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Cyclin is degraded by the ubiquitin pathway

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Cyclin degradation is the key step governing exit from mitosis and progress into the next cell cycle. When a region in the N terminus of cyclin is fused to a foreign protein, it produces a hybrid protein susceptible to proteolysis at mitosis. During the course of degradation, both cyclin and the hybrid form conjugates with ubiquitin. The kinetic properties of the conjugates indicate that cyclin is degraded by ubiquitin-dependent proteolysis. Thus anaphase may be triggered by the recognition of cyclin by the ubiquitin-conjugating system.

PROTEOLYSIS plays a critical part in mitosis of the eukaryotic cell cycle. Mitosis is induced by the activation of M-phase promoting factor (MPF), a protein kinase whose principal subunits are $p34^{cdc2}$, which has extensive homology to known protein kinases, and cyclin, a regulatory subunit whose abundance fluctuates throughout the cell cycle[1]. The transition from metaphase to anaphase which marks the end of mitosis is induced by the degradation of cyclin, which in turn leads to the inactivation of MPF. As cells enter interphase, cyclin degradation ceases, cyclin accumulates and, as a result of a complex series of post-translational reactions, MPF is activated[2] and another round of mitosis ensues. Cyclin degradation is thus the crucial event in exiting metaphase and entering the next interphase. As expected, cyclin mutants that retain the ability to activate MPF but cannot be degraded arrest the cell cycle in mitosis[3]. The basic features of this scheme appear to be conserved in all eukaryotic cells, although several other regulators, such as the products of the *Schizosaccharomyces pombe* genes *cdc25* and *wee1*, may also play a part in regulating the activity of MPF (ref. 4). The regulation of cyclin degradation is poorly understood, but it is clear that cyclin proteolysis is triggered by the presence of active MPF (ref. 5). Once initiated, cyclin degradation is rapid and highly specific.

Protein levels are determined by the balance between the rates of protein synthesis and protein degradation[6]. The half-lives of intracellular proteins vary over at least three orders of magnitude from minutes to days[6], and a number of distinct pathways of intracellular proteolysis have been described[7]. One prominent degradation pathway is mediated by conjugation of ubiquitin to unstable proteins[8–10]. Ubiquitin is a highly conserved protein of relative molecular mass 7,000(M_r 7K) which can be covalently linked through isopeptide bonds to lysine residues on proteins; when proteins are multiubiquitinated at a single site (by isopeptide linkage to lysine 48 of a ubiquitin molecule that is already ligated to a protein degradation substrate) they become targets for degradation[11]. The proteins involved in ubiquitin conjugation have been extensively characterized by both biochemical and genetic analysis[8–10,12–14]. However, there is only one case where a specific protein of physiological interest has been shown to be degraded by the ubiquitin pathway[15].

We have used crude extracts prepared from *Xenopus* eggs to examine several aspects of the degradation of cyclin. We have generated a stable metaphase state where cyclin is constitutively degraded. Using this system we have shown that the N terminus of cyclin is not only necessary but also sufficient for M-phase-specific degradation. Mutational analysis has localized the region of the N terminus that is required for cell-cycle-regulated destruction of cyclin. Finally, we have shown that cyclin degradation at mitosis is accompanied by the formation of cyclin–ubiquitin conjugates. A mutation within cyclin that inhibits degradation also inhibits ubiquitin conjugation. The measured flux through the cyclin–ubiquitin intermediate suggests that cyclin is degraded by way of the ubiquitin pathway. These results suggest that MPF regulates the conjugation of ubiquitin to cyclin, leading to the rapid destruction of cyclin by the constitutively active, ubiquitin-dependent proteolytic system.

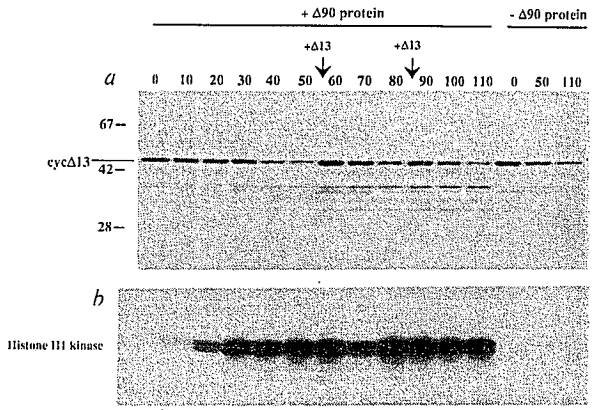

FIG. 1 Δ90 (stable mitotic) extracts constitutively degrade cyclin. Interphase extracts were prepared as described[2]. At 0 min, 1/10 volume of translation mix containing ^{35}S-labelled cycΔ13 was added, 1/20 volume of Δ90 protein was added to a portion of the extract, and the extracts were incubated at 22 °C. More cycΔ13 was added to the extracts containing Δ90 protein at 0, 59 and 89 min. At the indicated times samples were removed for analysis of ^{35}S-protein by SDS-PAGE and autoradiography, and samples were also analysed for histone H1 kinase activity as described[2]. It should be noted that cyclin destruction in mitotic extracts was biphasic; 80% of the cyclin was rapidly degraded but 20% was not. This is not seen *in vivo* and appears to be a consequence of a fraction of inactive cyclin molecules translated in the reticulocyte lysate.

