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The 'destruction box' of cyclin A allows B-type cyclins to be ubiquitinated, but not efficiently destroyed

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The destruction of mitotic cyclins by programmed proteolysis at the end of mitosis is an important element in cell cycle control. This proteolysis depends on a conserved motif of nine residues known as the 'destruction box', which is located 40–50 residues from the N-terminus. The sequences of the A- and B-type destruction boxes are slightly different, which might account for the differences in timing of their destruction. When the cyclin A-type destruction box was substituted for the normal one in cyclin B1 or B2, however, the resulting constructs were unexpectedly stable, although the converse substitution of B-type destruction boxes in cyclin A permitted normal degradation. We compared the ubiquitination of various cyclin constructs, and found that whereas mutation of the highly conserved residues in the destruction box strongly reduced the level of ubiquitinated intermediates, the stable destruction box 'swap' constructs did form such adducts. Thus, while ubiquitination is probably necessary for cyclin destruction, it is not sufficient. We also found that polyubiquitinated cyclin derivatives are still bound to p34^{cdc2}, which is not detectably ubiquitinated itself, raising the questions of how cyclin and cdc2 dissociate from one another, and at what stage, in the process of degradation.

Keywords: cell cycle/GST fusions/hexahistidine/proteolysis/ubiquitin

Introduction

Cell cycle transitions depend on cyclin-dependent protein kinases, whose activity rises and falls periodically during the growth and division of cells (Norbury and Nurse, 1992; Nasmyth, 1993; Ohtsubo and Roberts, 1993; Pines, 1994; Resnitzky *et al.*, 1994). These kinases are activated by combination with a cyclin subunit, but activity can be delayed or inhibited by appropriate phosphorylation events or by combination with inhibitory polypeptides (Coleman and Dunphy, 1994; Elledge and Harper, 1994). The abrupt fall in protein kinase activity that occurs around the time of the metaphase to anaphase transition depends on the destruction of mitotic cyclins, which is one of the key events in the eukaryotic cell cycle (Murray and Hunt, 1993). The protease responsible for this event is strikingly specific for cyclins, and highly regulated. It can turn on and off in the course of five minutes in the rapid cell cycles of fertilized clam eggs, and different cyclins are destroyed at slightly different times, cyclin A being degraded slightly ahead of cyclin B1 (Hunt *et al.*, 1992), and cyclin B3 lagging behind cyclin B1 (Kreutzer *et al.*, 1995).

A mutant B-type cyclin from the sea urchin *Arbacia* lacking the first 90 residues was found to be indestructible in frog egg extracts, and maintained these extracts in a mitotic state with high maturation promoting factor (MPF) activity (Murray *et al.*, 1989; Luca *et al.*, 1991; Holloway *et al.*, 1993). Point mutations revealed that a short, partially conserved sequence of nine amino acids located ~40–50 residues from the N-terminus, the so-called 'destruction box', is essential for cyclin proteolysis (Murray *et al.*, 1989; Glotzer *et al.*, 1991; Kobayashi *et al.*, 1992; Lorca *et al.*, 1992; Amon *et al.*, 1994). In the case of *Arbacia* cyclin B, short segments of the N-terminus containing a functional destruction box are themselves unstable, and in some cases can confer instability on other proteins when assayed in the *Xenopus* cell-free system (Glotzer *et al.*, 1991). Frog cyclins are different: their N-terminal fragments seem to be poor substrates for their own proteolysis system (Stewart *et al.*, 1994; Van der Velden and Lohka, 1994), and even full-length mutant cyclins that are unable to bind to p34^{cdc2} are either resistant to degradation, or only slowly destroyed (Stewart *et al.*, 1994).

Although many details remain to be discovered, there is persuasive evidence that cyclin degradation involves a specially regulated form of the ubiquitin-mediated proteolytic pathway (Glotzer *et al.*, 1991; Hershko *et al.*, 1991, 1994; Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995; Tugendreich *et al.*, 1995); for reviews see Hershko (1991), Rechsteiner (1991), Ciechanover (1994) and Jennissen (1995). Using a model construct containing the N-terminus of sea urchin cyclin B fused to protein A, ubiquitinated intermediates could be detected whose kinetics of formation and disappearance were consistent with those expected for obligatory intermediates in the cyclin destruction process (Glotzer *et al.*, 1991). Further, it was shown that methylated ubiquitin inhibited cyclin destruction in a cell-free extract of mitotic clam eggs, which could be restored by the addition of wild-type ubiquitin (Hershko, 1991). Yeast that contains only K48R ubiquitin, which cannot form long chains, arrest in late mitosis (Finley *et al.*, 1994). Quite recently, it has been found that a set of proteins, first identified in strains of yeast (*cdc16*, *23* and *27*) that undergo cell cycle arrest in late mitosis at the non-permissive temperature, form a complex with each other, and can enhance the activity of the ubiquitin ligation machinery in cell-free biochemical assays (Irniger *et al.*, 1995; King *et al.*, 1995; Tugendreich

et al., 1995). It is thought that ubiquitination of cyclins makes them substrates for the multicatalytic proteasome, and in support of this idea, mutations in certain proteasome subunits have been identified which show defects in cell cycle progression (Gordon *et al.*, 1993; Richter-Ruoff and Wolf, 1993), although they are probably rather pleiotropic.

