus, the timing of cyclin destruction is set by the cell cycle stage of the lysate, not by the cell cycle stage of the cyclins. If cyclins are in fact marked for destruction at a particular point in the cell cycle by some sort of posttranslational modification, that marking must be reversible or subservient to other cues. Furthermore, lysates and intact cells show the same requirements for protein synthesis to reach the cyclin destruction point. Most importantly, this requirement extends to substrate cyclins provided from cells that have progressed well beyond the commitment point for mitosis.

If protein synthesis is blocked past the commitment point, the cells proceed into and through M phase, destroy the cyclins on schedule, and arrest in the next interphase. Lysates made from these arrested cells were tested for proteolytic activity towards exogenous cyclin, which was provided as a dilute, radiolabeled M phase lysate. The emetine-arrested interphase lysates did not contain detectable cyclin proteolytic activity at any of the time points of the assay up to 5 h (not shown). This result establishes three points. First, it shows that ongoing protein synthesis is not needed to turn off cyclin

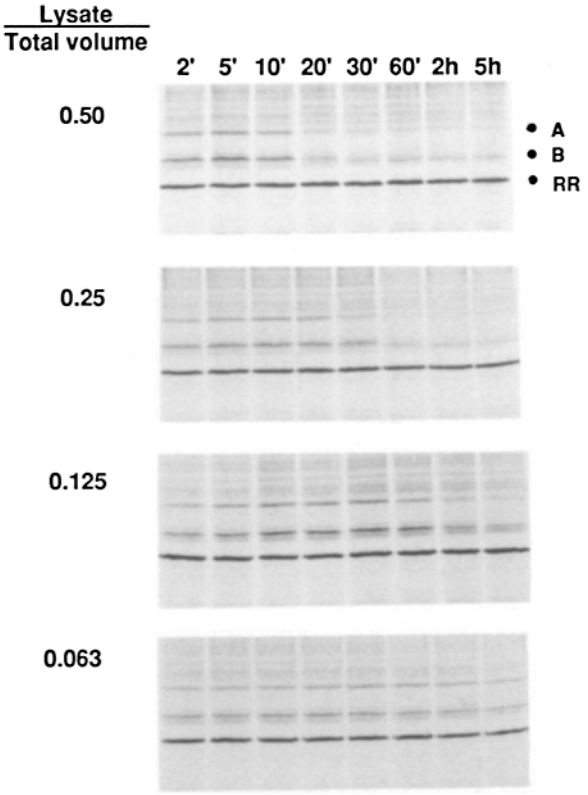

Figure 6. Small dilutions of concentrated lysates retard cyclin destruction; larger dilutions block cyclin destruction altogether. Embryos were labeled with [^{35}S]methionine during the first mitotic cell cycle and a concentrated lysate was prepared from early M phase cells as described in the text. One aliquot of the concentrated lysate was diluted with buffer to 0.50 of the reaction volume, which represented the standard lysate. Successively greater dilutions of the concentrated lysate were made to give reactions in which the lysate represented 0.25, 0.125, or 0.063 of the total incubation volume. Samples were taken from each reaction mix at the indicated times after the start of the incubation (t = 0 min) and analyzed by gel electrophoresis followed by autoradiography. To compare the kinetics of cyclin destruction in the standard lysate with those in the more dilute lysates, the autoradiograms were exposed for appropriately different times. The positions of cyclin A, cyclin B, and ribonucleotide reductase (*RR*) are indicated.

destruction activity, but is needed for its reappearance. Second, if cyclins need to be marked by a posttranslational modification in order to enter the cyclin destruction pathway, then that marking must be reversible and marked M phase cyclins must become unmarked in the emetine-arrested interphase lysates. Third, it rules out the idea that cells are maintained in interphase by a constitutively activated cyclin destruction pathway.

M Phase Lysates are Dominant over Interphase Lysates

The scheduled cyclin destruction process that operates in intact M phase cells, and in lysates made from them, is independent of ongoing protein synthesis. In contrast, when protein synthesis is blocked in early interphase cells taken before the commitment point, and in lysates made from them, cyclins fail to be destroyed. We exploited these features to ask if interphase cells contain a dominant inhibitor of cyclin protease activity. 1 vol of [^{35}S]methionine labeled, concentrated early interphase lysate (containing the labeled cyclin to be monitored) was mixed with one volume of concentrated, unlabeled M phase lysate. Emetine was added to the mix to block any new cyclin synthesis that might obscure the disappearance of old cyclins. The labeled cyclins (from the early interphase lysate) began to disappear with typical M phase kinetics, i.e., within 10–15 min (Fig. 8 *a*). When the concentrated, unlabeled M phase lysate was preincubated alone at 18°C for 5 min and then added to one volume of the radiolabeled early interphase lysate, the radiolabeled interphase cyclins were degraded within 5–10 min, i.e. 5 min faster (Fig. 8 *b*). These results indicate the M phase lysates are dominant over interphase lysates.

Inhibitors and Cyclin Destruction Activity

One useful feature of the cell-free system is that it provides the opportunity to test a wide range of protease inhibitors and other reagents that cannot enter intact cells. In most cases, emetine was added to lysates so that ongoing protein synthesis would not mask any weak proteolytic events. Most of the inhibitors tested had no effect on either the timing, duration or extent of cyclin destruction (see Table II). Only tosyllysine chloromethyl ketone (TLCK) unambiguously inhibited destruction (Fig. 9). TLCK is most commonly used as an irreversible inhibitor of trypsin-like serine proteases, but is known to block some cysteine proteases by alkylating sulfhydryl groups (see Table II for references to TLCK and other agents tested). Calpain inhibitor I, a cysteine protease inhibitor specific for calcium-activated calpain-like proteases, retarded cyclin degradation but did not completely block it (Fig. 9). Calpain inhibitor II had no effect. Freshly prepared PMSF (up to 2 mM), a general serine protease inhibitor, had no effect. Other common serine protease inhibitors such as 4-amidophenyl-methanesulfonyl fluoride (APMSF), aprotinin, chymostatin, hemin, and soybean trypsin inhibitor (SBTI) had no effect. α-2-macroglobulin, which is considered to be a "general" protease inhibitor, did not block cyclin destruction in vitro. The general cysteine protease inhibitor E64 (Fig. 9) and its lipid soluble analogue E64d also had no effect. However, several agents that interfere with sulfhydryl groups and are commonly used as inhibitors of cysteine proteases were very effective in blocking cyclin destruction. These included *p*-hydroxymercuribenzoic acid (pHMB), *n*-ethylmaleimide (NEM), $ZnCl_2$ (see below) and $CuCl_2$. Iodoacetamide, which also inhibits many cysteine proteases, had no effect on cyclin destruction. Taken together, these results are most consistent with the idea that both cyclin A and cyclin B are destroyed by cysteine proteases, but certainly do not rule out other explanations such as the involvement of a novel serine protease. We note that SBTI, which fails to block cyclin destruction in clam lysates, stabilizes MPF activity after microinjection into pre-meiotic oocytes (Picard et al., 1985) but, because of differences in experimental design, it is difficult to make meaningful comparisons.