METHODS. An expression vector for cycΔ90, pT7Δ90, was created by cutting cycΔ13 with *Bgl*II followed by partial digestion with *Bam*H1. The resulting 1.1-kb fragment was ligated into the T7 translation vector pET3b (ref. 38) linearized at the unique *Bam*H1 site. One litre of LB containing *E. coli* strain BL21(DE3)pLysS, pT7Δ90 was grown to 37 °C to OD_{600} 0.7. Δ90 protein was induced for 1 h with 0.08 mg ml^{-1} IPTG. The cells were pelleted, washed with 0.9% NaCl, pelleted and resuspended in 25 ml buffer A (10 mM Tris, 50 mM NaCl, 1 mM EDTA, pH 8.0) also containing 5 mM DTT, 0.05% Nonidet P-40, and 10 μg ml^{-1} chymostatin, pepstatin and leupeptin. The extract was sonicated for 2 min on ice and spun in a Sorvall SS-34 rotor at 12,000 r.p.m. for 15 min at 4 °C. The pellet was washed in buffer A containing 0.5 M NaCl (final), and resuspended in 20 ml buffer A containing 8 M urea and 5 mM DTT. Buffer B (20 ml; 50 mM Tris, 100 mM KCl, 5 mM $MgCl_2$, 5 mM DTT) was slowly added and the solution was spun 5 min 12,000 r.p.m. in a Sorvall HB-4 rotor at 4 °C. The supernatant was dialysed against buffer B. The resulting preparation contained ~0.5 mg ml^{-1} cycΔ90 protein, which was about 80% pure. ^{35}S-labelled cycΔ13 was produced by translation in a reticulocyte lysate system (Promega) of RNA transcribed by T7 RNA polymerase from FpΔ13TF1(–), a derivative of cycΔ13 (ref. 3), pGEM2 (Promega), pSP64T (ref. 39), and pUC-f1 (Pharmacia). It consists of the coding sequence of cycΔ13 flanked by the 5′ and 3′ untranslated regions of β globin originally derived from pSP64T (the 5′-untranslated region of β globin had been mutated to create a consensus ribosome binding site for *E. coli* translation) and inserted into the polylinker of pGEM2 that had the f1-ori inserted at the unique *Sph*1 site.

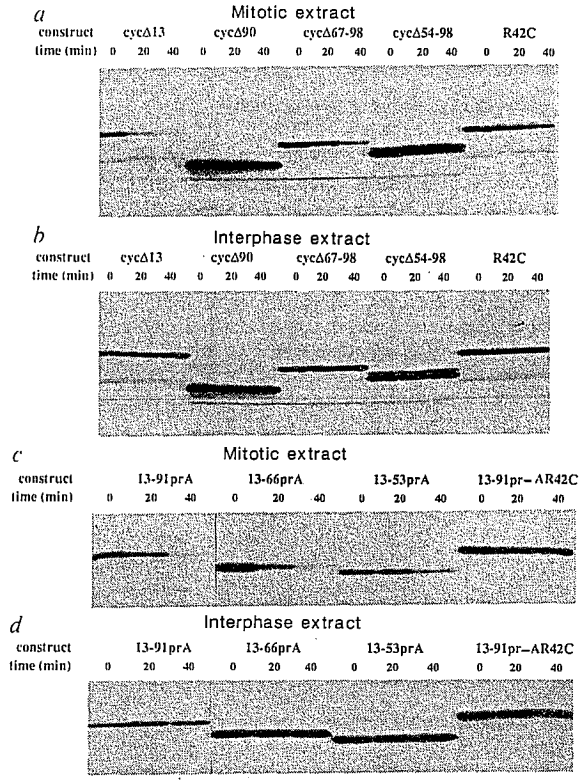

FIG. 2 Mutational analysis of the determinant required for cell-cycle-regulated proteolysis and alignment of the destruction box of mitotic cyclins. Radiolabelled cyclin derivatives, were added to mitotic (*a*, *c*) or interphase (*b*, *d*) extracts, and at the indicated times samples were removed, analysed by SDS-PAGE and subjected to autoradiography. *e*, Sequence of the first 100 amino acids of sea urchin (*Arbacia punctulata*) cyclin[40]. *f*, Other cyclins aligned to the *Arbacia* cyclin relative to the motif RxxLxyzxN (refs 17, 19, 40–48, S. Mackie and T. Hunt, personal communication). *g*, Summary of the results obtained with several constructs. Hatched box, destruction box; K, downstream lysine-rich region; c, cysteine replacement of arginine at position 42; filled box, protein A sequence.

METHODS. Mitotic extracts were generated by addition of 1/20 volume of Δ90 protein to fresh interphase extracts followed by incubation for 1 h at 22 °C. After 1 h sucrose was added to a final concentration of 150 mM and the extracts were frozen in small aliquots in liquid nitrogen. Frozen extracts were used in all experiments in this paper except those for Fig. 3. The ^{35}S-labelled derivatives containing the C-terminal half of cyclin (constructions outlined below) were transcribed *in vitro* and translated in reticulocyte extracts, and 1/5 volume was added to extracts. The ^{125}I-labelled derivatives, which are protein A fusions, were expressed in BL21(DE3)pLysS, purified by affinity chromatography with IgG–Sepharose 6 FF (Pharmacia) according to the manufacturer's instructions, and radiolabelled by iodination with 2 mM chloramine T (Eastman Kodak) with 0.5 mCi Na^{125}I (NEN) diluted to a specific activity of 10 mCi ml^{-1}. After 2 min the labelling reaction was stopped by addition of DTT to 0.1 M, carrier BSA was added to 0.2 mg ml^{-1} and the unincorporated radioactivity was removed by a G-25 Sephadex spin column equilibrated with 10 mM potassium phosphate, 2 mM DTT, pH 7.8. The only tyrosine residues that could be modified by the iodination reaction are on the protein A moiety. The iodinated proteins, labelled to a specific activity of 10^6 c.p.m. per μg, were added to extracts at a final concentration of 5,000 c.p.m. per μl. The constructs were generated as follows. pΔ13R42C was produced by oligonucleotide-directed mutagenesis[49] from FpΔ13TF1(−). pΔ54–98 was derived from FpΔ13TF1(−) by digesting with *Bst*B1, filling in with T4 DNA polymerase, digesting with *Hind*II and religating.