Biochemical fractionation of clam and frog egg extracts has revealed three components that are necessary for cyclin ubiquitination. These comprise ubiquitin-conjugating enzymes E1 and E2, together with a large cell cycle-regulated complex that seems to be an E3 enzyme (Hershko *et al.*, 1994; King *et al.*, 1995; Sudakin *et al.*, 1995). King *et al.* (1995) were able to further purify these components from frog egg extract, and to identify the *Xenopus* homologues of Cdc16 and Cdc27, which are mitotic arrest mutants in *Saccharomyces cerevisiae*, as part of a large E3 complex. They also identified the *Xenopus* E2 homologue of Ubc4 required for cyclin-specific ubiquitination (King *et al.*, 1995). However, deletion of Ubc4 and Ubc5 (closely related E2 enzymes) failed to arrest yeast cells in mitosis (Seufert and Jentsch, 1990), whereas a ts mutant strain of Ubc9, a recently described E2 enzyme, arrested in G_2/mitosis and failed to degrade cyclin B at the non-permissive temperature (Seufert *et al.*, 1995). Thus, some uncertainty remains about the identity of the essential components of the ubiquitination pathway and their role in cyclin degradation.

The metaphase arrest of *Xenopus* eggs is due to the activity of cytostatic factor (CSF), which delays the normal mitotic destruction of cyclins (Masui and Markert, 1971) through the protein kinase activity of the c-*mos* proto-oncogene (Sagata *et al.*, 1988). CSF is inactivated at fertilization by an increase in intracellular Ca^{2+}, which also leads to the inactivation of $p34^{cdc2}$ kinase by the destruction of its cyclin subunit(s) (Lorca *et al.*, 1991; Watanabe *et al.*, 1991). These effects can be induced in cell-free frog egg extracts by the addition of $CaCl_2$ (Murray, 1991). This Ca^{2+}-induced destruction appears to be mediated by the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), whose constitutively active form (Waldmann *et al.*, 1990) is able to initiate cyclin proteolysis in *Xenopus* egg extracts (Lorca *et al.*, 1993, 1994; Morin *et al.*, 1994). Somewhat paradoxically, $p34^{cdc2}$ kinase itself is necessary to activate cyclin degradation, although only the forms containing cyclin B and not the complexes containing cyclin A are active in this respect (Félix *et al.*, 1990; Luca *et al.*, 1991).

In this paper, we studied the differences in the kinetics of proteolysis of *Xenopus* mitotic cyclins by constructing cyclin chimeras in which the amino-termini of cyclin A1, B1 and B2 were exchanged. We found that the N-terminus of cyclin A1 conferred surprising stability to B-type cyclins, and when the destruction boxes of cyclins B1 and B2 were mutated to give the precise sequence of the A1 destruction box, they were essentially indestructible. Given the evidence described above that cyclins are destroyed by the ubiquitin-mediated proteolytic pathway, we tested whether these indestructible cyclin chimeras were ubiquitinated. We found that wild-type and chimeric cyclins were both ubiquitinated, irrespective of whether or not they were capable of being degraded. We conclude that, although ubiquitination is almost certainly necessary for cyclin degradation, it may not be sufficient.

