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Destruction of *Xenopus* cyclins A and B2, but not B1, requires binding to p34^{cdc2}

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The specific and rapid destruction of cyclins A and B during mitosis is their most remarkable property. A short peptide motif of ~10 amino acids near the N-terminus, known as the destruction box, is absolutely required for programmed proteolysis. In this paper we show that although the destruction box is necessary for the degradation of cyclin A, it is not sufficient. Mutant versions of cyclin A that cannot form complexes with p34^{cdc2} are stable, which we interpret to mean that this cyclin must bind to p34^{cdc2} in order to undergo programmed proteolysis. Thus, N-terminal fragments of cyclin A containing little more than the destruction box and its surroundings are indestructible. p34^{cdc2} binding also appears to be required for the destruction of cyclin B2. In contrast, cyclin B1 does not require p34^{cdc2} binding for specific proteolysis. The systems for the proteolysis of cyclins A, B1 and B2 thus appear to show important differences in the way they recognize their substrates.

Key words: cell cycle/phosphorylation/protease/protein kinase

Introduction

Cyclins were first identified in marine invertebrate eggs as newly synthesized proteins which accumulated rapidly during interphase and were abruptly destroyed in mitosis, just before the metaphase to anaphase transition (Evans *et al.*, 1983; Swenson *et al.*, 1986). These proteins have since been identified in eukaryotes from yeast to mammals (see Hunt, 1991). Cyclins appear to be activating subunits of protein kinases encoded by genes from the cdc2, or cyclin dependent kinase (cdk) family (see Nurse, 1990; Pines and Hunter, 1990). At least some of these kinases are important regulators of cell cycle transitions. The two best characterized groups of cyclins are those that regulate progression through mitosis (mitotic cyclins) and those that regulate progression through Start in budding yeast (G_1 cyclins).

The transition from interphase to mitosis requires the activation of maturation promoting factor (MPF) (Masui and Markert, 1971; Newport and Kirschner, 1984; Draetta and Beach, 1988; Langan *et al.*, 1989) which is a heterodimer composed of a p34^{cdc2} kinase subunit and a B-type cyclin (Dunphy *et al.*, 1988; Gautier *et al.*, 1988, 1990; Lohka *et al.*, 1988; Draetta *et al.*, 1989; Labbé *et al.*, 1989). The transition from metaphase to anaphase requires specific proteolysis of the mitotic cyclins and consequent loss of the kinase activity of p34^{cdc2} (Luca and Ruderman, 1989; Murray and Kirschner, 1989; Murray *et al.*, 1989; Luca *et al.*, 1991). Proteolysis can be activated by addition of MPF to interphase extracts (Félix *et al.*, 1990) and the protease remains active for as long as MPF remains active. Thus, protease-resistant mutants of cyclin B arrest cells in mitosis and maintain the protease in its active state (Murray *et al.*, 1989; Ghiara *et al.*, 1991; Glotzer *et al.*, 1991; Luca *et al.*, 1991; Gallant and Nigg, 1992). In cleavage stage embryos, the protease seems to be inactive in interphase and rapidly activated at the end of metaphase, for example, in clam embryos, where accurate measurements are possible, proteolysis is active for ~5 min, by which time most of the cyclin has been degraded and MPF turned off (Hunt *et al.*, 1992). The enzyme(s) responsible for the recognition of cyclin and initiation of its proteolysis have not been identified and the mechanism of their control is not known. Two lines of evidence, however, implicate the ubiquitin system in cyclin degradation. First, high molecular weight ubiquitinated intermediates of sea urchin cyclin B (and constructs derived from it) were detected in crude extracts of frog embryos undergoing cyclin proteolysis, and the flux through these intermediates was adequate to account for the total loss of cyclin (Glotzer *et al.*, 1991). Second, addition of carboxymethylated ubiquitin to clam extracts inhibited cyclin proteolysis (Hershko *et al.*, 1991). It is not clear, however, that polyubiquitination is the sole signal for cyclin destruction and it is also not known whether the abrupt onset of proteolysis is due to the sudden activation of a cyclin- and cell cycle stage-specific ubiquitin ligase or of the substrate, or both. Nor has it yet been possible to chart the biochemical route of destruction of the ubiquitinated cyclin, although it is generally thought that the multifunctional proteasome is responsible. At present, the one point that is clear is that the N-terminus of mitotic cyclins contains a short conserved region, no more than 10 residues long, known as the destruction box. Mutation of the conserved arginine or leucine residues in this motif inhibits the destruction of both cyclins A and B (Murray *et al.*, 1989; Glotzer *et al.*, 1991; Lorca *et al.*, 1991b, 1992a; Gallant and Nigg, 1992; Kobayashi *et al.*, 1992). In the case of cyclin B, such modifications also inhibit its polyubiquitination, consistent with the idea that the destruction domain is recognized by some component of the ubiquitin-conjugating system.

Although the degradation of cyclins A and B both require an intact destruction box and occur at about the same time in the cell cycle, there are distinct differences between some aspects of their proteolysis. For example, cyclin A is degraded slightly earlier in the cell cycle than cyclin B (Luca and Ruderman, 1989; Minshull *et al.*, 1990; Whitfield *et al.*, 1990; Hunt *et al.*, 1992), and whereas disruption of the mitotic spindle formation with colchicine strongly retards

cyclin B destruction, such treatment had no effect on cyclin A proteolysis (Whitfield *et al.*, 1990; Hunt *et al.*, 1992). Another curious difference between the two types of cyclin, both of which appear to be associated with $p34^{cdc2}$ at this juncture of the cell cycle, is that cyclin B−$p34^{cdc2}$ triggers cyclin destruction, whereas cyclin A does not; indeed, cyclin A tends to delay the destruction of both A- and B-type cyclins (Luca *et al.*, 1991; Lorca *et al.*, 1992b).

Certain other properties of cyclin proteolysis have been discovered during searches for inhibitors of the process. For example, the adenine analogue 6-dimethyl aminopurine stabilizes cyclins (Félix *et al.*, 1989, 1990; Luca and Ruderman, 1989), possibly by virtue of its ability to inhibit the protein kinase activity of MPF. The well-known ability of ATP-γ-S to stabilize MPF (Lohka *et al.*, 1988) is probably due to its ability to antagonize cyclin destruction. Other inhibitors include the trypsin inhibitor, tosyl-lysine chloromethyl ketone, certain sulfydryl-reactive agents, such as *N*-ethylmaleimide and $ZnCl_2$ and EDTA (Luca and Ruderman, 1989). Conversely, it has been reported that in *Xenopus* egg extracts cyclin destruction can be triggered by okadaic acid (Lorca *et al.*, 1991a).

Unfertilized *Xenopus* eggs are arrested in metaphase of meiosis II with high MPF activity (Masui and Markert, 1971; Lohka and Masui, 1983). Cell cycle arrest in these eggs is mediated by an activity known as cytostatic factor (CSF) and is thought to involve the c-*mos* protein kinase (Masui and Markert, 1971; Sagata *et al.*, 1989). Fertilization triggers a large increase in the intracellular Ca^{2+} levels (Busa and Nuccitelli, 1985), which leads to the specific proteolysis of the mitotic cyclins, MPF inactivation and resumption of the cell cycle (Newport and Kirschner, 1984; Murray *et al.*, 1989; Lorca *et al.*, 1991b). In this paper, we have used extract made from unactivated *Xenopus* eggs (CSF-arrested extract) in order to study cyclin destruction. For this analysis we used a number of deletion and point mutations of frog cyclin A, which we made recently to look at the requirements for binding to $p34^{cdc2}$ (Kobayashi *et al.*, 1992). We find that for the proteolysis of cyclin A to proceed with normal kinetics, it must be capable of binding to $p34^{cdc2}$. Surprisingly, cyclin B2 also needs to bind to $p34^{cdc2}$ for normal destruction, but cyclin B1 does not, confirming the results of Glotzer *et al.* (1991), who showed that constructs derived from sea urchin cyclin B containing a destruction box could be proteolysed even if they lacked the $p34^{cdc2}$ binding domain.

Results

The assay for cyclin destruction

In the experiments described below, we used concentrated extracts made from unactivated *Xenopus* eggs, called CSF-arrested extracts (Murray, 1991). Cyclin destruction can be triggered in these extracts by the addition of 0.4 mM $CaCl_2$ (Lohka and Maller, 1985; Murray *et al.*, 1989; Lorca *et al.*, 1992a; Vandervelden and Lohka, 1993), but if Ca^{2+} is not added, the cyclin proteins remain stable. CSF-arrested extract can be frozen in liquid nitrogen and retains the ability to translate added mRNA and degrade cyclins in response to added Ca^{2+} after thawing. In the experiments described in this paper, the standard assay for cyclin destruction was as follows. Synthetic mRNAs encoding wild type or mutant cyclins were translated in freshly thawed CSF-arrested extract in the presence of [^{35}S]methionine. Cycloheximide was then added to block further protein synthesis and Ca^{2+} (0.4 mM final concentration) was added to trigger cyclin destruction. Cyclin proteolysis was assayed by taking samples of the extract at various times after Ca^{2+} addition and analysing the ^{35}S-labelled products by SDS−PAGE and autoradiography (see Materials and methods). The intensities of the labelled cyclin bands on the autoradiograph were quantified by scanning densitometry.