e

Sea urchin *Arbacia punctulata* cyclin B (1–100)

```
1         10        20        30        40        50
MALGTRNMNMNLHGESKHTFNNENVSARLGGKSIAVQKPAQRAALGNISN

60        70        80        90        100
VVRTAQAGSKKVVKKDTRQKAMTKTKATSSLHAVVGLPVEDLPTEMRSTS
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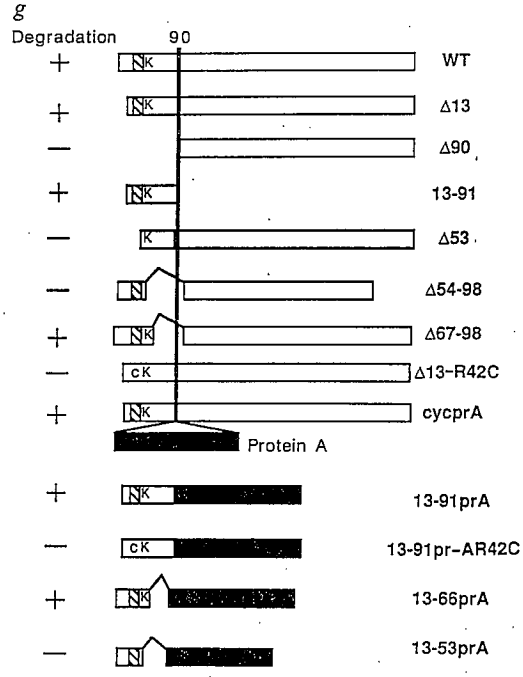
f

	42	43	44	45	46	47	48	49	50
Arbacia B	R	A	A	L	G	N	I	S	N
Strong. Purp B	R	A	A	L	G	N	I	S	M
Frog B2	R	A	A	L	G	E	I	G	N
Frog B1	R	T	A	L	G	D	I	G	N
Human B	R	T	A	L	G	D	I	G	N
Clam B	R	N	T	L	G	D	I	D	N
Cdc 13	R	H	A	L	D	D	V	S	N
Fly B	R	A	A	L	G	D	L	Q	N
Starfish B	R	G	A	L	E	N	I	S	N
Clam A	R	A	A	L	G	V	I	T	N
Fly A	R	S	I	L	G	V	I	Q	S
Frog A	R	T	V	L	G	V	I	G	D
Human A	R	A	A	L	A	V	L	K	S
Consensus	R		A	L	G		I		N
B-type						NDE			
A-type						V			

g



pΔ67–98 was generated by digesting pX13–91prAX (see below) with *Bss*HII, filling in with T4 DNA polymerase, digesting with *Bgl*I and ligating the fragment containing the N terminus of cyclin to FpΔ13TF1(−) digested with *Hind*II and *Bgl*I. *Construct pX13–91prAX is a derivative of FpΔ13TF1(−) in which a Sau3AI fragment containing the IgG-binding domain of protein A (see ref. 2 for details) was inserted at the unique Bgl*II site, and the C-terminus of cyclin was replaced with a stop codon introduced by PCR-directed mutagenesis. Construct pX13–66prAX was generated from pX13–91prAX by oligonucleotide-directed deletion with an oligonucleotide that replaced the sequences encoding amino acids 66–90 of cyclin and the first 29 amino acids of the protein A fragment with a *Bss*HII site; pX13–53prAX was generated by PCR-directed mutagenesis of pX13–66prAX; p13–91prA-R42CX was generated by inserting the *Nsi*1–*Bst*B1 fragment of pΔ13-R42C into the analogous sites in pX13–91prAX. The sequences of all constructs generated by means other than ligation of cohesive ends were verified by DNA sequencing using the Sequenase kit (USB).

Sequences necessary for cyclin degradation

The protein synthesis requirement of the early *Xenopus* cell cycles can be fulfilled by the translation of sea urchin cyclin B messenger RNA[16]. Translation of this mRNA drives both entry into and exit from mitosis. In cells and extracts that transit through the cell cycle, cyclin degradation is transient, making a detailed biochemical analysis of the mechanism and regulation of cyclin degradation difficult. However, a truncated cyclin protein, cycΔ90, that lacks the first 90 amino acids of cyclin, is not degraded at mitosis but is able to activate MPF (ref. 3). In the presence of this mutant a persistent metaphase state is established in which exogeneously added wild-type cyclins are degraded[3]. We have made use of the stable mitotic state induced by cycΔ90 to dissect the cyclin destruction pathway and characterize the domain of the cyclin molecule that confers cell-cycle-regulated proteolysis.

To prepare stable mitotically-arrested extracts we added bacterially expressed cycΔ90 to interphase *Xenopus* cell cycle extracts. Figure 1 shows the effect of adding cycΔ90 to these interphase extracts. Histone H1 kinase activity, a measure of MPF activity, peaked 30 minutes after addition of cycΔ90 and remained at high levels for the duration of the experiment. Cyclin proteolysis was first detectable between 40 and 50 minutes after the addition of cycΔ90. Cyclin added at later times was also degraded, showing that cyclin proteolysis activity is maintained at high levels for at least an hour. In the absence of added cycΔ90 protein, the extracts remained in interphase where H1 kinase activity was low and cyclin was stable. Thus interphase extracts activated by cycΔ90 generate a constitutively active cyclin proteolytic activity. We refer to these cycΔ90 extracts as mitotically arrested extracts.