Results

B-type cyclins are stabilized by the N-terminus of cyclin A1

The destruction of A- and B-type cyclins occurs with different timing in normal cell cycles, and requires the integrity of the nine-residue destruction box located ~50 residues from the N-terminus. To test whether the information for precise timing of cyclin destruction is contained in the destruction box and its surroundings, we constructed chimeric cyclins in which the N-termini of cyclins A1, B1 and B2 were exchanged at an *Nco*I site that was introduced at residue 137 in cyclin A1, 97 in cyclin B1 and 93 in cyclin B2. The start of the essential Cdc2-binding domain in these cyclins lies at residue 161 for cyclin A1, 134 for cyclin B1 and 128 for cyclin B2. Introducing the *Nco*I site into the constructs did not affect the destructibility of their translation products as compared with the original cyclin mRNAs (data not shown). Figure 1A shows the diagrams of the constructs and summarizes their properties in the destruction assay. The timing of destruction of these 'domain swap' constructs was assayed in *Xenopus* egg extracts to which $CaCl_2$ was added to trigger exit from metaphase. This provides a convenient cell-free assay for cyclin destruction, but it should be noted that cyclin A proteolysis typically lags behind that of the B-type cyclins in this case (Stewart *et al.*, 1994), unlike the situation in normal mitotic cycles (Minshull *et al.*, 1990; Hunt *et al.*, 1992). Figure 1B and C show that wild-type cyclins were proteolysed between 10 and 20 min after addition of Ca^{2+} to the extract, and that chimeras containing the N-terminus of either cyclin B1 or B2 were destroyed with similar kinetics. By contrast, however, chimeras containing the N-terminus of cyclin A and the C-terminus of either cyclin B1 or B2 were markedly resistant to proteolysis. While the degradation of cyclin A was almost complete within 30 min after $CaCl_2$ addition, the rate of proteolysis of cyclin A1–B1 was reduced by ~50% and was never completed, because eventually the protease system is inactivated. The stabilizing effect of the cyclin A1 N-terminus on cyclin B2 was even more striking. Wild-type cyclin B2 is almost completely destroyed within 20 min after the induction of degradation, whereas the A1–B2 chimeric cyclin remained stable after the addition of $CaCl_2$, similar to the control without added Ca^{2+} (compare the $+CaCl_2$ and $-CaCl_2$ panels of A1–B2 in Figure 1B). We were very surprised by these results, having expected relatively minor changes in the timing of cyclin destruction, rather than complete stabilization as a consequence of swapping wild-type cyclin N-termini. Although different extracts were used for the experiments shown in Figure 1B and C, constructs being compared were assayed in parallel using the same CSF extract supplemented with 10% reticulocyte lysate.

Chimeric cyclins can bind to and activate $p34^{cdc2}$

We previously found that cyclins A and B2 were strongly stabilized by mutations that impaired their ability to form complexes with $p34^{cdc2}$ (Stewart *et al.*, 1994). Thus, a possible explanation for the protease-resistance of the