Cyclin Destruction is an ATP-dependent Process and May Require Phosphorylation

Neither cyclin destruction nor resynthesis was affected by addition of ATP. When endogenous ATP stores were depleted, cyclin destruction was inhibited almost completely. Cyclin

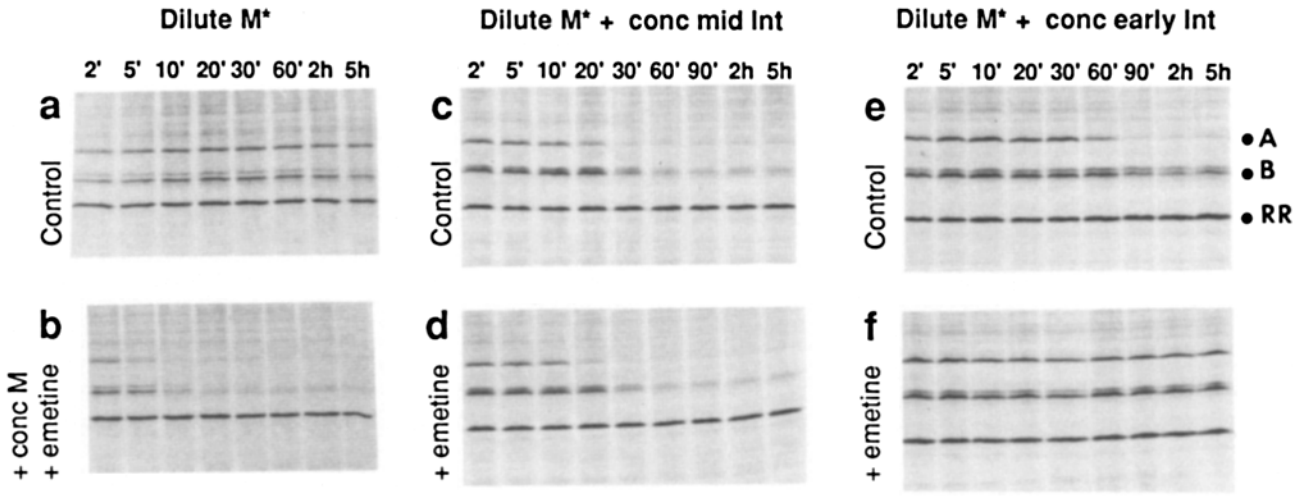

Figure 7. ^{35}S-cyclins in diluted M phase lysates are stable, but can serve as substrates for proteolysis when added to concentrated lysates. Embryos were labeled with [^{35}S]methionine during the first mitotic cell cycle. A concentrated lysate was made and diluted 16-fold to give a ratio of 0.063 lysate/total volume, as described in the text. The behavior of the radiolabeled cyclins in this dilute lysate (the "substrate" cyclins) is shown in *a*. The behavior of the radiolabeled substrate cyclins from the dilute lysate was then followed in the presence of: (*b*) concentrated M phase lysate and 100 μM emetine; (*c*) concentrated mid-interphase lysate; (*d*) concentrated mid-interphase lysate and 100 μM emetine; (*e*) concentrated early-interphase lysate; (*f*) concentrated early-interphase lysate and 100 μM emetine. After the start of the incubation (t = 0 min), samples were taken at the indicated times and analyzed by gel electrophoresis followed by autoradiography. The positions of cyclin A, cyclin B, and ribonucleotide reductase are indicated on the right side of *e*.

destruction was blocked by EDTA; this was partially reversed by the subsequent addition of excess Mg^{2+}. Addition of the kinase inhibitor 600 μM 6-DMAP (6-dimethylaminopurine) strongly inhibited cyclin destruction in vitro (Table II). These results indicate that there is at least one ATP-dependent step needed for cyclin destruction. Obvious candidates include ATP-dependent proteolysis, possibly mediated by ubiquitin, and ATP-dependent phosphorylation (reviewed by Bond and Butler, 1987; Rechsteiner, 1987).

1 mM $ZnCl_2$ Blocks Cyclin Destruction In Vitro

Immune precipitates of *S. cerevisiae* p34^{cdc28}, clam cyclin A, and clam cyclin B each have substantial kinase activity in vitro, and the patterns of phosphorylation are changed markedly when the in vitro kinase reactions are carried out in the presence of 1 mM $ZnCl_2$ (Reed et al., 1985; Swenson et al., 1989; Westendorf et al., 1989). Furthermore, zinc strongly inhibits the activity of histone H1 kinase (Pelech et al., 1987) which, at least under some circumstances, appears to be the same as MPF which, in turn, appears to be a complex of cyclin, p34$^{cdc\,2/28}$ protein kinase, and a small number of other proteins (reviewed by Featherstone, 1989, but see also Labbé et al., 1989). These observations prompted us to test the effect of zinc on cyclin destruction in vitro. When 1 mM $ZnCl_2$ was added to M phase lysates, cyclin destruction was inhibited severely (Fig. 10 *a*). 250 μM $ZnCl_2$ was equally effective; 125 μM $ZnCl_2$ delayed destruction by 30–

Table I. Fates of Substrate Cyclins Added to Concentrated Lysates from the Same or Different Stages of the Cell Cycle