In this assay system the cyclins are destroyed specifically and rapidly within ~30 min of Ca^{2+} addition to the extract and when the same CSF-arrested extract is used for a series of assays, the results are highly reproducible. However, batches of extract prepared from different females vary considerably both in their ability to translate added mRNA and in their ability to destroy cyclin proteins. The length of time required for the complete destruction of wild type cyclin, for example, varied between 15 and 40 min (compare Figure 1A, lanes 1−6 and Figure 6, top panel, first six lanes). For these reasons, wild type cyclins were included in every assay as positive controls and comparisons between the rate and extent of destruction of mutant constructs were always performed at the same time in the same batch of extract. Some cyclin A constructs carried an N-terminal c-*myc* tag, while others did not. In all experiments, the appropriate wild type control was used, although there is no difference between the destruction of c-*myc* tagged and non-c-*myc* tagged cyclin A (data not shown).

Cyclin A destruction at the end of M phase requires $p34^{cdc2}$ binding

Figure 1A shows that in the standard destruction assay wild type cyclin A was rapidly destroyed between 30 and 60 min after Ca^{2+} addition (compare the intensity of lanes 4 and 5). In striking contrast to the wild type protein, however, two cyclin A mutants in which single highly conserved residues within the cyclin box had been conservatively

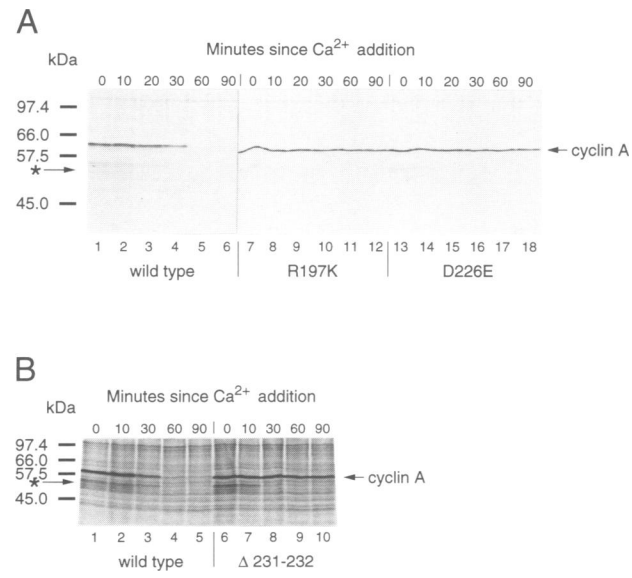

Fig. 1. Destruction of cyclin box mutants of cyclin A. (**A**) Destruction of R197K and D226E cyclin A. Lanes 1−6, c-*myc* tagged wild type cyclin A; lanes 7−12, R197K cyclin A; lanes 13−18, D226E cyclin A. (**B**) Destruction of Δ 231−232 cyclin A. Lanes 1−5, wild type cyclin A; lanes 6−10, Δ 231−232 cyclin A. Asterisk on left indicates position of the endogenous B-type cyclins. The standard destruction assay was used (see Materials and methods).

mutated (R197 to K or D226 to E), were still present in the extract 90 min after Ca^{2+} addition (Figure 1A, lanes 12 and 18). These mutants are unable to bind to $p34^{cdc2}$ (data not shown). Two other point mutations, R197A and D226A, also do not bind to $p34^{cdc2}$ (Kobayashi *et al.*, 1992) and are not destroyed (see Table I). Figure 1B shows that another mutant (Δ 231−232), which cannot bind to $p34^{cdc2}$ (Kobayashi *et al.*, 1992), is also indestructible in the CSF-arrested extract. This construct contains a deletion of two amino acids (C 231 and M 232) in the heart of the conserved cyclin box. It is unlikely that such conservative point mutations or such a small deletion seriously distort the structure of the protein. These results suggested that it may be necessary for cyclin A to bind to $p34^{cdc2}$ in order to undergo programmed proteolysis. It has previously been shown that the destruction box of cyclin A is necessary for programmed proteolysis (Kobayashi *et al.*, 1992; Lorca *et al.*, 1992a).

In order to confirm this conclusion, we next studied the destruction of a number of C-terminally deleted cyclin A mutants (Figure 2A) which cannot bind to $p34^{cdc2}$ (Kobayashi *et al.*, 1992 and data not shown). As shown in Figure 2B, C and D, and summarized in Table I, constructs lacking 14, 79, 139, 194, 221 or 295 residues from the C-terminus of cyclin A are all much more stable in destruction assays than their wild type parent. CΔ194 cyclin A showed slight instability in the destruction assay (Figure 2D) and for this reason a parallel construct in which the essential residues within the destruction box of CΔ194 were mutated to alanine (see Materials and methods) was made (CΔ194-ATVA). CΔ194-ATVA showed similar instability in the destruction assay to CΔ194 (Figure 2D), thus the gradual proteolysis of CΔ194 appears to be non-specific, probably due to slight misfolding of the protein.

Table I. Summary of the properties of the cyclin A constructs described in this paper

Mutant	$p34^{cdc2}$ binding	Destruction box	Destruction
Wild type	+	+	+
c-*myc* tagged cyclin A	+	+	+
FVD mutant	+	+	+
Δ 98−100	ND	+	+
S136A	+	+	+
CΔ14	−	+	−
CΔ24	−	+	−
CΔ50	−	+	−
CΔ79	−	+	−
CΔ139	−	+	−
CΔ194	−	+	−
CΔ221	−	+	−
CΔ295	−	+	−
CΔ194-ATVA	−	−	−
CΔ221-ATVA	−	−	−
CΔ295-ATVA	−	−	−
NΔ133	+	−	−
Δ 88−144	+	+	−
Δ 90−147	+	+	−
Δ 102−158	+	+	−
Δ 109−161	+	+	±
Δ 101−169	−	+	−
Δ 80−201	−	+	−
Δ 36−245	−	−	−
Δ 109−161-ATVA	ND	−	−
Δ 101−169-ATVA	ND	−	−
Δ 231−232	−	+	−
R197A	−	+	−
D226A	−	+	−
R197K	−	+	−
D226E	−	+	−
ATVA	+	−	−

ND, not determined.

The result that cyclin A destruction requires $p34^{cdc2}$ binding was confirmed by the analysis of two internal deletion mutants of cyclin A, Δ 101−169 and Δ 80−201 (formerly NΔ169 and NΔ201). These constructs are also unable to bind to $p34^{cdc2}$, have intact destruction boxes (Kobayashi *et al.*, 1992) and again are much more stable than wild type cyclin A (data not shown). Δ101−169 does display a slight instability in the destruction assay, like CΔ194, but this instability is not affected by mutation of the destruction box (data not shown). Therefore the instability of this mutant is probably due to non-specific proteolysis, as it is for CΔ194. These data and those from the degradation of cyclin B2-CΔ24 (see next section) suggest that some C-terminally truncated cyclins may be misfolded and are recognized by a constitutive cytoplasmic proteolysis pathway. This pathway is not dependent on the cyclin destruction box and occurs even when the specific cyclin proteolysis pathway is not activated (see next section).

From these results we conclude that the cyclin A destruction at the end of M phase requires $p34^{cdc2}$ binding.

Cyclin B2 destruction also requires $p34^{cdc2}$ binding, but cyclin B1 destruction does not

At first sight, the results described in the previous section appear to disagree with those of Glotzer *et al.* (1991), who showed that short segments of the N-terminus of sea urchin cyclin B, which do not contain the $p34^{cdc2}$ binding domain, could be destroyed in a cell cycle-regulated manner. To investigate further this apparent contradiction, we made mutants of cyclins B1 and B2 in which the final 24 residues of each protein were deleted. This removes part of the region shown to be required for the binding of $p34^{cdc2}$ to cyclin A (Kobayashi *et al.*, 1992). As expected, neither of these mutants could bind to $p34^{cdc2}$ (data not shown). In agreement with the results of Glotzer *et al.* (1991), cyclin B1-CΔ24 was destroyed rapidly, albeit slightly later than wild type cyclin B1, when Ca^{2+} was added to the CSF-arrested extract, but remained stable in the absence of Ca^{2+} (Figure 3A). Remarkably, however, cyclin B2-CΔ24 was much more stable than wild type cyclin B2 when Ca^{2+} was added to the CSF-arrested extract (Figure 3B) and although this construct does show gradual proteolysis, this is unlike the rapid destruction of wild type cyclin B2. We believe this gradual proteolysis to be due to non-specific degradation, since cyclin B2-CΔ24 was destroyed at a similar rate in CSF-arrested extract in the absence of Ca^{2+}. To check that the inability of cyclin B2-CΔ24 to undergo programmed proteolysis was due to its failure to bind to $p34^{cdc2}$, and not simply because it folds incorrectly, we mutated R163 (the equivalent of R197 in cyclin A) to an alanine residue in full-length cyclin B2. This cyclin box mutant did not bind to $p34^{cdc2}$ (data not shown) and was extremely stable in the standard destruction assay (Figure 4). Thus it appears that cyclins A and B2 require $p34^{cdc2}$ binding in order to be destroyed at the end of M phase, whereas *Xenopus* cyclin B1 and sea urchin cyclin B do not.