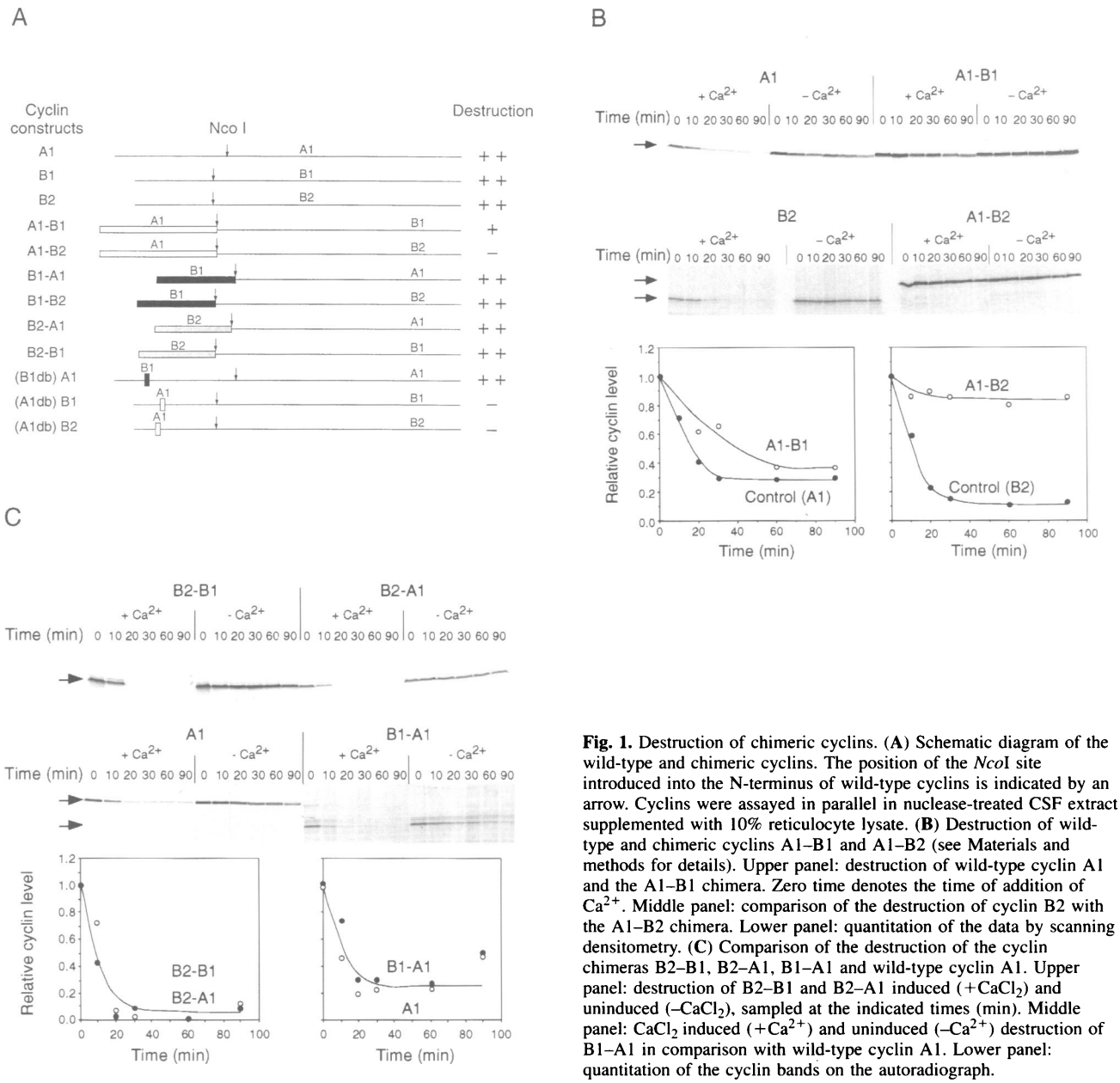

Fig. 1. Destruction of chimeric cyclins. (**A**) Schematic diagram of the wild-type and chimeric cyclins. The position of the *Nco*I site introduced into the N-terminus of wild-type cyclins is indicated by an arrow. Cyclins were assayed in parallel in nuclease-treated CSF extract supplemented with 10% reticulocyte lysate. (**B**) Destruction of wild-type and chimeric cyclins A1–B1 and A1–B2 (see Materials and methods for details). Upper panel: destruction of wild-type cyclin A1 and the A1–B1 chimera. Zero time denotes the time of addition of Ca^{2+}. Middle panel: comparison of the destruction of cyclin B2 with the A1–B2 chimera. Lower panel: quantitation of the data by scanning densitometry. (**C**) Comparison of the destruction of the cyclin chimeras B2–B1, B2–A1, B1–A1 and wild-type cyclin A1. Upper panel: destruction of B2–B1 and B2–A1 induced ($+CaCl_2$) and uninduced ($-CaCl_2$), sampled at the indicated times (min). Middle panel: $CaCl_2$ induced ($+Ca^{2+}$) and uninduced ($-Ca^{2+}$) destruction of B1–A1 in comparison with wild-type cyclin A1. Lower panel: quantitation of the cyclin bands on the autoradiograph.

chimeric cyclins A1–B1 and A1–B2 could be misfolding that prevented their binding to $p34^{cdc2}$. Accordingly, we assayed all the cyclin chimeras for their ability to bind to and activate $p34^{cdc2}$. Synthetic mRNAs for the various constructs were translated in a 1:1 mixture of RNase-treated *Xenopus* egg extract and reticulocyte lysate in the presence of [^{35}S]methionine. Bacterially produced GST–$p34^{cdc2}$ was added at the start of the incubation, and after translation, the labelled cyclins bound to GST–$p34^{cdc2}$ were recovered using GSH–Sepharose beads (Kobayashi *et al.*, 1994). The cyclins recovered from the affinity matrix were visualized by SDS–PAGE and autoradiography. Figure 2 shows that the indestructible cyclin A1–B1 and A1–B2 chimeras bound strongly to GST–$p34^{cdc2}$ (lanes 4 and 5). The chimeras showed similar histone H1 kinase activity to their corresponding wild-types (data not shown). As a negative control, lane 13 shows that a cyclin A1 mutant R197K, which is unable to bind to $p34^{cdc2}$, did not bind to GSH–Sepharose. Immunoprecipitation with anti-$p34^{cdc2}$ antibodies confirmed that all the chimeras were able to bind to endogenous $p34^{cdc2}$ (data not shown).

B-type cyclins are indestructible upon substitution of an A-type destruction box

In the chimeric cyclin constructs, it is reasonable to suppose that the immediate surroundings of the destruction box are correctly folded, but as can be seen in Figure 1, the distance between the destruction box and the cyclin box (which binds to $p34^{cdc2}$) is altered. We previously found that this spacing can be critical, for deletions that bring the destruction box too close to the cyclin box strongly stabilize cyclin A1 (Stewart *et al.*, 1994). We therefore tested the effects of mutating the three or four residues in the destruction boxes of cyclins B1 and B2 to create the cyclin A1 destruction box sequence in an otherwise unaltered B-type context (see Figure 3A). These constructs were translated in *Xenopus* egg extract containing 10% reticulocyte lysate by volume to improve