We have previously shown that amino acids 13–90 of sea urchin cyclin B are essential for cell-cycle-regulated destruction[3]. We asked whether the N-terminal region of the molecule was sufficient to confer M-phase-specific degradation to another protein. We used the heterologous sea urchin cyclin protein for these studies for reasons of convenience; all discussion of specific sequences and positions refer to this cyclin. We constructed a hybrid protein, 13–91prA, which consists of amino acids 13–91 of sea urchin cyclin B fused to a region of *Staphylococcus aureus* protein A that binds IgG. This fusion protein was expressed in *Escherichia coli*, purified by affinity chromatography and radio-iodinated. The 13–91prA protein was efficiently degraded in mitotic extracts but was stable in interphase extracts (Fig. 2*c*, *d*), establishing that residues 13–91 of cyclin contain sequences that are sufficient for cell-cycle-regulated proteolysis.

When we compared the N-terminal 90 amino acids of 13 cyclins from eight species we found only 10–20% sequence conservation. However, two features stood out: first, there is a 9-amino-acid region, RAALGNISN (single-letter amino-acid code), the 'destruction box', which contains two invariant residues, four highly conserved residues, and one residue (position 47) that serves to differentiate A and B type cyclins as it is always asparagine, aspartic acid or glutamic acid in B-type cyclins, and always valine in A-type cyclins (see Fig. 2*f*). In addition, there are three positions where there is weak conservation of a two- or three-amino-acid motif, whose functional significance is unclear. Second, we noted that although residues 54–90 show virtually no conservation across all species analysed, there is a pronounced enrichment for lysine residues. The number of lysine residues in this region ranges from 1 to 13, with a median of six.

To test whether these structural elements have functional significance, the proteolysis of additional cyclin mutants was analysed. Plasmids encoding mutant cyclins were transcribed *in vitro* and translated in a reticulocyte lysate system and the translation products were added to mitotic or interphase extracts. Deleting residues 66–98 from cycΔ13, a derivative of cyclin that lacks the first 13 amino acids, produced a derivative that contained the destruction box, the isolated small motifs, and a portion of the lysine-rich region. This derivative was degraded in mitotic extracts and stable in interphase extracts (Fig. 2*a*, *b*). By contrast, a slightly larger deletion spanning residues 54–98 produced a derivative stable in both mitotic and interphase extracts (Fig. 2*a*, *b*, *g*). Similarly, using *E. coli* expressed proteins, we found that fusing residues 13–66 to protein A was sufficient to confer cell-cycle-regulated proteolysis on protein A, but residues 13–53 were insufficient (Fig. 2*c*, *d*). The results suggest that residues 54–66 contain a determinant required for cyclin proteolysis. Furthermore, deletion of residues 1–53 blocked degradation in mitotic extracts (data not shown), suggesting a role for the destruction box in cyclin proteolysis. To test this, we converted the invariant arginine at position 42 of cycΔ13 to a cysteine to create the derivative cycΔ13-R42C. Figure 2*a*, *c* shows that this mutant protein cannot be degraded, and that the same mutation in a cyclin–protein A fusion also blocks degradation. Figure 2*g* summarizes the behaviour of the mutant constructs.

To explore further the relationship between cyclin proteolysis and exit from mutosis we tested the ability of cycΔ13-R42C to drive extracts in and out of mitosis by adding cycΔ13-R42C mRNA to nuclease-treated cell-cycle extracts containing sperm nuclei. Accumulation of cycΔ13-R42C, as well as the control cyclin cycΔ13, activated histone H1 kinase (Fig. 3) and caused breakdown of the nuclear envelope and chromosome condensation. In the cycΔ13-containing extract, cyclin degradation was induced 40 min after the beginning of the reaction and H1 kinase activity fell shortly afterwards. By contrast, in the cycΔ13-R42C-containing extract histone H1 kinase and cyclin protein levels stayed high, and nuclei remained in mitosis, supporting the argument that cyclin destruction is essential for exit from mitosis.

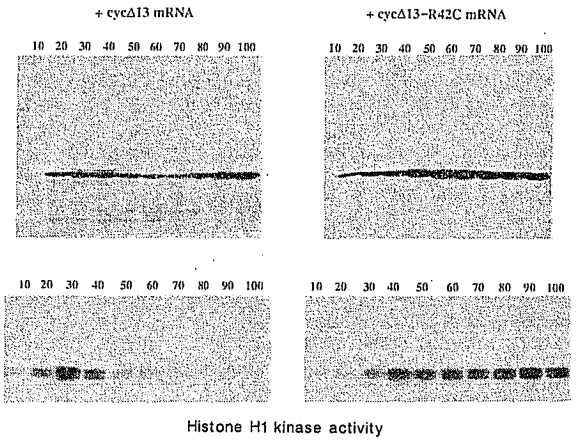


FIG. 3 CycΔ13-R42C causes metaphase arrest and a degradation intermediate is detectable during the degradation of cycΔ13. Activated cycling extracts, containing ^{35}S-methionine, were programmed with mRNA for cycΔ13 or cycΔ13-R42C and at the indicated times samples were removed for analysis of ^{35}S-labelled proteins and histone H1 kinase activity. METHODS. Extracts were prepared and nuclease-treated as described[16]. mRNA was generated by *in vitro* transcription of FpΔ13TF1(−) and pΔ13-R42C (see Fig. 2). ^{35}S-labelled proteins and histone H1 kinase activity were analysed as described in Fig. 1.