The assay used by Glotzer *et al.* (1991) for studying cyclin

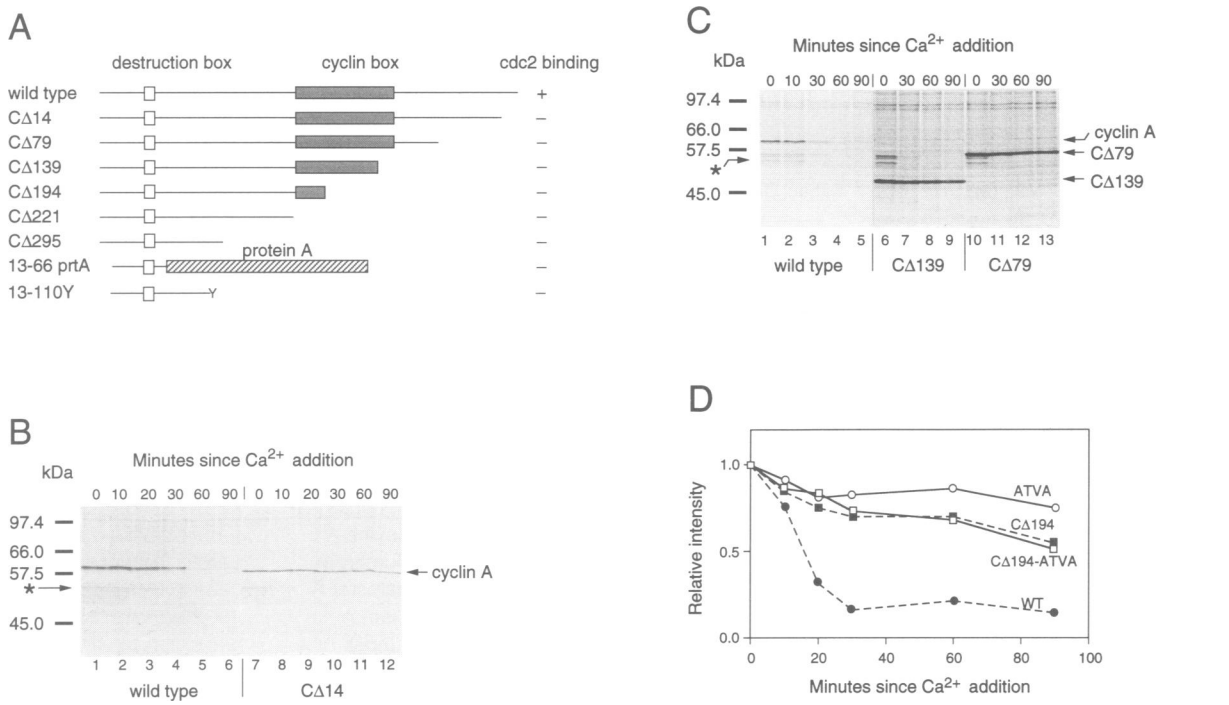

Fig. 2. Destruction of C-terminally deleted mutants of cyclin A. (**A**) Schematic diagram of C-terminally deleted cyclin A mutants CΔ14, 79, 139, 194, 221 and 295 and the sea urchin cyclin B constructs 13−66 protein A (sea urchin cyclin B residues 13−66 fused to protein A) and 13−110Y (sea urchin cyclin B residues 13−110 followed by a single Y residue and no protein A) (Glotzer *et al.*, 1991). (**B**) Destruction of CΔ14 cyclin A. Lanes 1−6, c-*myc* tagged wild type cyclin A; lanes 7−12 CΔ14 cyclin A. Asterisk on left indicates position of the endogenous B-type cyclins. The standard destruction assay was used (see Materials and methods). (**C**) Destruction of CΔ79 and CΔ139 cyclin A. Lanes 1−5, wild type cyclin A; lanes 6−9, CΔ139 cyclin A; lanes 10−13, CΔ79 cyclin A. Asterisk on left indicates position of the endogenous B-type cyclins. The standard destruction assay was used (see Materials and methods). (**D**) Destruction of CΔ194 cyclin A. The mRNAs encoding full-length c-*myc* tagged cyclin A (●); full-length c-*myc* tagged cyclin A with a mutated destruction box, ATVA (○); CΔ194 cyclin A (■) and CΔ194 cyclin A with a mutated destruction box, CΔ194-ATVA (□) were translated in nuclease treated CSF-arrested extract and the intensities of the cyclin bands on the autoradiograph were determined by scanning densitometry. A graph was plotted of the proportion of the cyclin A protein remaining against time (see Materials and methods).

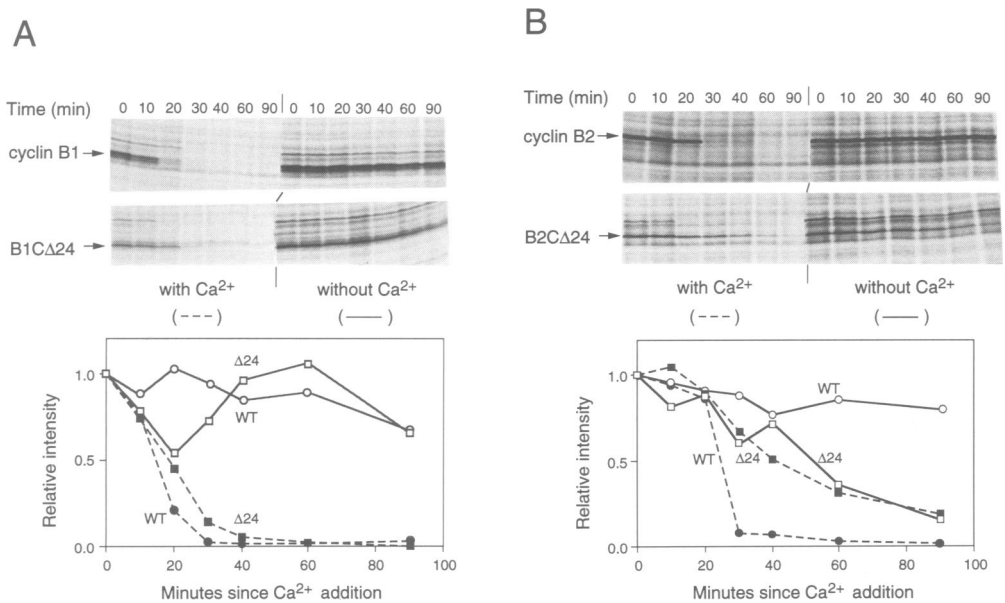

Fig. 3. (**A**) Destruction of cyclin B1-Δ24. Top panel, wild type cyclin B1. Middle panel, cyclin B1-CΔ24: left-hand seven lanes, Ca^{2+} added at time zero; right-hand seven lanes, no Ca^{2+} added to assay (i.e. no cyclin destruction triggered). Bottom panel, quantification by scanning densitometry: wild type cyclin B1 with Ca^{2+} added to assay (●), wild type cyclin B1 without Ca^{2+} added to assay (○), cyclin B1-CΔ24 with Ca^{2+} added to assay (■) and cyclin B1-CΔ24 without Ca^{2+} added to assay (□). (**B**) Destruction of cyclin B2-CΔ24. Top panel, wild type cyclin B2. Middle panel, cyclin B2-CΔ24: left-hand seven lanes, Ca^{2+} added at time zero; right-hand seven lanes, no Ca^{2+} added to assay (i.e. no cyclin destruction triggered). Bottom panel, quantification by scanning densitometry: wild type cyclin B2 with Ca^{2+} added to assay (●), wild type cyclin B2 without Ca^{2+} added to assay (○), cyclin B2-CΔ24 with Ca^{2+} added to assay (■) and cyclin B2-CΔ24 without Ca^{2+} added to assay (□). The standard destruction assay was used and the intensities of the cyclin bands on the autoradiographs were determined by scanning densitometry. Graphs were plotted of the proportion of the cyclin protein remaining against time (see Materials and methods).