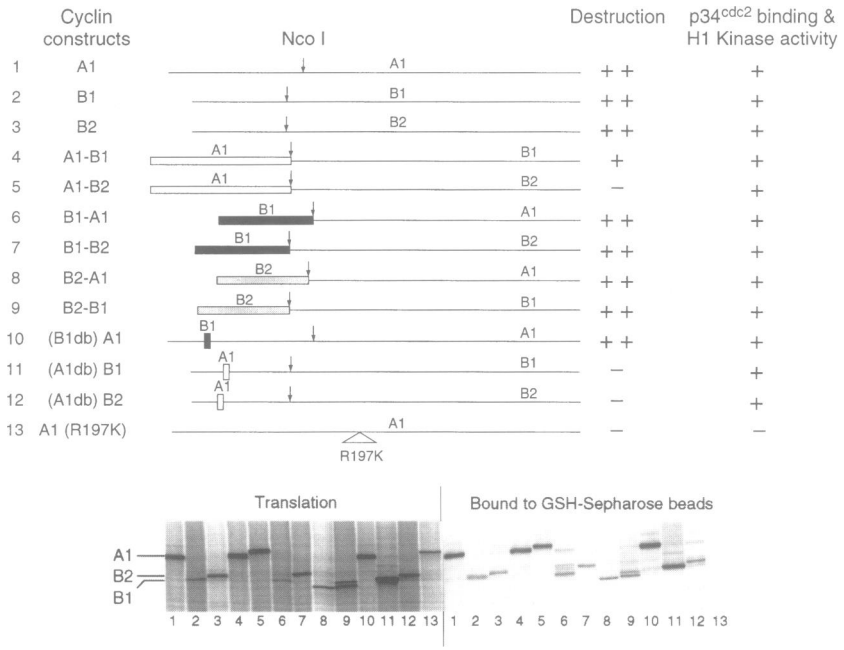

Fig. 2. Binding of chimeric cyclins to p34^{cdc2}. Upper panel: schematic diagram of wild-type and chimeric cyclins as well as a cyclin A1 mutant (cyclin box mutant A1 R197K). The triangle at the A1 R197K construct marks residue 197 where the conserved arginine in the cyclin box was exchanged for a lysine. Complete destruction is indicated as (++), binding to p34^{cdc2} and H1 kinase activity are indicated by (+). Lower panel: constructs were translated in the presence of [^{35}S]methionine and bacterially expressed GST–p34^{cdc2} in a 1:1 mixture of RNase-treated, CSF-arrested egg extract and reticulocyte lysate. Cyclins bound to p34^{cdc2} were recovered by GSH–Sepharose and analysed by SDS–PAGE and autoradiography. The left-hand side of the autoradiograph shows the translation; the right-hand side shows the affinity chromatography. The numbers below each lane indicate the construct. Only construct 13 (A1 R197K) showed no detectable binding to GST–p34^{cdc2}.

translation, and tested in the standard destruction assay. Figure 3B shows that cyclin B1 with an A1-type destruction box [(A1db)B1] was strongly stabilized as compared with wild-type cyclin B1. Similarly, Figure 3C shows that changing the destruction box of cyclin B2 to the A1 type destruction box [(A1db)B2] had an even more pronounced stabilizing effect than it did for cyclin B1. On the other hand, the constructs in which the B1-type destruction box was substituted for the destruction box of cyclin A1 [(B1db)A1] showed normal proteolysis, as might have been expected from the domain swap constructs (data not shown). We therefore did not construct (B2db)A1, and have not identified the residue(s) responsible for the stabilizing effect of the A-type destruction box on B-type cyclins.

Cyclin ubiquitination

As previously discussed in the Introduction, there is much evidence to suggest that the ubiquitin degradation pathway is involved in the proteolysis of mitotic cyclins (Glotzer *et al.*, 1991; Hershko, 1991; King *et al.*, 1995; Seufert *et al.*, 1995; Sudakin *et al.*, 1995). A cyclin-specific ubiquitin ligase is activated in mitosis and covalently links ubiquitin chains to the N-terminus of mitotic cyclins to mark the cyclin as a substrate for the multicatalytic proteasome.

To investigate whether or not the indestructible cyclin chimeras were ubiquitinated, we developed a simple assay for ubiquitination that could be used in crude egg extracts. We constructed a version of *Xenopus* ubiquitin with an N-terminal hexahistidine tag (H_6-Ub) which could be purified from bacteria or frog egg extracts by affinity chromatography on Ni^{2+}-NTA–agarose (Beers and Callis, 1993). The purified H_6-Ub was added to *Xenopus* egg extracts supplemented with reticulocyte lysate (50% v/v final concentration, see Materials and methods) in the presence of [^{35}S]methionine and the appropriate synthetic mRNA. When sufficient synthesis of the labelled substrate

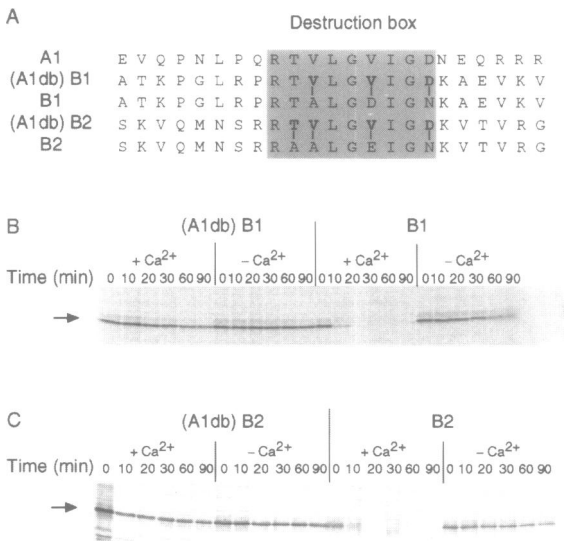

Fig. 3. Degradation of destruction box chimeras. (**A**) Diagram to compare the amino acid sequences of the destruction boxes and flanking sequences of cyclin A1, B1 and B2. The destruction box swaps (A1db)B1 and (A1db)B2 show the amino acids (in bold) that were changed to create the A1-type destruction box. (**B**) Degradation of (A1db)B1 in comparison with wild-type cyclin B1. (**C**) Destruction of (A1db)B2 in comparison with wild-type cyclin B2. Arrows indicate the unmodified cyclins.