Component 1*	Component 2	Emetine	Fate of radiolabeled substrate cyclins
Conc. M	–	±	Destroyed with M-phase kinetics (*5–15 min*)
Dilute M	–	±	Stable
Dilute M	Conc. M	±	Destroyed with M-phase kinetics
Dilute M	Conc. mid Int	±	Destroyed with mid interphase kinetics (*10–20 min*)
Dilute M	Conc. early Int	–	Destroyed with early interphase kinetics (*30–60 min*)
Dilute M	Conc. early Int	+	Stable
Dilute M	Conc. em. arr. 2-cell	±	Stable
Conc. mid Int	–	±	Destroyed with mid interphase kinetics
Dilute mid Int	–	±	Stable
Dilute mid Int	Conc. M	±	Destroyed with M phase kinetics
Dilute mid Int	Conc. mid Int	±	Destroyed with mid-interphase kinetics
Dilute mid Int	Conc. em. arr. 2-cell	±	Stable
Conc. early Int	–	–	Destroyed with early interphase kinetics
Conc. early Int	–	+	Stable
Conc. early Int	Conc. M	+	Destroyed with M phase kinetics
Dilute early Int	–	±	Stable

Component 1, which contained radiolabeled substrate cyclins (*), and component 2, concentrated "host" lysates, were prepared at the indicated cell cycle stages, mixed as described in the text, and the fate of the radiolabeled cyclins were followed. Abbreviations: conc., concentrated; early Int, early interphase; em. arr. 2-cell, emetine arrested 2-cell; M, M phase; mid Int. mid interphase.

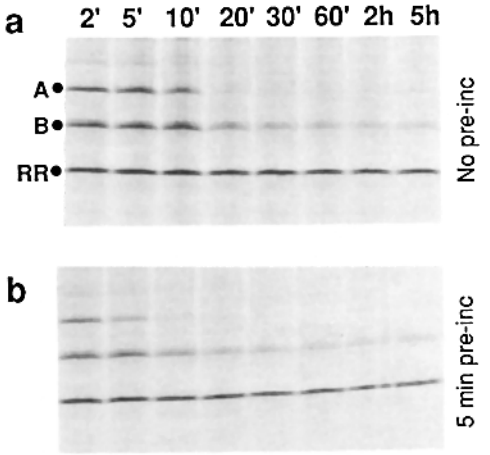

Figure 8. M phase lysate is dominant over interphase lysate. Embryos were labeled with [^{35}S]methionine during the first mitotic cell cycle and a concentrated lysate was prepared from early interphase cells. (*a*) One volume of unlabeled M-phase lysate was mixed with one volume of the ^{35}S-labeled interphase lysate, 100 μM emetine was added to prevent the early interphase from proceeding independently to the cyclin destruction point (see text) and, after the start of the incubation (t = 0 min), samples were taken at the indicated times. (*b*) 1 vol of the unlabeled M phase lysate was preincubated alone at 18°C for 5 minutes. 1 vol of the ^{35}S-labeled early interphase lysate and 100 μM emetine was then added. Time zero indicates the time at which the early interphase and M phase lysates were mixed together. Samples were taken at the indicated time and analyzed by gel electrophoresis followed by autoradiography. The positions of cyclin A, cyclin B, and ribonucleotide reductase (*RR*) are indicated.

60 min (not shown). Zinc inhibition was blocked when 5 mM EGTA was added at the same time (Fig. 10 *a*) and was reversed when EGTA was added later (Fig. 10 *b*). The kinetics of cyclin destruction after EGTA "rescue" were delayed only slightly relative to the control.

Calcium and Cyclin Destruction

The addition of 5 mM EGTA, an effective calcium chelator (Caldwell, 1970), had no effect on either the timing or extent of cyclin destruction in vitro, suggesting that calcium was not involved in cyclin destruction. However, in view of numerous reports of transient rises of intracellular free calcium around the time of nuclear envelope breakdown and the metaphase-anaphase transition (e.g., Poenie et al., 1985, 1986; Twigg et al., 1988) and the report that microinjection of the calcium-activated protease calpain advances the onset of both M phase and anaphase in tissue culture cells (Schollmeyer, 1988), we assayed the effect of added calcium in vitro.

Addition of up to 1 mM $CaCl_2$ had no effect on the onset, duration, or extent of cyclin destruction in vitro, although it did block further protein synthesis (Fig. 11 *a*). We point out, however, that the absence of a response to 1 mM $CaCl_2$ could be due to the presence of effective endogenous calcium chelating systems in the concentrated lysates (see review by Borle, 1981). In contrast, the addition of 5 mM $CaCl_2$ resulted in a very rapid degradation of the cyclins (Fig. 11 *b*): by the first time point taken (2 min), almost all of cyclin A and much of cyclin B were gone. This was quickly followed by the loss of several other prominently labeled proteins, including ribonucleotide reductase. Most other radiolabeled proteins were completely resistant to this calcium-activated protease activity, as were the bulk of the proteins seen on Coomassie brilliant blue-stained gels. Thus, while calcium did not trigger widespread, nonspecific proteolysis, more than just the cyclins were destroyed. One obvious possibility is that this represents the combined effects of a calcium-activated cyclin protease and a second, calcium-activated protease that recognizes a different, restricted set of substrates.

To ask if cyclin is indeed destroyed by a calcium-activated cyclin protease (or marked for proteolysis by a calcium-activated event), we added calcium to lysates in which cyclin destruction had been blocked by various inhibitors. When cyclin destruction was blocked by adding 6-DMAP or depleting ATP, subsequent addition of 5 mM $CaCl_2$ overcame

Table II. Effect of Various Treatments on Cyclin Destruction In Vitro

Inhibitors of cyclin destruction in vitro		References
ATP depletion	–	
6-DMAP	600 μM	1, 2, 3
EDTA	5 mM	
$CuCl_2$	1 mM	
$ZnCl_2$	1 mM	
Calpain inhibitor I	100 μg/ml	4
pHMB	5 mM	5, 6
NEM	5 mM	5, 6
TLCK	300 μg/ml	7, 8, 9
No effect on cyclin destruction in vitro		**References**
ATP	500 μM	
ATP regen system	–	
ATP-γ-S	10-500 μM	
EGTA	10 mM	
$CaCl_2$	up to 1 mM	
A23187	10 μM	
LiCl	1 mM	
NaF	500 μM	
α-2-macroglobulin	100 μg/ml	10
Aprotinin	200 μg/ml	11
Calpain inhibitor II	100 μg/ml	4, 5
Chymostatin	100 μg/ml	12
E64	100 μg/ml	13, 14
E64d	500 μg/ml	15
Leupeptin	100 μg/ml	12
Hemin	200 μM	16, 17
PMSF	5 mM	18
APMSF	100 μg/ml	19
SBTI	250 μg/ml	20, 21
β-mercaptoethanol	1 mM	
DTT	5 mM	
Iodoacetamide	5 mM	
Colcemid	300 μM	
Emetine	100 μM	