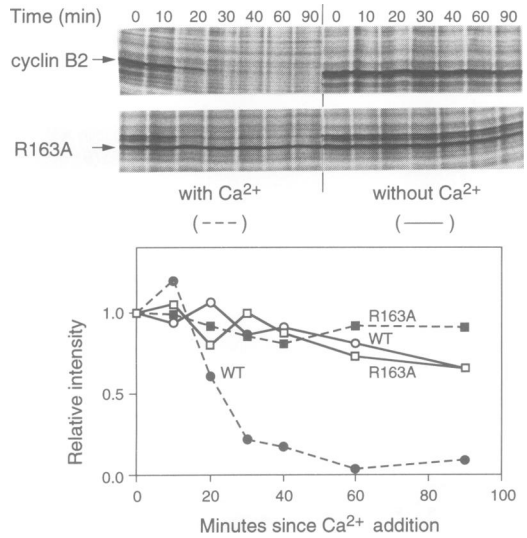

Fig. 4. Destruction of R163A cyclin B2. Top panel, wild type cyclin B2. Middle panel, R163A cyclin B2: left-hand seven lanes, Ca^{2+} added at time zero; right-hand seven lanes, no Ca^{2+} added to assay (i.e. no cyclin destruction triggered). Bottom panel, quantification by scanning densitometry: wild type cyclin B2 with Ca^{2+} added to assay (●), wild type cyclin B2 without Ca^{2+} added to assay (○), R163A cyclin B2 with Ca^{2+} added to assay (■) and R163A cyclin B2 without Ca^{2+} added to assay (□). The standard destruction assay was used and the intensities of the cyclin bands on the autoradiograph were determined by scanning densitometry. A graph was plotted of the proportion of the cyclin protein remaining against time (see Materials and methods).

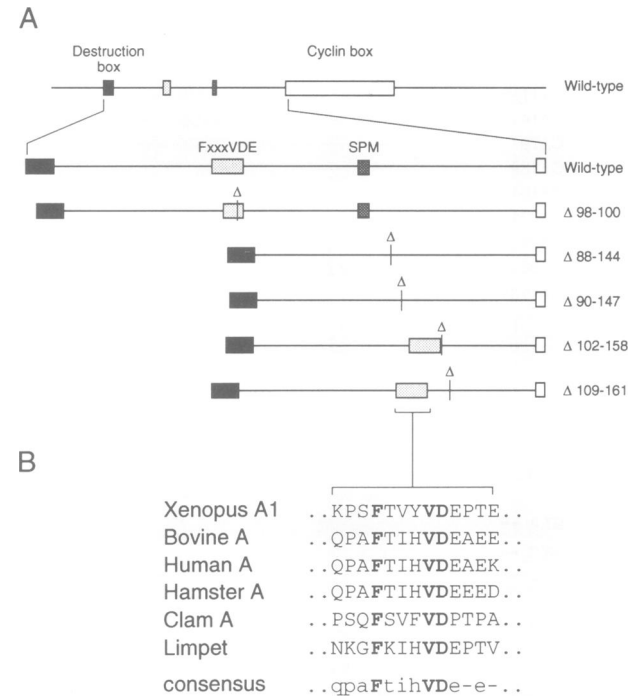

Fig. 5. Schematic diagram of internal deletions of cyclin A and identification of an N-terminal conserved sequence motif. (**A**) Diagram of position and extent of deletions from five internally deleted cyclin A mutants. (**B**) Conservation of the FxxxVDE motif in cyclin A of six different animals. The motif does not occur in precisely the same position within each cyclin A protein, but all lie between the destruction box and the start of the cyclin box.

destruction differed from the assay system used here in two important respects. First, Glotzer *et al.* used bacterially synthesized substrates labelled with ^{125}I and secondly, destruction was assayed in interphase frog egg extracts in which cyclin destruction was promoted by addition of bacterially synthesized indestructible sea urchin cyclin B constructs. It was therefore important to test these constructs in our assay system. One of the constructs contained sea urchin cyclin B residues 13–66 fused to protein A and the other contained residues 13–110 of sea urchin cyclin B without protein A, but with a single C-terminal tyrosine residue (to allow iodination) (Glotzer *et al.*, 1991) (see Figure 2A for a scale diagram of these constructs compared with the cyclin A deletion series). Parallel constructs in which the destruction boxes were mutated served as controls. We obtained these constructs from M.Glotzer and tested their destruction in an assay system in which their mRNAs were translated in a 1:1 (v/v) mixture of *Xenopus* CSF-arrested extract and nuclease-treated rabbit reticulocyte lysate. A portion of this reaction (1/5 part by volume) was then added to freshly thawed CSF-arrested extract, and cyclin proteolysis was triggered by adding Ca^{2+} (see Materials and methods). While the constructs with the wild type cyclin destruction boxes were rapidly destroyed after adding Ca^{2+}, the constructs with the mutated destruction boxes were stable (data not shown). Both constructs were stable in the absence of added Ca^{2+} (data not shown). Thus, our assay system gave exactly the same results as Glotzer *et al.* (1991). We recently learned that Zhang and Ruderman (personal communication) tested the destruction of human cyclin B1, and found that mutants that were unable to bind to $p34^{cdc2}$ were perfectly well destroyed. We therefore conclude that there are important differences in the requirements for destruction of cyclins B1, B2 and A. Cyclin B1 can be recognized and destroyed as such, whereas cyclins B2 and A are only destroyed when they are bound to $p34^{cdc2}$.

Some cyclin A mutants that have an intact destruction box and can bind to $p34^{cdc2}$ are nevertheless indestructible

The properties of some other mutant cyclin A constructs complicated the simple rule described above. Three clear examples were provided by the constructs Δ 88–144, Δ 90–147 and Δ 102–158 cyclin A [previously named NΔ144, NΔ147 and NΔ158 (Kobayashi *et al.*, 1992)] which have deletions of 57 or 58 residues between the destruction box and the start of the cyclin box (see Figure 5A). These three mutants were degraded extremely slowly compared with wild type cyclin A when Ca^{2+} was added to CSF-arrested extract (Figure 6, first six lanes, and see Table I), although this destruction is specific since it did not occur in the assay in the absence of Ca^{2+} (Figure 6, last six lanes). All three constructs have been shown to bind and activate $p34^{cdc2}$ with similar affinities and kinetics to wild type cyclin A (Kobayashi *et al.*, 1992 and data not shown) and all contain intact destruction boxes (Figure 5A). A similar, but not identical result was obtained for the construct Δ 109–161 cyclin A (previously NΔ161). This construct lacks 53 residues just upstream of the region of cyclin A required for binding to $p34^{cdc2}$ (see Figure 5A); it can activate $p34^{cdc2}$ (Kobayashi *et al.*, 1992) and contains an intact destruction box. However, its proteolysis was 'unreliable' when compared with wild type cyclin A. In some assays, Δ 109–161 was almost completely stable, like Δ 88–144, Δ 90–147 and Δ 102–158 cyclin A, while in

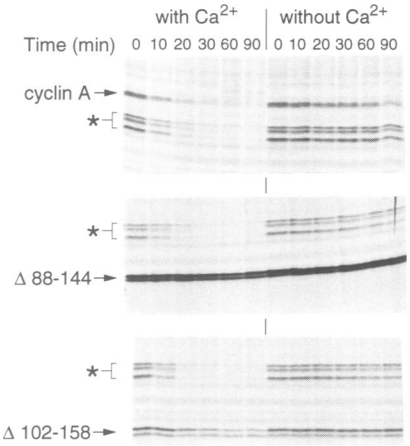

Fig. 6. Destruction of internally deleted cyclin A mutants. Top panel, wild type cyclin A. Middle panel, Δ 88−144 cyclin A. Bottom panel, Δ 102−158: left-hand six lanes, Ca^{2+} added at time zero; right-hand six lanes, no Ca^{2+} added to assay (i.e. no cyclin destruction triggered). Asterisks on left indicate positions of the endogenous B-type cyclins. The standard destruction assay was used (see Materials and methods).

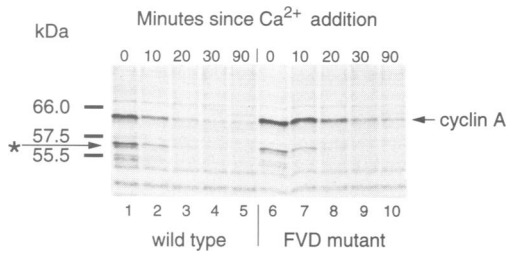

Fig. 7. Destruction of the FVD mutant of cyclin A. Lanes 1−5, c-*myc* tagged wild type cyclin A; lanes 6−10, FVD mutant of cyclin A. Asterisk on left indicates position of the endogenous B-type cyclins. The standard destruction assay was used (see Materials and methods).

others it was destroyed, although more slowly and later than wild type cyclin A (data not shown).