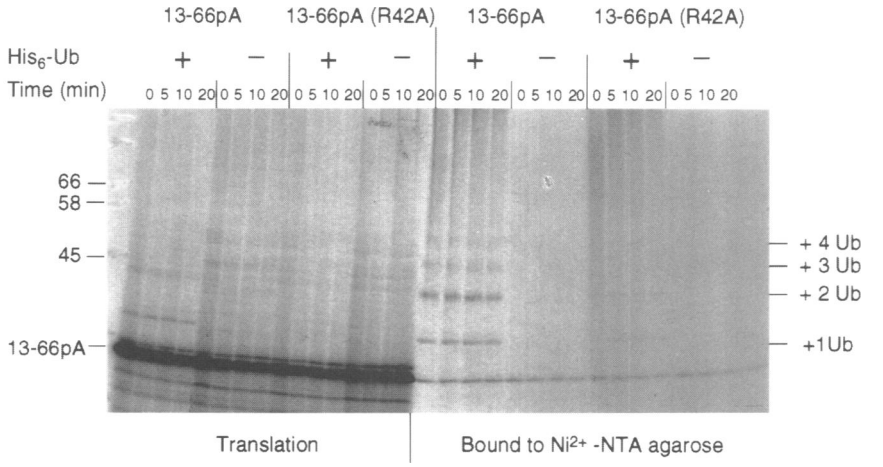

Fig. 4. Ubiquitination of sea urchin cyclin B 13-66pA and 13-66pA (R42A). The test constructs were translated in the presence of [^{35}S]methionine in a 1:1 mixture of RNase-treated egg extract and reticulocyte lysate in the presence or absence of H_6-ubiquitin. $CaCl_2$ was added to induce destruction and samples taken for analysis at the indicated times. Left: [^{35}S]methionine-labelled 13-66pA and 13-66pA (R42A) translated in the presence (+) or absence (–) of added H_6-ubiquitin (H_6-Ub). Right: affinity chromatography with Ni^{2+}-NTA–agarose to recover H_6-ubiquitin.

had occurred, destruction was induced by adding $CaCl_2$. The reaction was stopped at various times after addition of the Ca^{2+} by sampling into binding buffer (see Materials and methods), so that any labelled proteins that had become covalently linked to the histidine tagged ubiquitin could be recovered with Ni^{2+}-NTA–agarose.

Since this assay system differs from that used by Glotzer *et al.* (1991), we first tested if we could reproduce their results when using their constructs. We translated the mRNA of the sea urchin cyclin B–protein A construct (13-66pA) and its destruction box mutant [13-66pA (R42A)] in our assay system and recovered the proteins bound to histidine-tagged ubiquitin with Ni^{2+}-NTA–agarose. The left side of Figure 4 shows the autoradiogram of the translation products of both constructs during a time-course between 0 and 20 min after the addition of $CaCl_2$. The right-hand side shows the proteins that were recovered by Ni^{2+}-NTA–agarose. Without added H_6-Ub, there was very slight retention of the labelled 13-66 protein A translation product by the affinity matrix, but no sign of ubiquitin adducts. When H_6-Ub was present, however, at least two clear bands, together with a higher-molecular weight smear, were retained by the Ni^{2+}-NTA–agarose. The discrete bands presumably represent adducts with one and two ubiquitin molecules. As expected from the results of Glotzer *et al.* (1991) the intensity of these discrete bands retained by the affinity resin was much lower when the destruction box mutant form of 13-66pA was used in a parallel incubation. Indeed, addition of the bacterially expressed ubiquitin led to the appearance of one or two new higher-molecular weight bands that could be detected without the need for affinity chromatography, which were not detected in the case of the destruction box mutant. These results suggested that H_6-Ub and chromatography on Ni^{2+}-NTA–agarose could be used as an assay for ubiquitination in the crude cell-free system.