The concentrations listed for "inhibitors of cyclin destruction" were the minimum ones needed inhibit cyclin destruction in M phase lysates. The concentrations listed for reagents having no effect on cyclin destruction were the highest ones tested. References: (1) Rebhun et al., 1973; (2) Neant and Guerrier, 1988; (3) Neant et al., 1989; (4) Crawford et al., 1988; (5) Shaw and Green, 1981; (6) Bond and Butler, 1987; (7) Shaw et al., 1965; (8) Penn et al. 1976; (9) Kinzel and Konig, 1980; (10) Barret, 1981; (11) Kassel, 1970*a*; (12) Umezawa, 1976; (13) Hanada et al., 1978; (14) Sugita et al., 1980; (15) Shoji-Kasai et al. 1988; (16) Etlinger and Goldberg, 1980; (17) Haas and Rose, 1981; (18) James, 1978; (19) Laura et al., 1980; (20) Kassel, 1970*b*; (21) Picard et al., 1985.

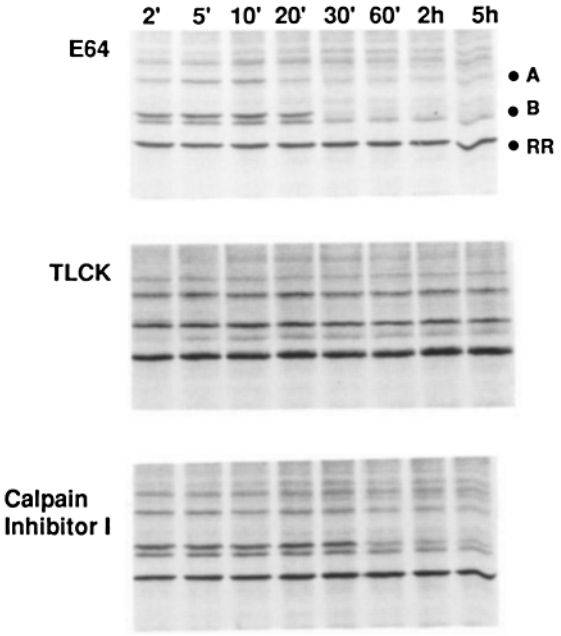

Figure 9. Effect of protease inhibitors on cyclin destruction in vitro. Embryos were labeled with [^{35}S]methionine during the first mitotic cell cycle and a lysate was prepared from early M phase cells. Aliquots of this lysate were mixed with 100 μM E64d, 300 μg/ml TLCK, or 100 μg/ml calpain inhibitor I, as described in the text, and the fate of the labeled cyclins were analysed by gel electrophoresis followed by autoradiography. The positions of cyclin A, cyclin B, and ribonucleotide reductase are indicated.

the inhibition and led to proteolysis of cyclins and the other subset of proteins (not shown). Because this calcium-activated protease activity was not ATP-dependent, it seems unlikely to be the same entity as cyclin protease. (It is, however, possible that high calcium levels override the requirement for an ATP-dependent step). In contrast, calcium-activated protease activities towards both cyclins and non-cyclin proteins were inhibited by zinc (Fig. 11 *c*). Thus, the evidence on the involvement of calcium is ambiguous.

Clam Cyclin B Made in Reticulocyte Lysates Is Destroyed in the Clam Cell-free System, But Cyclin A Is Not

When SP6 cyclin B translation products made in reticulocyte lysate were added to a clam M phase lysate, most cyclin B was destroyed on schedule. Sea urchin B-type cyclin reticulocyte translation product was similarly destroyed in a cell-free system from frog eggs (Murray and Kirschner, 1989). In contrast, SP6 cyclin A translation products remained stable for >5 h (Fig. 12). Various control experiments showed that the presence of reticulocyte lysate itself, either dilute or concentrated, had no effect on the onset, duration, or extent of destruction of endogenous or exogenous cyclin A or B (not shown). To test the idea that cyclin A destruction might require cyclin B, or some other newly synthesized protein, we asked about the behavior of the cyclin A present in the translation products programmed by total two-cell RNA. Out of all the abundant proteins encoded by two-cell RNA, only cyclin B was selectively destroyed (Fig. 12). Improper folding or posttranslational modifications of cyclin A produced in