Cyclin is conjugated to ubiquitin

When we analysed long exposures of the gels of the labelled cycΔ13 protein during the experiment described above, we noticed that just before the onset of cyclin degradation a small amount of cyclin was apparently converted to a faint ladder of higher M_r forms, which disappeared with destruction of the unmodified cyclin (see Fig. 3). These forms were not observed with the non-degradable cycΔ13-R42C. Because the average difference in M_r between the bands forming this ladder was

~7K, the M_r of ubiquitin, we asked whether these bands were conjugates of cyclin and ubiquitin. We first sought a more sensitive means for visualizing the putative cyclin-ubiquitin conjugates. The cyclin-protein A fusion protein, 13-91prA, could be radio-iodinated to high specific activity and showed cell-cycle-specific degradation. As shown in Fig. 4*a*, when the labelled 13-91prA was added to mitotic extracts and samples removed at various times during degradation, a ladder of high M_r forms with a spacing of ~7K was visible on overexposed gels. These forms decreased in amount as the cyclin-protein A product was degraded. As the only labelled protein in the extract was the iodinated 13-19prA and lower M_r degradation products derived from it, the high M_r ladder must have derived from the cyclin-protein A fusion. To enhance our ability to follow these forms we purified and concentrated 13-19prA and its complexes using the protein A epitope tag and IgG-Sepharose; under these conditions the high M_r ladder is easily detected (Fig. 4*b*).

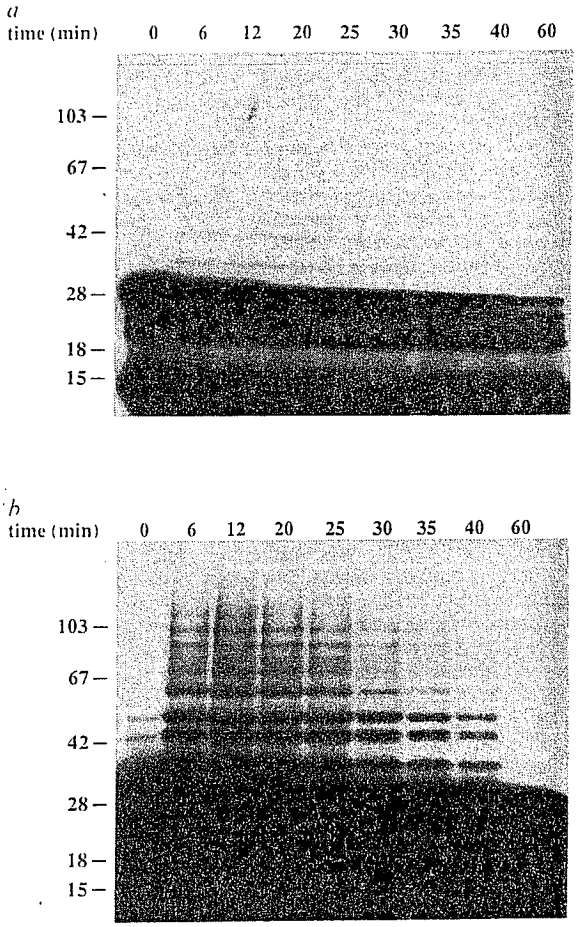


FIG. 4 Kinetic analysis of degradation of 13-91prA and of the abundance of high-M_r forms of 13-91prA. Labelled 13-91prA was added to mitotic extracts. At the indicated times samples were quenched with sample buffer (*a*) or precipitated with IgG-Sepharose (*b*), and analysed by SDS-PAGE and autoradiography.

METHODS. Extracts and proteins were prepared as described in Fig. 2. ^{125}I-labelled 13-91prA was added to mitotic extracts at 5,000 c.p.m. per ml. 0.5 ml samples were quenched with sample buffer and electrophoresed directly, or 10 ml samples were quenched by addition of 10 volumes RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing 10 mM *N*-ethylmaleimide. The solution was incubated for 10 min on ice before cysteine was added to 0.1%. IgG-Sepharose beads were added and incubated for 1 h at 4 °C on a rotating wheel. Beads were pelleted by centrifugaton and washed three times in RIPA buffer. The precipitated proteins were recovered by boiling for 2 min in sample buffer before analysis by SDS-PAGE and autoradiography.

To ascertain whether these high M_r forms were indeed ubiquitin adducts to cyclin, we added radio-iodinated ubiquitin to interphase and mitotic extracts in the presence or absence of 13-91prA. After a 10-min incubation the 13-91prA and any conjugated products were affinity-purified with IgG-Sepharose beads. An autoradiograph of the affinity-purified material is shown in Fig. 5. In mitotic extracts, a ladder of labelled bands was precipitated only when 13-91prA was present (compare lanes 3 and 4). These bands precisely coincided with the ladder of bands generated from ^{125}I-labelled 13-91prA in mitotic extracts (compare lanes 4 and 5). Precipitation of ^{125}I-labelled ubiquitin conjugated to 13-91prA and producton of slowly migrating forms of [^{125}I] 13-91prA were also detected in interphase extracts (lanes 2 and 6; see weak bands at 31 and 38K), but at very much reduced levels. These experiments show unambiguously that the high M_r products formed from 13-91prA in mitotic extracts contain ubiquitin and most likely represent multi-ubiquitinated 13-91prA, and that the conjugation of ubiquitin to cyclin derivatives is regulated during the cell cycle. When a similar experiment was performed with the protein A fusion protein containing the arginine to cysteine mutation, 13-91prA-R42C, some of the ubiquitinated forms (31 and 38K) appear specifically in mitons (lane 11) but none of the higher, multi-ubiquitinated forms were observed (Fig. 5, lanes 11-12). Thus a cyclin derivative that cannot be degraded cannot be multi-ubiquitinated, supporting the hypothesis that multi-ubiquitination of cyclin is required for its destruction.