To check that this assay was specific for proteins undergoing destruction, we tested some stable proteins. We looked for ubiquitination of $p34^{cdc2}$ and $p33^{cdk2}$ after translation of their mRNAs as described above, or used [^{35}S]methionine-labelled proteins expressed in bacteria as substrates for the assay. Figure 5 summarizes the results of these experiments, in which Ca^{2+} was added at time 0 in all cases. The left side of Figure 5A shows the total translation products in CSF extract; wild-type cyclin B1 or $p33^{cdk2}$ were translated in the presence or absence of H_6-Ub. After the addition of $CaCl_2$, cyclin B1 was rapidly degraded whereas $p33^{cdk2}$ remained stable. After affinity purification with Ni^{2+}-NTA–agarose, H_6-Ub conjugates were recovered in the case of cyclin B1, but not in the reactions with labelled Cdk2. Similar results were obtained for $p34^{cdc2}$ (data not shown). As shown on the left side of Figure 5B, bacterially expressed [^{35}S]methionine-labelled GST–protein was stable during the time-course of incubation, and no GST–ubiquitin conjugates were detected. In order to check that the cyclin-specific degradation and ubiquitination had been switched on during the assays in which the stable proteins were unmodified, Figure 5C compares bacterially expressed [^{35}S]-labelled maltose-binding protein (MBP) with cyclin B1 produced by translation in the extract. MBP was stable during the time-course of 60 min, whereas cyclin B1 was rapidly degraded (Figure 5C, left lanes). The recovery with Ni^{2+}-NTA–agarose shows that only cyclin B1 formed the ladder of higher-molecular weight polypeptides (as well as a higher-molecular weight smear), whereas the MBP protein showed neither a ubiquitination ladder nor a high molecular weight smear containing MBP protein. The retention of MBP on the Ni^{2+}-NTA when H_6-Ub was added seems to represent a non-covalent interaction between the two proteins that was not seen in other cases.

Cyclin ubiquitination also occurs in indestructible cyclins

We next tested the ubiquitination of the cyclin domain swap construct A1–B2 that had proven to be surprisingly resistant to proteolysis. The left side of Figure 6A shows the translation of the constructs in the presence or absence of H_6-Ub. The right half of Figure 6A shows the labelled proteins that were recovered by Ni^{2+}-NTA–agarose. The destruction of the wild-type cyclin A1 was slow, probably because the translation reactions contained 50% v/v reticulocyte lysate in order to increase the translation efficiency. Scanning densitometry showed that about half