A conserved sequence motif is missing from Δ 88−144 and Δ 90−147 cyclin A

To understand the reason for the abnormal destruction of the internal deletion mutants of cyclin A, Δ 88−144, Δ 90−147, Δ 102−158 and Δ 109−161, we inspected the amino acid sequence of the region that had been deleted from these constructs. The region that was deleted in Δ 88−144 and Δ 90−147 cyclin A included a conserved motif, **FTVYVDE**, which is present in the N-terminus of all known cyclin A sequences, except that of *Drosophila* cyclin A (Figure 5B). The conservation of this motif, and its absence from Δ 88−144 and Δ 90−147 cyclin A, suggested that it might represent a 'secondary destruction box' that was required for the proteolysis of cyclin A. This conserved motif was present in Δ 102−158 and Δ 109−161 cyclin A, but lay close to the start of the deleted regions (see Figure 5A), perhaps interfering with its function, giving the altered destruction kinetics of these two mutants. We tested the role of the FxxxVDE motif in two ways. First, the F, V and D residues were mutated to alanine residues (see Materials and methods). This construct ('FVD mutant' of cyclin A) was tested in a destruction assay. Although in repeated assays the mutation showed slightly slower proteolysis than wild type cyclin A, it was nevertheless destroyed rapidly (Figure 7). To confirm this result, a second cyclin A mutant was constructed in which the V, D and E residues were simply deleted (construct Δ 98−100, see Materials and methods). The degradation of Δ 98−100 cyclin A was very similar to that of wild type cyclin A when tested in a destruction assay (data not shown). Thus, the presence or absence of the FxxxVDE motif did not explain the patterns of stability or destructibility of Δ 88−144, Δ 90−147, Δ 102−158 and Δ 109−161 cyclin A proteins in a simple way.

A major p34^{cdc2} phosphorylation site is missing from Δ 88−144 and Δ 109−161 cyclin A

Another feature of the anomalously stable internal deletion constructs is that all four lack the tripeptide sequence SPM, a well-conserved motif ~30 residues upstream of the start of the p34^{cdc2} binding region of almost all known cyclin A sequences, from molluscs to humans (see Figure 5A). Since this represented a potential phosphorylation site for proline-directed protein kinases, another hypothesis to explain the stability of some of the cyclin A mutant proteins was that phosphorylation at this S_{136}PM site was required for normal rapid destruction. Mutant cyclins would be stabilized in one of two ways: inability to bind to p34^{cdc2}, or lack of the phosphorylation site. According to this model, phosphorylation of cyclin A would 'tag' it for destruction. If the protein were not tagged, it could not be destroyed. To investigate this hypothesis, we compared the phosphorylation of wild type cyclin A and mutants lacking the SPM motif. *In vitro*-transcribed mRNA was translated in a mixture of CSF-arrested extract and rabbit reticulocyte lysate. The cyclin A was harvested with an anti-*Xenopus* cyclin A polyclonal antiserum and incubated with [γ-^{32}P]ATP. The products were analysed by SDS−PAGE followed by autoradiography, shown in Figure 8A; the ^{35}S-labelled cyclin A is shown in lanes 1−6 and the ^{32}P-labelled products in lanes 7−12.

The newly translated cyclin A binds to and activates p34^{cdc2}, which is present at ~0.8 μM in egg extracts. This activated p34^{cdc2} can then phosphorylate the cyclin subunit to which it is bound, so that the immunoprecipitates incorporate label from [γ-^{32}P]ATP into cyclin A and into other tightly bound proteins. Since the egg extracts contained low levels of endogenous cyclin A protein and mRNA, these immunoprecipitates incorporated some ^{32}P even without added mRNA (Figure 8A, lane 12). When mRNA for cyclin A was added to these extracts, however, the total concentration of cyclin A protein was increased >15-fold after a 2 h incubation (determined by quantitative immunoblotting, data not shown). This accounts for the greatly increased ^{32}P labelling seen in lane 7 of Figure 8A compared with lane 12. By contrast, the cyclin A mutants, Δ 101−169 and CΔ79, which cannot bind to p34^{cdc2}, were not detectably phosphorylated in this assay (Figure 8A, lanes 10 and 11). Only the endogenous wild type cyclin A was labelled with ^{32}P in these lanes. Thus, the ^{32}P incorporation seen in the immunoprecipitates of kinase-binding constructs can probably be ascribed to p34^{cdc2} or p33^{cdk2}.

Two of the internal deletion constructs, Δ 88−144 and Δ 109−161, which lack the SPM motif, can bind to p34^{cdc2} and are phosphorylated in these reactions (Figure 8A, lanes 8 and 9), but they incorporated only ~10% as much ^{32}P as did wild type cyclin A (compare lanes 7, 8 and 9,

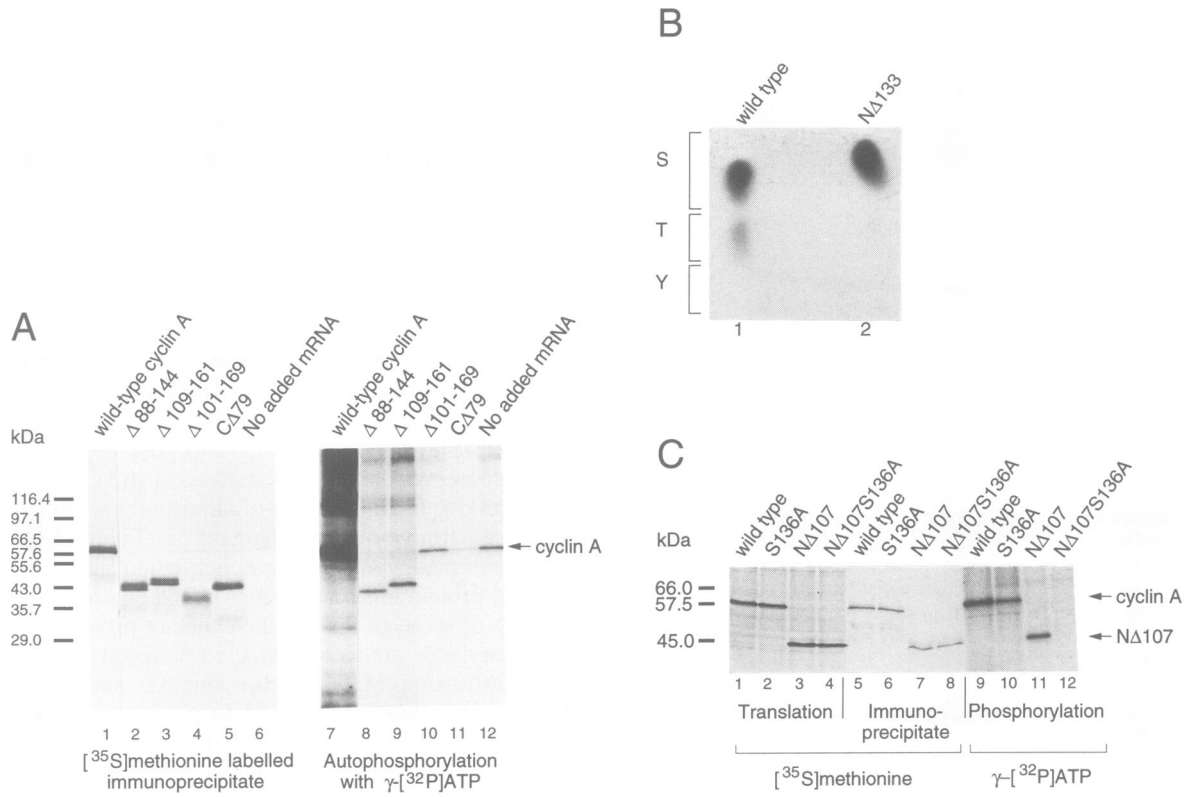

Fig. 8. Analysis of phosphorylation of cyclin A. **(A)** Phosphorylation of cyclin A mutants. Translations of cyclin A constructs were immunoprecipitated with anti-cyclin A antibodies. Left-hand panel, translation was carried out in the presence of [^{35}S]methionine. The immunoprecipitates were eluted from the protein A−Sepharose with SDS sample buffer and analysed by SDS−PAGE and autoradiography (see Materials and methods). Right hand panel, translation was carried out in the absence of [^{35}S]methionine. The immunoprecipitates were incubated with [γ-^{32}P]ATP before elution with SDS sample buffer and analysis by SDS−PAGE and autoradiography. **(B)** Phosphoamino acid analysis of wild type and c-*myc* NΔ133 cyclin A. The residue(s) that are phosphorylated by p34^{cdc2} when p34^{cdc2} bound wild type cyclin A or c-*myc* NΔ133 cyclin A is immunoprecipitated and exposed to [γ-^{32}P]ATP were analysed by acid hydrolysis of the phosphorylated proteins followed by thin layer electrophoresis in pH 3.5 buffer (see Materials and methods). The positions of the amino acid standards are indicated to the left of the figure. Lane 1, wild type cyclin A; lane 2, c-*myc* NΔ133 cyclin A. **(C)** Phosphorylation of S136A mutant of cyclin A. Translations of c-*myc* tagged cyclin A constructs were immunoprecipitated with the anti c-*myc* monoclonal antibody, 9E10 (see Materials and methods). Left panel, [^{35}S]methionine-labelled translations of cyclin A constructs; middle panel, immunoprecipitates of [^{35}S]methionine-labelled translations; right panel, unlabelled immunoprecipitates of cyclin A translations were incubated with [γ-^{32}P]ATP, eluted with SDS sample buffer and analysed by SDS−PAGE and autoradiography.

quantified by scanning densitometry). These results suggest that there are at least two phosphorylation sites in the N-terminus of *Xenopus* cyclin A, of which the major one is deleted in the mutant cyclin As, Δ 88−144 and Δ 109−161.