Kinetics of cyclin ubiquitination

The ubiquitinated cyclin adducts could represent intermediates on the pathway of cyclin destruction in mitosis or non-productive side-products that accumulate in mitosis while cyclin is being degraded by a different pathway. As no specific inhibitors of ubiquitin conjugation or of the protease system that degrades ubiquitinated proteins are known, we could not demonstrate directly that the ubiquitin-cyclin conjugates are intermediates in cyclin degradation. Therefore, we attempted to demonstrate that at steady state the flux of cyclin through the ubiquitin-containing intermediates is equal to the overall rate of cyclin degradation.

To model the kinetics of cyclin degradation we first estimated the percentage of 13-91prA present as ubiquitin conjugates during the course of degradation in mitosis. From the time course in Fig. 4 we estimate that ~3% of the input protein is present as conjugates between 0 and 35 min. We determined the half-life of these conjugates by a 'pulse-chase' experiment. Radiolabelled 13-91prA was allowed to accumulate into ubiquitin-13-91prA conjugates in a mitotically arrested extract. After 10 min unlabelled 13-91prA was added at levels that saturated the cyclin destruction pathway and the time course of the loss of radiolabelled ubiquitinated conjugates was analysed by gel electrophoresis. Radioactivity in the entire collection of ubiquitin conjugates decayed to less than half the original amount within 120 s (Fig. 6*a*). An exponential curve was fitted to data representing the concentration of total labelled ubiquitin conjugates with time and from this the half-life of the conjugates was determined to be 90 s.

Assuming that the ubiquitination and subsequent destruction reactions follow first order kinetics, the concentration of ubiquitin intermediates, $I(t)$, is given by the equation

$$I(t) = k_1 A(t) - k_2 I(t)$$

where $A(t)$ represents the concentration of the unconjugated cyclin, and k_1 and k_2 are first-order rate constants. The value of k_2 was estimated from the pulse-chase experiment. Because the intermediates do not accumulate appreciably, we argue that the initial conjugation step must be rate limiting. Thus k_1 is

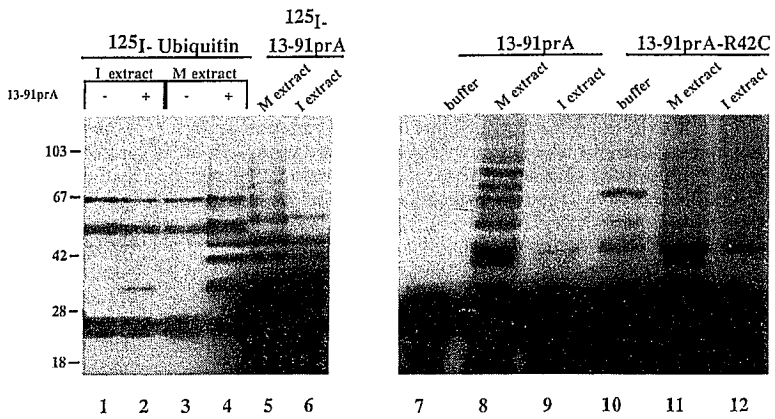


FIG. 5 The high-M_r forms of cyclin are ubiquitin conjugates. Lanes 1–4 contain IgG-binding proteins labelled with ^{125}I-labelled ubiquitin that are generated in interphase (lanes 1–2) or mitotic (lanes 3–4) extracts in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of unlabelled 13–91prA. Lanes 5–8 contain the IgG-binding proteins generated from ^{125}I-13–91prA (lanes 5–6) or ^{125}I-13–91prA-R42C (lanes 7–8) in mitotic (lanes 5 and 7) and interphase (lanes 6 and 8) extracts. METHODS. Ubiquitin was purchased from Sigma. Proteins were radiolabelled and extracts prepared as described in Fig. 2. Lanes 1–4, ubiquitin (105 c.p.m. per mg) was added to extracts at a concentration of 2×10^4 c.p.m. per ml, and incubated 10 min. To lanes 2 and 4, 3 μg of 13–91prA was added, incubated 10 min, and the reaction stopped as described in Fig. 4. In lanes 5–8, 5×10^4 c.p.m. of labelled 13–91prA or 13–91prA-R42C (both at 10^6 c.p.m. per mg) was added to 10 ml of extracts and incubated for 10 min then the reaction was quenched. Proteins that bind to IgG–Sepharose were recovered and analysed as described in Fig. 4.

roughly equal to the overall reaction rate, which was estimated by cutting out the bands containing the unmodified cyclin from the gel shown in Fig. 4*b*, counting them, and fitting the data to an exponential decay function. Experimental values of $I(t)$ were determined by cutting out the regions of the gel that contain the modified forms of cyclin from Fig. 4*b* and counting them. Figure 6*b* compares the values of concentration of unconjugated cyclin and cyclin–ubiquitin conjugates measured from Fig. 4*b* with the values predicted by mathematical simulation using the derived values of k_1, and k_2. The close agreement strongly suggests that all of the cyclin that is degraded passes through a ubiquitinated intermediate.