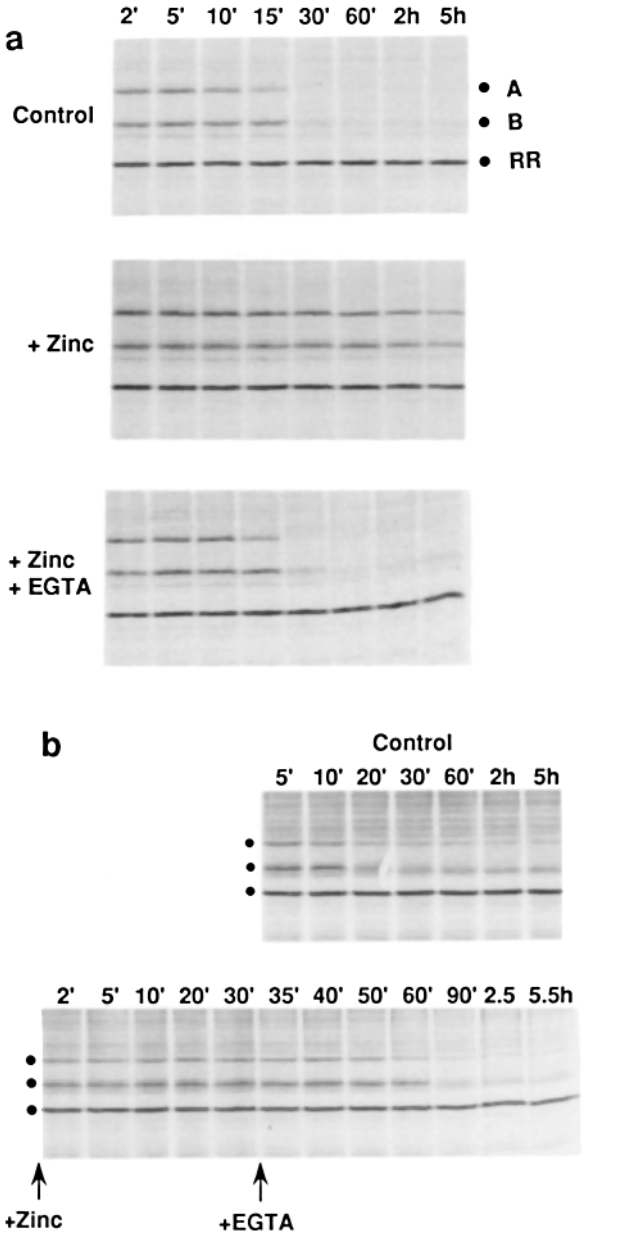

Figure 10. 1 mM $ZnCl_2$ inhibits cyclin destruction in vitro; this inhibition can be rescued by the subsequent addition of EGTA. Embryos were labeled with [^{35}S]methionine during the first mitotic cell cycle and a lysate was prepared from early M phase cells. The protein synthesis inhibitor emetine was added to prevent new cyclin synthesis from masking the disappearance of prelabeled cyclins. The fates of the radiolabeled cyclins in the lysate were monitored under the following conditions: (*a*) Control, lysate alone; + Zinc, lysate plus 1 mM $ZnCl_2$ added at 0 min; + Zinc + EGTA, lysate plus 1 mM $ZnCl_2$ and 5 mM EGTA, both added at 0 min. (*b*) Control, lysate alone; + Zinc + EGTA, lysate plus 1 mM $ZnCl_2$ (added at 0 min), plus 5 mM EGTA (added at 31 min). The positions of cyclin A, cyclin B, and ribonucleotide reductase are indicated by dots on the left of *a* and *b*.

reticulocyte lysates could explain its resistance to proteolysis but, at this point, we cannot say how cyclin A made in reticulocyte lysate differs from that made in vivo or in clam lysates in vitro. Nevertheless, these results add to the rapidly growing body of evidence that the two cyclin types differ in several fundamental ways.

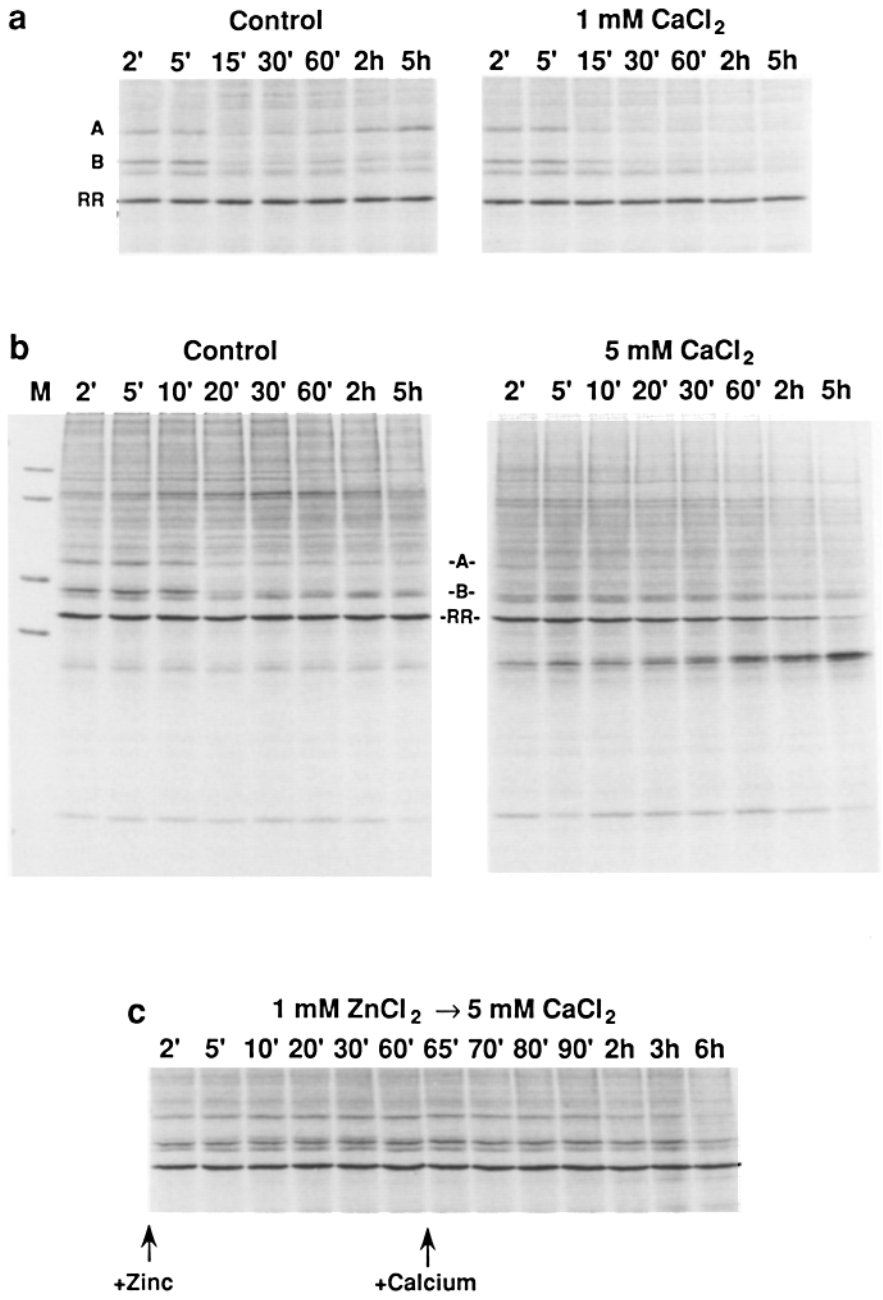

Figure 11. Effects of calcium on cyclin destruction in vitro. Embryos were labeled with [^{35}S]methionine during the first mitotic cell cycle and a lysate was prepared from early M phase cells. The fates of the cyclins during the incubations in vitro were monitored as follows. (*a*) Under standard conditions (control) and in the presence of an additional 1 mM $CaCl_2$ (added at 0 min); (*b*) under standard conditions (Control) and in the presence of an additional 5 mM $CaCl_2$ (added at 0 min); (*c*) 1 mM $ZnCl_2$ (added at 0 min) followed by 5 mM $CaCl_2$ (added at 60 min). The positions of cyclin A, cyclin B, and ribonucleotide reductase are indicated on *a* and *b*. Lane *M* of the control of *b* contains the molecular weight markers; these are, from top to bottom: 116, 94, 56, and 40 kD.