Although $S_{136P}M$ is both a conserved motif and a potential site for proline directed phosphorylation, other serine and threonine residues are also deleted from Δ 88−144 and Δ 109−161. For this reason, it was necessary to check that the phosphorylation detected in the assay described above was indeed due to phosphorylation on S136, and not on some other nearby serine or threonine.

Xenopus cyclin A is phosphorylated on S136

To check that the phosphorylated residue in these assays was serine, we carried out phosphoamino acid analysis of phosphorylated wild type cyclin A. Figure 8B (lane 1) shows that the phosphoamino acid in cyclin A is indeed mainly serine, with only minor phosphorylation of threonine. The phosphorylated threonine residue was in the N-terminus of the cyclin A protein, since the N-terminally deleted construct c-*myc* NΔ133 (see Materials and methods) contained only phosphoserine (Figure 8B, lane 2).

To check that S136 in the sequence SPM was the major *in vitro* phosphorylation site in *Xenopus* cyclin A, it was replaced with an alanine in a c-*myc* tagged wild type cyclin A (S136A cyclin A). Replacement of S136 with alanine strongly reduced the phosphorylation of this construct but did not eliminate it (Figure 8C, compare lanes 9 and 10). This is probably due to phosphorylation at the other site(s) in the N-terminus of cyclin A. To check this, the same serine (136) to alanine mutation was made in the construct c-*myc* NΔ107, which lacked the 107 N-terminal residues of cyclin A. This construct was not phosphorylated in the immunoprecipitation assay (Figure 8C, compare lanes 11 and 12).

To confirm that S136 was the major phosphorylation site in cyclin A, wild type cyclin A protein was doubly labelled with [γ-^{32}P]ATP and [^{35}S]methionine, isolated and digested with V8 endopeptidase as described in Materials and methods. The resulting peptides were resolved by thin layer electrophoresis followed by chromatography. Wild type cyclin A gave two major [γ-^{32}P]ATP-labelled spots on the autoradiograph, which were eluted from the TLC plate and used for progressive Edman chemistry to identify the positions of both the phosphorylated residue and any ^{35}S-labelled residues within the peptide (see Materials and methods). The data from this analysis (data not shown, but see Materials and methods for a more detailed description)

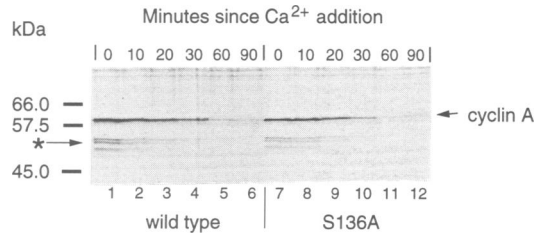

Fig. 9. Destruction of the S136A mutant of cyclin A. Lanes 1–6, c-*myc* tagged wild type cyclin A; lanes 7–12, c-*myc* tagged S136A cyclin A. Asterisk on left indicates position of the endogenous B-type cyclins. The standard destruction assay was used (see Materials and methods).

confirmed that the site in cyclin A that is phosphorylated by the assay described above, is indeed S136.

Phosphorylation on S136 is not required for cyclin destruction

To test whether phosphorylation on S136 was required for the degradation of cyclin A, mRNA encoding full-length S136A mutant cyclin A protein was translated in CSF-arrested extract, followed by addition of Ca^{2+} to trigger cyclin destruction. Proteolysis of the mutant followed the same kinetics as wild type cyclin A (Figure 9). Thus, S136 is not required for the destruction of cyclin A. This eliminates the hypothesis that phosphorylation at this site could account for the requirement for binding to $p34^{cdc2}$.

Discussion

The major finding of these studies is that mutant cyclin A proteins that cannot bind to $p34^{cdc2}$ are stable when cyclin proteolysis is triggered by the addition of Ca^{2+} to *Xenopus* egg extracts. It is possible that these mutations cause severe conformational changes in the cyclin A protein which simultaneously prevent them binding to $p34^{cdc2}$ and impair their recognition by the cyclin destruction machinery. However, it is highly unlikely that all the mutations introduced into cyclin A which prevent binding to $p34^{cdc2}$ (Kobayashi *et al.*, 1992) cause serious conformational changes. Two constructs in particular, one in which R197 is mutated to a lysine residue (R197K) and the other in which D226 is mutated to glutamic acid (D226E), neither bind to $p34^{cdc2}$ nor get destroyed. It seems improbable that these extremely conservative changes would have such a drastic effect on cyclin A conformation as to render the mutant proteins unrecognizable by both $p34^{cdc2}$ and the cyclin destruction machinery. This suggests either that the recognition of cyclin A by the destruction machinery requires the simultaneous recognition of $p34^{cdc2}$, or alternatively that when cyclin A binds to $p34^{cdc2}$ it alters its conformation so as to make the destruction box accessible for recognition by the proteolytic system. Our data do not allow us to distinguish between these hypotheses.

We were somewhat surprised that cyclin A must bind to $p34^{cdc2}$ in order to be destroyed, as previous studies on the stability of sea urchin cyclin B in *Xenopus* extracts did not suggest any such requirement (Glotzer *et al.*, 1991). To investigate this point further, we made C-terminal deletions of both *Xenopus* cyclins B1 and B2, which removed their ability to bind to $p34^{cdc2}$. As might have been expected from the results of Glotzer *et al.* (1991), cyclin B1-CΔ24 underwent quite normal programmed proteolysis upon addition of Ca^{2+} and was stable in the absence of added Ca^{2+}. To our surprise, however, cyclin B2-CΔ24 behaved more like non-$p34^{cdc2}$ binding mutants of cyclin A, and did not show the rapid destruction of wild type cyclin B2. Cyclin B2-CΔ24 appeared to undergo slow, non-specific proteolysis which occurred at a similar rate in the presence or absence of Ca^{2+}. This suggests that cyclin B2-CΔ24 may not be folded correctly, which could explain its indestructibility. However, a point mutant of cyclin B2 (R163A) that did not bind to $p34^{cdc2}$ was completely stable, both in the presence and absence of Ca^{2+}. This mutation is unlikely to cause great conformational changes to the protein and it therefore appears that like cyclin A, cyclin B2 must be bound to $p34^{cdc2}$ in order to be destroyed at the end of M phase. This result is in agreement with the results of Vandervelden and Lohka (1993) who showed that the N-terminus of cyclin B2 was not destroyed when Ca^{2+} was added to a CSF-arrested extract, in contrast to wild type *Xenopus* cyclin B2. We thus conclude that the recognition of cyclins A and B2 for destruction differs from that of cyclin B1. Differences in the proteolysis of cyclins A and B have been noted previously; the destruction of cyclin A normally occurs before that of cyclin B *in vivo* (Luca and Ruderman, 1989; Minshull *et al.*, 1990; Whitfield *et al.*, 1990; Hunt *et al.*, 1992) and colchicine arrests clam embryos in mitosis at a point where cyclin A is being continually destroyed but the destruction of cyclin B is delayed (Whitfield *et al.*, 1990; Hunt *et al.*, 1992). It is quite surprising that the proteolysis of cyclins B1 and B2 should show such a marked difference, considering how similar they are. It should, however, be noted that the N-termini of *Xenopus* cyclins B1 and B2 show only 35% identity up to the beginning of the cyclin box (and only 23% in the first 80 residues), compared with 62% identity throughout the rest of the protein, which may explain the differences. Further mutational studies will be required to understand this.

Cyclin A, however, needs more than an intact destruction box and the ability to bind to $p34^{cdc2}$ for normal proteolysis. Three mutant cyclin A proteins, Δ 88–144, Δ 90–147 and Δ 102–158, possess intact destruction boxes and can bind to (and activate) $p34^{cdc2}$; however, all three constructs get destroyed extremely slowly compared with wild type cyclin A. We estimate that the rates of destruction (taken at the point of most rapid destruction, see Materials and methods) of these mutants are $<10\%$ of that of wild type cyclin A. Δ 109–161 cyclin A can also bind to $p34^{cdc2}$ and contains an intact destruction box, but it shows slower and less reliable proteolysis compared with wild type cyclin A protein. Two hypotheses could account for these anomalous examples. (i) Given that dual recognition of the destruction box and $p34^{cdc2}$ is required for the correct degradation of cyclin A, the spatial relationship between the two recognition elements might be important. (ii) The deleted region in these constructs may contain additional element(s) whose presence is important for programmed proteolysis. We tested the second hypothesis quite extensively, because the region deleted in the anomalously stable cyclins contained at least two conserved motifs which might have been important for destruction. The first motif has the sequence **FTVYVDE** (the residues in bold are conserved in all examples of cyclin A except for *Drosophila*), and is situated 40–50 amino acids downstream of the destruction box. It is deleted in Δ 88–144 and Δ 90–147. When the conserved residues F, V and D

were mutated to alanine, or residues V, D and E were deleted, however, the mutant proteins were destroyed with near normal kinetics.