Discussion

Progression through the cell cycle depends on the specific proteolysis of cyclin at the end of mitosis. To study the mechanism of cyclin degradation we have generated a *Xenopus* extract arrested in metaphase that constitutively degrades cyclin. Our system avoids the complications of standard cell-cycle extracts that only degrade cyclin transiently. We used this extract to map the sequences that confer cell-cycle-regulated degradation upon cyclin and to show that cyclin–ubiquitin conjugates appear during cyclin degradation. These conjugates accumulate and disappear with kinetics that are consistent with their being intermediates in the course of cyclin degradation, and do not form with cyclin derivatives that cannot be degraded. This evidence strongly suggests that the cell-cycle-regulated degradation of cyclin occurs by way of the ubiquitin pathway.

An N-terminal domain of cyclin is sufficient to confer cell-cycle-regulated degradation. With the demonstration that cycΔ90 cannot be degraded, this shows that the N terminus of cyclin is necessary and sufficient for cyclin degradation. By fusing this domain to protein A we created a hybrid protein that could be easily recovered from reactions by virtue of its binding to IgG. An isolated domain of cyclin, consisting of residues 13–91, which is entirely separate from the domain required for the activation of $p34^{cdc2}$ (ref. 3), can also be degraded in a cell-cycle-regulated fashion (data not shown). The region between amino acids 13 and 66 is sufficient to confer mitotic-specific degradation to the C-terminal portion of the cyclin molecule or to a foreign protein, protein A. Within this region there are two conserved features. The first is a sequence of nine amino acids (42–51) that we term the destruction box. Mutation of the invariant arginine residue at position 42 prevents the degradation of cyclin or a cyclin–protein A fusion carrying the mutation. We suggest that this region is recognized by some component of the ubiquitin-conjugating system. Although the destruction boxes of A and B type cyclins are similar, at one position there is a difference between A and B cyclins which may correlate with the earlier degradation of the A cyclins found in many organisms[17–20]. One of the *Saccharomyces cerevisiae* G1 cyclins, CLN2[23], contains the sequence RMGLVINAK, which has weak homology to the destruction box. The G1 cyclins have been identified only in budding yeast and though they are unstable it is not clear whether their stability is regulated during the cell cycle. Similarly, the yeast transcriptional regulator α2 has the motif RDILVFLSR[21] in a domain involved in destabilizing the protein[22]. The significance of these weak homologies to the destruction box remains to be established.

Sequences outside the destruction box are also required for cyclin degradation. Deletion derivatives of cyclin and of cyclin–protein A fusions that lack residues 54–66 are stable. In sea urchin cyclin B, residues 54–66 contain 4 lysine residues. The region between residues 51–90 is not conserved among cyclins, but is generally rich in lysine (17% averaged over all cyclin sequences). Gonda and coworkers showed that the ubiquitin-mediated destruction of proteins whose N-terminal amino acid is recognized by ubiquitin-conjugating enzymes requires a nearby lysine residue to act as an acceptor site for ubiquitin conjugation[11,24,25]. We speculate that the lysine residues C-terminal to the destruction box are the sites for ubiquitin conjugation. Interestingly, cyclins from many organisms show cell-cycle-dependent degradation in the *Xenopus* extracts (our unpublished results). This suggests that there is a minimal structural requirement for degradation.

Though cyclin and proteins containing cyclin peptides are clearly ubiquitinated, the ubiquitinated conjugates might not be intermediates in the pathway of cyclin degradation at mitosis. Although ubiquitin-mediated degradation has been well-studied in *in vitro* systems (for example, RNase A, casein, bovine serum albumin and lysozyme[26]), many of the substrates have been denatured or chemically-modified proteins and it is possible that the ubiquitin pathway serves principally to target misfolded proteins for destruction. Several properties of the ubiquitination of cyclin suggest that it does indeed represent the pathway of destruction. First, the observed ubiquitination of cyclin shows a tight dependence on the cell cycle. In mitotic extracts, 3% of 13–91prA is found as ubiquitin conjugates; in interphase extracts the level of ubiquitination of this protein is reduced about 10-fold. Second, a non-degradable mutant cyclin, cycΔ13-R42C, is not detectably multi-ubiquitinated under conditions where cyclin multi-ubiquitination was easily detected. A similar mutant

in the cyclin-protein A conjugate also blocks both degradation and ubiquitination. Third, the kinetics of the ubiquitination and degradation reactions are consistent with a requirement that all of the cyclin must pass through ubiquitin conjugates before degradation and are inconsistent with a model in which only a small amount of degradation occurs through ubiquitin and the majority by a different pathway. Figure 6*b* shows experimentally determined levels of ubiquitin conjugates and cyclin protein at various times fitted to a model for cyclin degradation in which ubiquitination of cyclin is a prerequisite for its degradation.