Discussion

The Cell-free System

We have developed a cell-free system from early *Spisula* embryos that reproduces several aspects of the programmed cyclin destruction and reappearance that occurs during each cell cycle in vivo. Cyclins in interphase or M phase lysates were stable for relatively appropriate lengths of time before undergoing destruction. Cyclin proteolysis did not require ongoing protein synthesis, and proceeded on schedule even in lysates that had been frozen and thawed several times. Once past the destruction point, cyclins accumulated again. Their reappearance was dependent on new protein synthesis, as expected, but the extent of protein synthetic activity varied among different lysates and was depressed by freezing and thawing. Just as in intact cells, the in vitro destruction of cyclin A began first and was followed soon thereafter by the destruction of cyclin B. Once initiated, cyclin A destruction proceeded rapidly and efficiently. In almost all cases, cyclin A was completely destroyed within a period of 5 min. The behavior of cyclin B was more variable. Its destruction often took longer and the extent of proteolysis was more variable among different preparations. No proteolytic intermediates were seen for cyclin A; smaller (45–50 kD) breakdown products of cyclin B were sometimes apparent in immune precipitates but, for technical reasons, it was hard to judge whether these arose from specific or nonspecific proteolytic activity.

Setting the Timetable for Cyclin Destruction

In both intact embryonic cells and in the cell-free system, cyclin levels are controlled by alternating between long periods

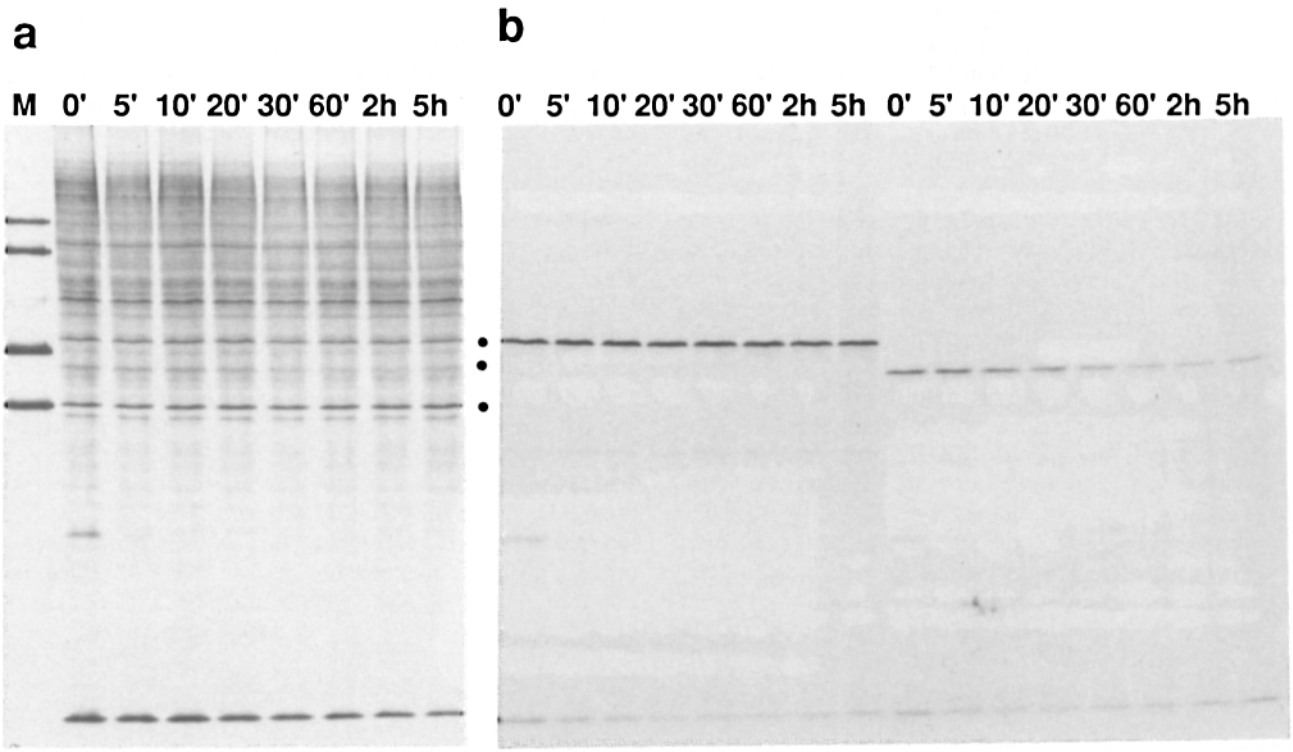

Figure 12. In vitro translated cyclin B but not cyclin A can be used as substrate for the in vitro cyclin destruction assay. (*a*) Total two-cell embryo RNA was translated in a reticulocyte cell-free system in the presence of [^{35}S]methionine and the labeled products were added to an unlabeled, standard M phase lysate. (This lysate was known, from previous experiments, to carry out programmed destruction of exogenous substrate cyclins within 10–15 min.) Emetine was added at 0 min to block further protein synthesis. Samples were taken at the indicated times and analyzed by gel electrophoresis followed by autoradiography. (*b*) SP6-cyclin A or SP6-cyclin B mRNAs were translated in a reticulocyte cell-free system in the presence of [^{35}S]methionine. The labeled translation mixes were added to aliquots of the same unlabeled M-phase lysate used in part *a*. Samples were taken at the indicated times and analyzed as above. Lane M of *a* contains the molecular weight markers; these are, from top to bottom: 116, 94, 56, and 40 kD. Dots, from top to bottom: cyclin A, cyclin B, and RR.

of stability, during which the cyclins accumulate steadily, and brief bursts of proteolysis, during which the cyclins disappear over a period of just a few minutes. How is the oscillation between these two states controlled and what regulates the timing of the switches? Several lines of evidence show that (*a*) cells must build up a certain level of cyclin to proceed at a later time into M phase (*b*) entry into M phase is a prerequisite for proceeding to the cyclin destruction point near the end of M phase.