The tripeptide sequence, SPM, is the second conserved motif deleted from the cyclin A mutants Δ 99−144, Δ 90−147, Δ 102−158 and Δ 109−161. This S_{136P} motif is conserved in all known examples of cyclin A at similar (although not identical) positions in the molecule, ~30 residues upstream of the start of the $p34^{cdc2}$ binding domain. We showed here that S136 is a major *in vitro* site for phosphorylation by $p34^{cdc2}$. When we mutated S136 to an alanine residue, however, the mutant cyclin A protein was destroyed at exactly the same time and with exactly the same kinetics as wild type cyclin A. Thus phosphorylation on this residue is clearly not required for the destruction of cyclin A. It has previously been shown that phosphorylation of *Xenopus* cyclins B1 and B2 is not required for destruction (Izumi and Maller, 1991), but the relationship between phosphorylation and destruction has not been studied for cyclin A. As the requirements for cyclin A and B2 destruction differ from those of cyclin B1, we considered it important to investigate this point. It should be noted that phosphorylation on S136 and the minor threonine phosphorylation in the N-terminus of cyclin A are not necessarily the only phosphorylations to take place on cyclin A *in vivo*. Our assay, which was performed on immunoprecipitates, relied on *de novo* phosphorylation or the turnover of a pre-existing phosphate. If a site were stably phosphorylated by endogenous (and therefore non-radioactive) ATP in the CSF-arrested extract during translation of cyclin A mRNA, it would not incorporate [γ-^{32}P]ATP after immunoprecipitation. In addition, our assay only reliably detects the phosphorylation of cyclin A by the $p34^{cdc2}$ to which it is bound. We cannot exclude the possibility that cyclin A is phosphorylated by other protein kinases, some of which may target it for destruction.

What other features necessary for destruction might be deleted in Δ 88−144, Δ 90−147 and Δ 102−158? Glotzer *et al.* (1991) found evidence that the region between residues 54 and 66 in sea urchin cyclin B was necessary for its degradation, in addition to an intact destruction box, possibly because the lysine residues in this region were required as ubiquitin acceptor sites. Two lysine residues were deleted from cyclin A mutants Δ 88−144 and Δ 90−147 and one from Δ 102−158, but we do not consider this very significant, for the following reasons. First, the positioning of lysine residues in this region is not conserved between different species of cyclin A and moreover, two lysine residues remain between the destruction box and the deleted region in these constructs. Finally, chicken cyclin A does not have any lysine residues in the 50 amino acids following its destruction box, and only two in the next 77 residues, although presumably it is degraded in the usual fashion.

Thus, we did not identify any conserved motifs or residues between the destruction box and the start of the cyclin box whose presence was essential for normal rapid proteolysis. It is possible that the precise spacing of the destruction box with respect to the cyclin box is important, because Δ 109−161 cyclin A, which can be degraded (although not in an entirely wild type manner), has the destruction box slightly further away from the cyclin box than those of the other anomalously stable mutants. It would, perhaps, be worthwhile to insert new sequences into these mutants to place the destruction box further away from the cyclin box. However, deciding what sequences to insert would be difficult, as we do not know the structure of the cyclin protein and one would not know whether the sequence inserted was causing distortion of the protein. It is also possible that the conformation of the destruction box and its surroundings are crucial and are significantly altered by the exact location of the deletions in Δ 88−144, Δ 90−147, Δ 102−158 and Δ 109−161 cyclin A mutants.

We had not expected to find that cyclin A needed to be bound to $p34^{cdc2}$ in order to be destroyed, and are still confused by the anomalous stability of some of the internal deletion mutants, even after many experiments to test what we considered to be reasonable explanations. It is difficult to distinguish between a model in which the destruction apparatus recognizes both cyclin and $p34^{cdc2}$, and one in which binding to $p34^{cdc2}$ causes some sort of conformational change in the presentation of the destruction box. If the latter is true, small changes in sequence could have large effects on stability. The problem in this case is to explain why Δ 109−161 is, at times, destructible and why the cyclin B1 proteolysis system shows so much less stringent requirements for specific recognition than those of cyclins A and B2. Elucidation of these points will probably require both structural information about the conformation of cyclins bound to $p34^{cdc2}$ and a more detailed knowledge of the recognition and effector components of the mitotic protease. Perhaps the most intriguing question of all is this: what is the normal signal for cyclin A destruction? Does the disappearance of cyclin A mark the successful passage through a checkpoint, the completion of some essential cellular process? Failure to destroy cyclin A certainly delays the onset of anaphase (Luca *et al.*, 1991), but would its premature loss accelerate passage through mitosis?

Materials and methods

Cyclin A constructs

The construction and structure of N- and C-terminal deletion mutants of cyclin A were described by Kobayashi *et al.* (1992).

c-*myc* NΔ107 cyclin A was constructed using PCR. A 5′ primer was constructed with a *Bsm*I site upstream of the desired start of the protein and this was used in conjunction with a 3′ primer which recognized a sequence downstream of the naturally occurring *Nsi*I site in cyclin A. The fragment of cyclin A thus generated was digested with *Bsm*I and *Nsi*I and inserted between the *Bsm*I and *Nsi*I sites of c-*myc* tagged cyclin A (Kobayashi *et al.*, 1992). The N-terminus of this construct therefore had the following sequence: MDP**MEQKLISEEDL**NSAF, followed by residue 107 of cyclin A and the rest of the cyclin A protein. The bold portion is the epitope recognized by the 9E10 monoclonal antibody (Evan *et al.*, 1986). c-*myc* NΔ133 was constructed in a similar way using non-c-*myc* tagged NΔ133, and its N-terminus had the following sequence: MDP**MEQKLISEEDL**-NSAFMDPM, followed by residue 133 of cyclin A.

Mutagenesis of individual residues within cyclin A was carried out using PCR essentially as described by Horton and Pease (1991). The altered segments were checked by sequencing the double-stranded template DNA with the United States Biochemicals Sequenase kit (Cleveland, OH).

The long C-terminal deletions of cyclin A, CΔ194, CΔ221 and CΔ295, were constructed using PCR. 3′ primers were constructed to produce a stop codon at the desired place, followed by a *Bam*HI restriction enzyme site and were used in conjunction with 5′ primers which annealed to the T7 RNA polymerase promoter upstream of the parent cyclin A construct. The PCR product was digested with *Nco*I and *Bam*HI and inserted between the *Nco*I and *Bcl*I sites of c-*myc* tagged cyclin A (Kobayashi *et al.*, 1992). The amino acid sequences of the C-termini of the constructs are as follows: CΔ295: ...DSNIV*; CΔ221: ...ITSAM*; CΔ194: ...YLAMN*.

The destruction box mutants of these constructs were made using the same primers, but instead of using wild type c-*myc* tagged cyclin A, the template used for the PCR was a c-*myc* tagged cyclin A construct in which the invariant residues within the destruction box, R41 and L44, had been mutated to alanine residues as previously described (Kobayashi *et al.*, 1992).

The Δ 98−100 mutant of cyclin A was constructed using the Amersham oligonucleotide-directed *in vitro* mutagenesis kit (Amersham, UK). The oligonucleotide:

GGTTTCTGTTGG$_{|}$ATAGACTGTAAAAC

was used to delete nucleotides GTGGATGAG (position of the deletion is indicated by the arrow) from c-*myc* tagged cyclin A. This deletes the tripeptide V98 D99 E100 from the protein.

Cyclin B constructs

CΔ24 cyclins B1 and B2 were constructed using PCR. 3′ primers were constructed to produce a stop codon at the desired place, followed by a *Bam*HI restriction enzyme site and were used in conjunction with 5′ primers that produced an *Nco*I site at the start of the coding sequence of the cyclin protein. The PCR product was digested with *Nco*I and *Bam*HI and inserted between the *Nco*I and *Bcl*I sites of c-*myc* tagged cyclin A, thus giving the cyclin B mutants the 5′ and 3′ untranslated regions of this construct, which gives good translation of the proteins (Kobayashi *et al.*, 1992). The amino acid sequences of the C-termini of the constructs are as follows: cyclin B1-CΔ24: ...KYASS*; cyclin B2-CΔ24: ...KYASS*.