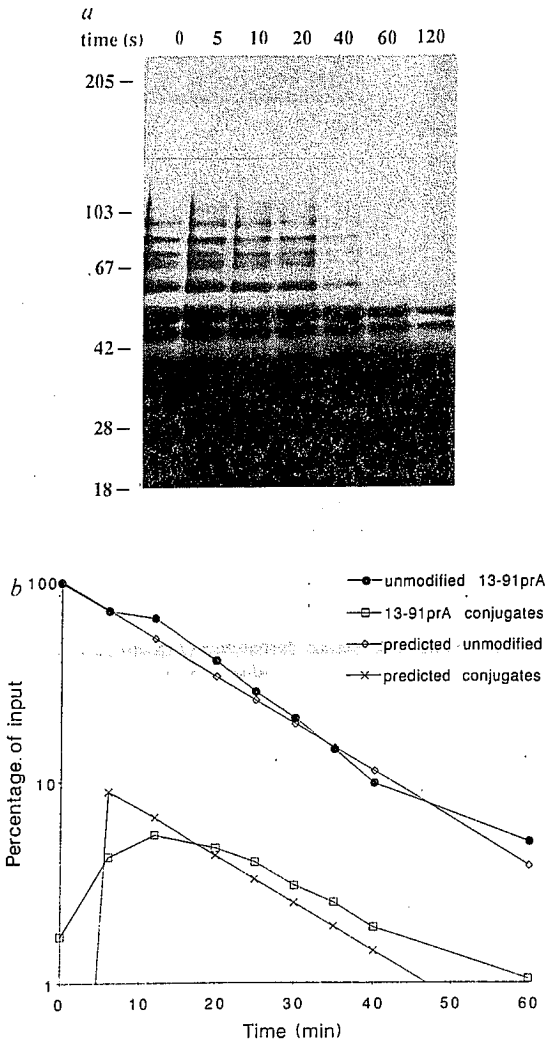


FIG. 6 Pulse-chase analysis of the stability of the ubiquitin conjugates. *a*, ^{125}I-labelled 13-91prA was added to a mitotically arrested extract. After 10 min, aliquots were removed and added to a 16.5×mass excess of unlabelled 13-91prA. At the indicated times the reaction was quenched and the IgG-binding species were analysed as described in Fig. 4. The decrease in radioactivity in the conjugates was quantitated by γ counting, plotted as a function of time and fitted to an exponential decay function to give $k_2=0.46\ \text{min}^{-1}$ ($R=0.97$), corresponding to a half-life of 1.5 min. *b*, The amount of modified and unmodified cyclin in the experiment shown in Fig. 4*b* plotted and compared with the values predicted from the mathematical model described. The value used for k_1 in the model was $0.053\ \text{min}^{-1}$ ($R=0.99$), which corresponds to $t_{1/2}=13$ min.

METHODS. Extracts and 13-91prA were prepared as described in Fig. 2, and the proteins were recovered and analysed as Fig. 4. The modelling was performed using the Stella software package.

Given the measured rate of degradation of cyclin, the measured half-life of the ubiquitin-cyclin conjugates and a reaction sequence in which the ubiquitin conjugates are intermediates in the destruction pathway, this model yields a level of ubiquitin conjugates of cyclin found during the degradation reaction that is almost exactly equal to that observed.

As the ubiquitin pathway seems to be responsible for cyclin proteolysis in mitosis, some aspect of the recognition of cyclin by the ubiquitin-conjugating system must be regulated by MPF. Several possibilities exist. MPF could activate a cyclin-specific enzyme that is directly involved in conjugating ubiquitin to cyclin. Several forms of ubiquitin-conjugating enzymes have been characterized[10,27], so it is conceivable that there is a form of these enzymes specific for cyclin. Alternatively, the ubiquitin-conjugating enzymes may be active throughout the cell cycle and MPF might stimulate an activity that makes cyclin a better substrate for ubiquitination. Examples of such activities are protein kinases that phosphorylate cyclin; cyclin-binding proteins that interact with the ubiquitination machinery; or a cyclin-specific endoprotease, activated by MPF, that could cleave cyclin and expose a destabilizing amino terminus[28]. Although, we cannot so far distinguish between these possibilities, the biochemical system we have established should permit the fractionation and characterization of the ubiquitin-conjugating activity and the identification of those activities that are triggered by MPF. There is one other known example of a specific protein of physiological interest that shows a regulated degradation by the ubiquitin pathway: the light-activated degradation of phytochrome in plants[15,29] and this system has interesting parallels with cyclin. In both cases the protein whose degradation is regulated accumulates under one set of conditions and is degraded under another. Active molecules are generated when light interacts with phytochrome or p34^{cdc2} associates with cyclin and undergoes specific post-translational modifications. The duration of the physiological effects of the activated molecule is limited because it induces its own ubiquitin-mediated degradation. In both systems neither the identity of the ubiquitin conjugating enzyme nor the nature of the event that allows recognition of the substrate by the ubiquitin conjugating system is known. However, there are a number of mutants in ubiquitin-conjugating enzymes that lead to interesting phenotypes including cell cycle arrest[12,13,30–34]. Of particular interest is the result that mutations in a specific ubiquitin ligase in budding yeast, *CDC34*, lead to arrest of the cell cycle at the G1 to S transition[13], an arrest which could be due to a failure to degrade one or more of the G1 cyclins.

Cyclin degradation at the metaphase-anaphase transition in the mitotic cycles of the early frog embryo is not regulated by the state of the mitotic spindle[35,36]. However, in many other cells the metaphase to anaphase transition is tightly regulated by feedback controls that prevent the onset of anaphase until the mitotic spindle has been fully assembled. In most cells the molecules that mediate the feedback control are not known. One exception is unfertilized frog eggs, which are arrested in metaphase of meiosis II by an activity named cytostatic factor (CSF), one of whose components is the *c-mos* proto-oncogene[37]. In CSF-arrested cells, cyclin is stable even though MPF levels are high, suggesting that the key event regulating the onset of anaphase may be the degradation of cyclin[3]. The experiments reported here suggest that the degradation of cyclin is a consequence of its recognition by the ubiquitin-conjugating system. An understanding of this recognition step may be required for a complete understanding of the mechanisms regulating the completion of mitosis and meiosis. □

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