First, introducing cyclin into G2-arrested frog oocytes drives them into M phase in a dose-dependent manner (Swenson et al., 1986; Pines and Hunt, 1987; Westendorf et al., 1989) and translation of endogenous frog oocyte cyclin mRNAs is essential for entry into mitosis (Minshull et al., 1989). Using an mRNA-dependent cell-free system from frog eggs, Murray and Kirschner (1989) have shown that cyclin is the only newly synthesized protein needed to drive nuclei into M phase. In apparent contrast with this idea, Lehner and O'Farrell (1989) found that mutant *Drosophila* embryos with lower cyclin A levels progressed through the later cell cycles with wild-type kinetics and concluded that cyclin is not the rate limiting component. It is important to point out, however, they did not consider contributions by cyclin B, also present in *Drosophila* embryos (Whitfield et al., 1989) and that cyclin B can substitute for cyclin A as an inducer of M phase, at least under some circumstances (Westendorf et al., 1989).

Second, if protein synthesis is blocked before a certain point in early interphase, clam embryos fail to enter mitosis and fail to degrade the cyclins. If protein synthesis is blocked after this point, the "commitment point," cells will proceed through mitosis and degrade the cyclins on schedule (Hunt, T., and J. Ruderman, unpublished, reviewed in Swenson et al., 1989). This protein synthesis requirement is reproduced in the cell-free system described here. Lysates made from cells taken before the commitment point required further protein synthesis to proceed to the cyclin destruction point, whereas lysates made after the commitment point went on to destroy the cyclins. Furthermore, lysates from emetine-arrested interphase cells never went on to develop cyclin destruction activity. Perhaps the most convincing evidence that entry into M phase is required for the subsequent destruction of the cyclins comes from the recent work of Murray and Kirschner (1989), who found that low amounts of cyclin both failed to drive nuclei into M phase and failed to be destroyed.

Concentrated lysates destroyed cyclins on schedule. Successive dilutions led to successive delays in reaching the cyclin destruction point, as would be expected in a simple bimolecular reaction involving a substrate and its protease. At high dilutions, cyclins were stable for many hours. This feature provided the opportunity to use the radiolabeled cyclins present in these very dilute lysates as substrates in other reactions. When substrate cyclins from one stage of the cell cycle were mixed with concentrated lysates made at a different

stage of the cell cycle, the timing of the destruction of the radiolabeled substrate cyclins was always determined by the cell cycle stage of the concentrated "host" lysate, not by the cell cycle stage of the substrate cyclins. These results further show that even if posttranslational modifications such as phosphorylation (see below) are used to tag cyclins for subsequent destruction, this tagging is either readily reversible or is, by itself, insufficient to trigger cyclin destruction.

When equal volumes of two different concentrated lysates, one from early interphase and the other from M phase, were mixed together, cyclin destruction proceeded with M phase kinetics, rather than with kinetics intermediate between early interphase and M phase. The outcome of this simple experiment reveals two new pieces of information. First, interphase lysates do not contain a dominant inhibitor of cyclin protease. Second, even though cyclin levels control the timing of entry into M phase, and entry into M phase is required for subsequent cyclin destruction, cyclin levels themselves do not determine when destruction occurs. If cells did initiate cyclin destruction upon reaching a critical cyclin concentration, then the mixed lysate with intermediate cyclin levels would have required further cyclin protein synthesis to reach the cyclin destruction point. They do not. Thus we can conclude that, once in M phase, the timing of cyclin destruction is set by something other than accumulation of cyclin.

A particularly intriguing question concerns the sequential proteolysis of the two different cyclins. Why is cyclin A destroyed first, then cyclin B? One obvious possibility is that there are two different, sequentially activated proteases. Every one of the protease inhibitors and other agents tested affected the destruction of cyclin A and cyclin B equally. This indicates that, if there are two different cyclin proteases, they are remarkably similar and probably arose from a common ancestor. The converse explanation is, of course, that both cyclins are degraded by a single protease, and that the timing of degradation is controlled at the level of substrate accessibility. At this point, we cannot distinguish between these two alternatives.

Calcium and Cyclin Destruction In Vitro

Sparked by Weisenberg's (1972) observation that calcium can depolymerize microtubules, Kiehart's (1981) demonstration that microinjection calcium can dissociate mitotic spindles and numerous indications of intracellular calcium bursts at key morphological transitions during the cell cycle, including nuclear envelope breakdown, entry into mitosis, and anaphase onset (e.g. Keith et al., 1985; Hepler, 1985; Poenie et al., 1985; 1986; Steinhardt and Alderton, 1988; Twigg et al., 1988), there has been persistent enthusiasm for the idea that these calcium bursts actually drive transitions from one stage of the cell cycle to the next. Calcium sequestering vesicles associated with the mitotic spindle (Silver et al., 1980) could supply the calcium for triggering anaphase, although it should be pointed out that there are several conflicting observations on the temporal order of the calcium burst and anaphase onset. Schollmeyer (1988) has reported that injections of calpain, a calcium-activated cysteine protease, accelerates passage into and through mitosis, but certain technical aspects of those experiments preclude a firm conclusion on this point (see Lee, 1989, for discussion).