Mutagenesis of R163 to alanine in cyclin B2 was carried out using PCR essentially as described by Horton and Pease (1991). The altered segments were checked by sequencing the double-stranded template DNA with the United States Biochemicals Sequenase kit (Cleveland, OH).

Standard cyclin destruction assays

Cyclin destruction assays were carried out as previously described by Kobayashi *et al.* (1992). Briefly, mRNA for the test constructs was translated for 1−2 h in the presence of [^{35}S]methionine in cell-free extracts of unactivated frog eggs (CSF-arrested extract) (Murray, 1991), at which time cycloheximide (100 μg/ml final concentration) was added to block further protein synthesis and 0.4 mM Ca^{2+} (final concentration) was added to initiate cyclin proteolysis. Samples of the reactions were analysed by SDS−PAGE and the intensities of the labelled cyclin bands on the autoradiograph were quantified by scanning densitometry.

Cyclin destruction assays using proteins made in mixtures of frog egg extract with rabbit reticulocyte lysate

Some mRNAs are very poorly translated in pure frog egg extracts. To circumvent this problem, these mRNAs were translated in a 1:1 (v/v) mixture of rabbit reticulocyte lysate and CSF-arrested extract (Kobayashi *et al.*, 1992). Cycloheximide (100 μg/ml final concentration) was added at the end of the incubation to block further translation, and 2 μl of this mixture were added to 8 μl of freshly thawed CSF-arrested extract. Destruction of cyclins was triggered by the addition of $CaCl_2$ to 0.5 mM final concentration (compensating for the 1 mM EGTA present in nuclease-treated reticulocyte lysate). Samples were taken at intervals after adding the $CaCl_2$ for analysis on SDS−PAGE and autoradiography, and the intensities of the labelled cyclin bands on the autoradiograph were quantified by scanning densitometry.

Cyclin destruction assays using RNase A treated CSF-arrested extract

The long C-terminal deletions of cyclin A contain very few methionine residues. When mRNA encoding these proteins was translated in CSF-arrested extracts in the presence of [^{35}S]methionine, the proteins were therefore not labelled very strongly. This made them very difficult to visualize on autoradiographs due to the translation of endogenous mRNA contained within the extract. To overcome this problem, a 1/10 vol of a 10 μg/ml RNase A solution was added to the CSF-arrested extract for 15 min at 10°C and then inactivated by the addition of a 1/10 vol of a 20 000 U/ml RNAGuard (Pharmacia, Uppsala, Sweden) for 15 min at 10°C. To this RNase treated extract was added a 1/10 vol of reticulocyte lysate, 1/20 vol of [^{35}S]methionine and 1/10 vol of *in vitro* transcribed mRNA encoding the protein to be tested. After 90 min at 23°C, the same volume of newly thawed (non-RNase A treated) CSF-arrested extract, containing 100 μg/ml cycloheximide, was added to the translation and cyclin destruction triggered by the addition of 0.45 mM $CaCl_2$ (final concentration). Samples were taken at intervals for analysis by SDS−PAGE and autoradiography and the intensities of the labelled cyclin bands on the autoradiograph were quantified by scanning densitometry.

Quantitation of cyclin destruction using scanning densitometry

The intensities of the cyclin bands on the autoradiograph were determined by scanning densitometry. The intensity of the cyclin band at time zero (the point of Ca^{2+} addition to the extract) was taken as one and the values for subsequent time points converted to a proportion of this value. Graphs were plotted of the proportion of ^{35}S-labelled cyclin protein remaining against time. To analyse the differing rates of cyclin destruction, the slopes of the graphs at the points of most rapid destruction were determined.

Immunoprecipitations

The mRNAs encoding the cyclin A constructs to be tested were translated in 1:1 mixtures of CSF-arrested extract and nuclease-treated rabbit reticulocyte lysate for 2 h at 23°C. If the products were to be ^{35}S-labelled, then 1 mCi/ml (final concentration) of [^{35}S]methionine was included in the incubation. Either 2 μl of polyclonal rabbit anti-cyclin A serum (Kobayashi *et al.*, 1991) or 1 μl of protein A-purified monoclonal anti c-*myc* antibody, 9E10 (17 mg/ml) (Evan *et al.*, 1986), was then added to 5 μl of the extract, which was diluted 4- to 6-fold with bead buffer (10 mM Tris−Cl pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml soybean trypsin inhibitor and 1 mM benzamidine). This was incubated on ice for 1 h. The immunocomplexes were diluted with 100−200 μl of bead buffer and recovered on 15−20 μl of protein A−Sepharose (Pharmacia, Uppsala, Sweden) for 30 min at room temperature. The immunocomplex loaded beads were washed four times with bead buffer and transferred into a fresh tube. [^{35}S]methionine-labelled immunocomplexes were eluted with SDS sample buffer and analysed by SDS−PAGE and autoradiography. Unlabelled immunocomplexes were used for phosphorylation assays as described below.

Phosphorylation assays

Unlabelled immunocomplexes bound to protein A−Sepharose (see above) were washed twice with Cicerelli buffer (50 mM β-glycerophosphate, 7 mM NaF, 0.3 mM EDTA, 15 mM $MgCl_2$ and 2 mM DTT, pH 7.3) before incubation in 10 μl Cicerelli buffer with 0.5 μl [γ-^{32}P]ATP (Amersham, Amersham, UK) for 30 min at 20°C. The bound proteins were eluted with SDS sample buffer and analysed by SDS−PAGE and autoradiography.

Phosphoamino acid analysis

The mRNA encoding c-*myc* tagged wild type cyclin A and c-*myc* NΔ133 was translated, without [^{35}S]methionine, in 1:1 mixtures of frog egg extract and nuclease-treated reticulocyte lysate for 2 h at 23°C. The translation products were immunoprecipitated with the anti c-*myc* antibody 9E10 (Evan *et al.*, 1986) and collected on protein A−Sepharose beads (Pharmacia, Uppsala, Sweden). The bead-bound immunoprecipitates were washed twice with Cicerelli buffer and incubated with [γ-^{32}P]ATP in 10 μl Cicerelli buffer for 30 min at 20°C. The proteins were eluted with SDS sample buffer and analysed by SDS−PAGE, followed by electrophoretic transfer to Immobilon membrane (Millipore, Bedford, MA). Phosphoamino acid analysis was carried out according to Kamps (1991), using electrophoresis at pH 3.5 on silica thin layer plates to resolve the phosphoamino acids.

Phosphopeptide mapping

Phosphopeptide mapping was carried out essentially to the protocol of Luo *et al.* (1991) for tryptic peptide mapping of immobilized proteins, except that the protein was digested with 6 μg of staphylococcal V8 protease in 50 mM ammonium bicarbonate−5% acetonitrile overnight. The resulting peptides were analysed by electrophoresis on thin layer silica plates in pH 4.72 buffer and chromatography in phospho chromatography buffer, according to Boyle *et al.* (1991). The plate was then autoradiographed. To ensure that only [γ-^{32}P]ATP-labelled peptides and not [^{35}S]methionine-labelled spots were detected on the autoradiograph, a sheet of paper was placed between the silica plate and the film.

Edman degradation of phosphorylated peptides

The silica containing the phosphorylated peptides was scraped off the plate and the peptides were extracted by incubation in 100 μl of 7% ammonia−50% methanol for 2 h. This was filtered through a 0.45 μm Ultrafree unit and freeze dried. The freeze-dried material was dissolved in 30 μl of 1:1 (v/v) acetonitrile−water and covalently attached to an aryl amine coated PVDF membrane (Coull *et al.*, 1991). Edman chemistry was performed for 20 cycles with the resulting phenyl thiohydantoin residue being diverted for collection in a fraction collector. The radioactivity in these fractions was determined in a scintillation counter.

When this procedure was carried out on the spots resulting from V8 endopeptidase digestion of phosphorylated cyclin A, one of these spots released ~30% of its radioactive material at position number 2 in the peptide (20 cycles in total). The procedure was repeated with protein labelled with both [γ-^{32}P]ATP and [^{35}S]methionine. Edman degradation of the same phosphorylated peptide showed that there was a methionine residue at position number 4. There are only two predicted peptides in V8-digested c-*myc* tagged cyclin A with a serine residue at position number 2, only one of which has a methionine residue at position number 4. This peptide contains S136 as its second residue. Thus we concluded that S136 is the major site of

phosphorylation in cyclin A in the assay described above and in the results section.

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Note added in proof

Since the experiments described in this paper were performed, we have made a *Xenopus* cyclin B1 mutant in which arginine 168 (equivalent to R197) in cyclin A and R163 in cyclin B2) was changed to an alanine residue (cyclin B1 R168A). This mutant does not bind to $p34^{cdc2}$. Much to our surprise, and in contrast to the CΔ24 cyclin B1 mutant described in this paper, cyclin B1 R168A is not destroyed in our standard destruction assay.