We found that calpain inhibitor 1, an inhibitor of certain calcium-activated cysteine proteases, slowed cyclin destruction but did not completely block it. We were not able to assay calpain itself. As a more direct test of the idea that calcium is a physiological trigger of cyclin destruction, we looked at the effect of depleting calcium or adding extra calcium to the cell-free system. High concentrations of EGTA had no effect on timing of the onset, duration or extent of cyclin proteolysis, and adding up to 1 mM $CaCl_2$ failed to trigger or accelerate cyclin destruction. However, the addition of even higher levels of $CaCl_2$ (5 mM) resulted in the breakdown of a small set of proteins which included the cyclins and ribonucleotide reductase. Calcium-activated cyclin breakdown was rapid and complete; loss of reductase and a few other proteins proceeded more slowly (see Fig. 11 *b*). Although the consequence of calcium addition is obvious and dramatic, the interpretation is not. Various approaches failed to indicate whether this calcium-activated proteolysis was artifactual or represented the combined effect of two different calcium-activated pathways, one for cyclins and one for second restricted set of substrates. More information, especially on the rate of calcium chelation by EGTA and the effectiveness of calcium sequestering systems in these very concentrated lysates, will be needed to sort this out.

The Cyclin Destruction Pathway

Is periodic cyclin destruction controlled by periodic activation of cyclin proteases or by period changes in the accessibility of the substrate cyclins themselves? Soon after we began this work, it became obvious that it would be difficult to answer this question without knowing something about cyclin protease. Hoping to learn which class of protease is involved, we tested a wide variety of protease inhibitors in the cell free system. Only one of these, TLCK, completely blocked cyclin destruction. TLCK, a chloromethyl ketone derivative of lysine, irreversibly inhibits trypsin and trypsin-like serine proteases by alkylating the histidine residue in the active site (Shaw et al., 1965) TLCK is not, however, a perfect diagnostic tool as it can also alkylate thiol groups and block the activity of certain cysteine proteases (e.g. Chou et al., 1974; Arnon, 1970) and some kinases are inactivated by TLCK alkylation of their sulfhydryl groups (Kinzel and Konig, 1980). TLCK thus could be blocking one or more of several different possible steps in the cyclin destruction pathway, such as phosphorylation or conformation changes in the cyclins themselves, $p34^{cdc\ 2/28}$ protein kinase, and other cyclin-associated proteins, and cyclin protease itself, as well as directly inhibiting the active site of cyclin protease.

Penn et al. (1976) found that adding a low concentration of TLCK to cultures of sea urchin embryos delayed the cell cycle in an interesting way: cells passed through interphase and entered M phase on schedule, but anaphase onset was delayed considerably. We argued that TLCK was acting as a specific inhibitor of a protease involved in the maintenance of cell cycle times rather than a general inhibitor of macromolecular synthesis. A closer inspection of the delay in clam embryos shows that TLCK arrests cells in late metaphase (Luca, F., and J. Ruderman, unpublished). Taken with the demonstration in this paper that TLCK inhibits cyclin destruction, we make two conclusions. First, maintaining high cyclin levels keeps cells in M phase. Second, cyclin destruction, which temporally precedes anaphase onset, is probably a requirement for it.

Like TLCK, the ATP requirement for cyclin destruction

could be involved at one or more possible points in the cyclin destruction pathway. Obvious candidates include substrate or protease phosphorylation, ATP-dependent ubiquitin conjugation and ATP-dependent proteolysis of ubiquitin conjugates. 6-DMAP, a puromycin analogue that has no effect on protein synthesis but is an effective kinase inhibitor; (Rebhun et al., 1973; Neant and Guerrier, 1988; Neant et al., 1989; Rime et al., 1989), completely blocks cyclin destruction in vitro. (Curiously, Neant et al. [1989], have reported that addition of 6-DMAP to sea water blocked embryonic cells in interphase but, in contrast to our findings, did not interfere with a protein thought to be cyclin. We have no explanation for this discrepancy. Although cyclin phosphorylation (Swenson et al., 1989; Westendorf et al., 1989) may be involved in tagging the cyclins for proteolysis, it is unlikely by itself to be the trigger for cyclin destruction, because phosphorylated cyclins appear long before they are destroyed (cited in Minshull et al., 1988). It is, of course, possible that destruction is initiated when a threshold level of phosphocyclin is reached.

While phosphorylation cycles and the role of phosphorylation in the cell cycle have received considerable attention over the past several years, early reports of thiol cycles have not been followed up at the molecular level (e.g. Rapkine, 1931; Rapkine et al., 1931; Sakai and Dan, 1959; Mazia et al., 1960; 1981). Among the nine treatments found to block cyclin destruction in the clam cell free system, six have the potential to act by interfering with sulfhydryl-mediated functions. Four of these (NEM, pHMB, $ZnCl_2$, $CuCl_2$) have direct effects on sulfhydryl groups. It is particularly interesting to consider that 1 mM $ZnCl_2$ has profound effects on several cyclin-associated activities: it changes the substrate specificity of cdc28 protein kinase (Reed et al., 1985) and cyclin-associated kinases in vitro (Swenson et al., 1989; Westendorf et al., 1989), it changes the activity of MPF-related histone H1 kinase activity (Pelech et al., 1987) and it stabilizes cyclins in the cell-free system (this paper). The other two inhibitors of cyclin destruction in vitro (TLCK, calpain inhibitor I) could involve effects on sulfhydryl groups, as pointed out earlier.

There is now considerable evidence that the rise in cyclin levels leads to the generation of active MPF, either by providing an essential subunit to cdc2/28 protein kinase or catalytic activator of this kinase, and that the scheduled destruction of cyclin near the end of each M phase leads to the loss of MPF activity (Draetta et al., 1989; Westendorf et al., 1989; Murray and Kirschner, 1989). We strongly suspect that cyclins present in concentrated extracts of other cell types will be seen to undergo the same programmed destruction reported here for concentrated clam embryos lysates. Such behavior would readily explain the notorious instability of MPF activity in vitro.

We thank the staff of the Marine Biological Laboratory, Woods Hole, MA, where many of these experiments were carried out; Joanne Westendorf, Ellen Shibuya, Robert Palazzo, Peter Helper, David Adelson and Alfred Goldberg for helpful discussions; Ellen LeMosy for cyclin A antibodies; Kazutomo Imahori for a generous gift of E64d; and Marc Kirschner and David Beach for communicating their results before publication.

F. C. Luca was supported by National Institutes of Health (NIH) predoctoral training grant GM-07754 to Duke University. This work was supported by NIH grant HD-23696 to J.V. Ruderman.

Received for publication 25 May 1989 and in revised form 12 July 1989.

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