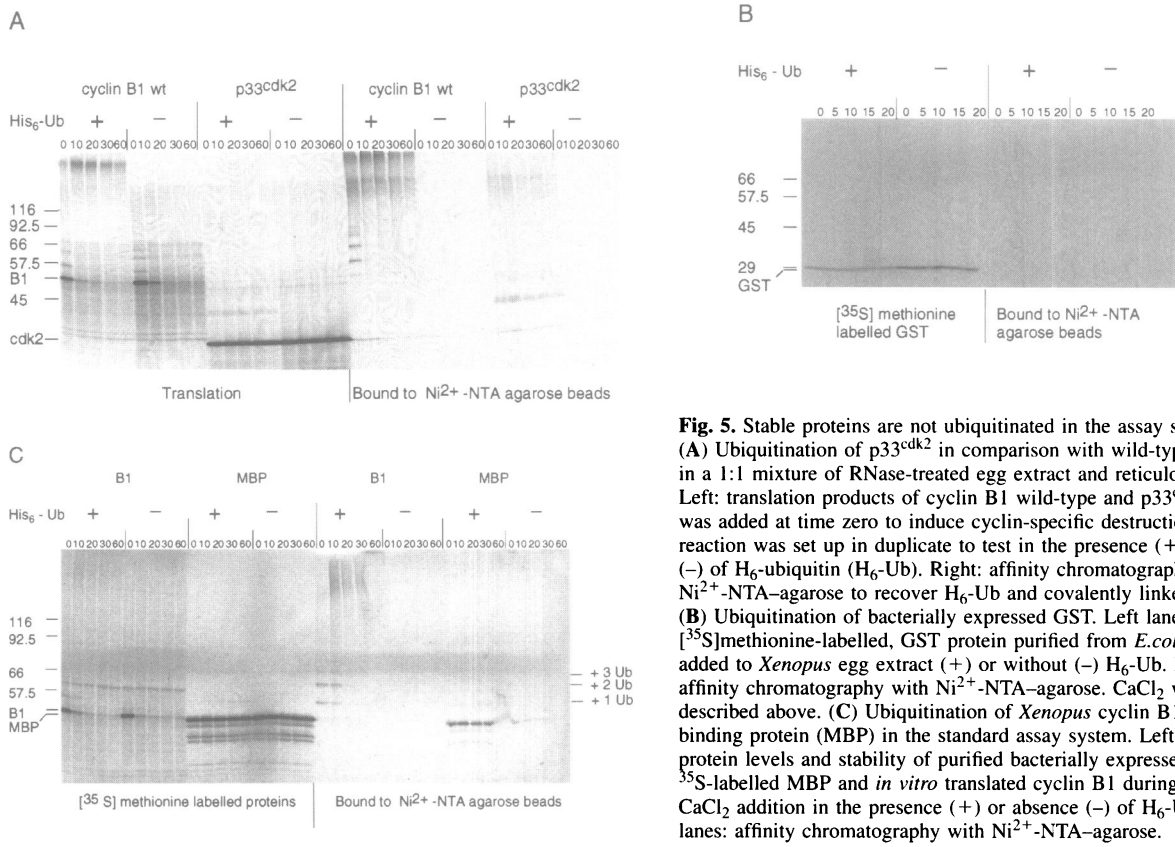

Fig. 5. Stable proteins are not ubiquitinated in the assay system. (**A**) Ubiquitination of $p33^{cdk2}$ in comparison with wild-type cyclin B1 in a 1:1 mixture of RNase-treated egg extract and reticulocyte lysate. Left: translation products of cyclin B1 wild-type and $p33^{cdk2}$; $CaCl_2$ was added at time zero to induce cyclin-specific destruction. Each reaction was set up in duplicate to test in the presence (+) or absence (–) of H_6-ubiquitin (H_6-Ub). Right: affinity chromatography with Ni^{2+}-NTA–agarose to recover H_6-Ub and covalently linked proteins. (**B**) Ubiquitination of bacterially expressed GST. Left lanes: [^{35}S]methionine-labelled, GST protein purified from *E.coli* BL21 was added to *Xenopus* egg extract (+) or without (–) H_6-Ub. Right lanes: affinity chromatography with Ni^{2+}-NTA–agarose. $CaCl_2$ was added as described above. (**C**) Ubiquitination of *Xenopus* cyclin B1 and maltose binding protein (MBP) in the standard assay system. Left lanes: protein levels and stability of purified bacterially expressed, ^{35}S-labelled MBP and *in vitro* translated cyclin B1 during 60 min after $CaCl_2$ addition in the presence (+) or absence (–) of H_6-Ub. Right lanes: affinity chromatography with Ni^{2+}-NTA–agarose.

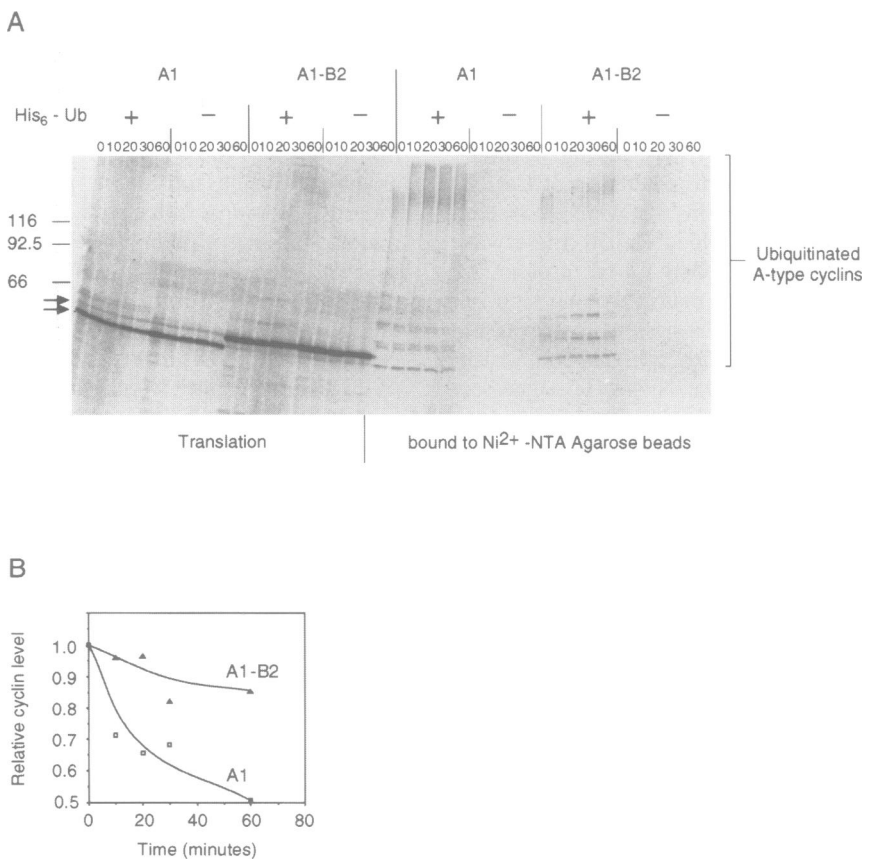

Fig. 6. Ubiquitination of the indestructible cyclin chimera A1–B2 in comparison with wild-type cyclin A1. (**A**) Left lanes: destruction of [^{35}S]methionine-labelled A1–B2 and cyclin A1 translated in the presence (+) or absence (–) of H_6-ubiquitin (His_6-Ub). Right lanes: affinity chromatography with Ni^{2+}-NTA–agarose. The arrows indicate the unmodified cyclin. (**B**) Quantitation of the cyclin bands during the destruction.

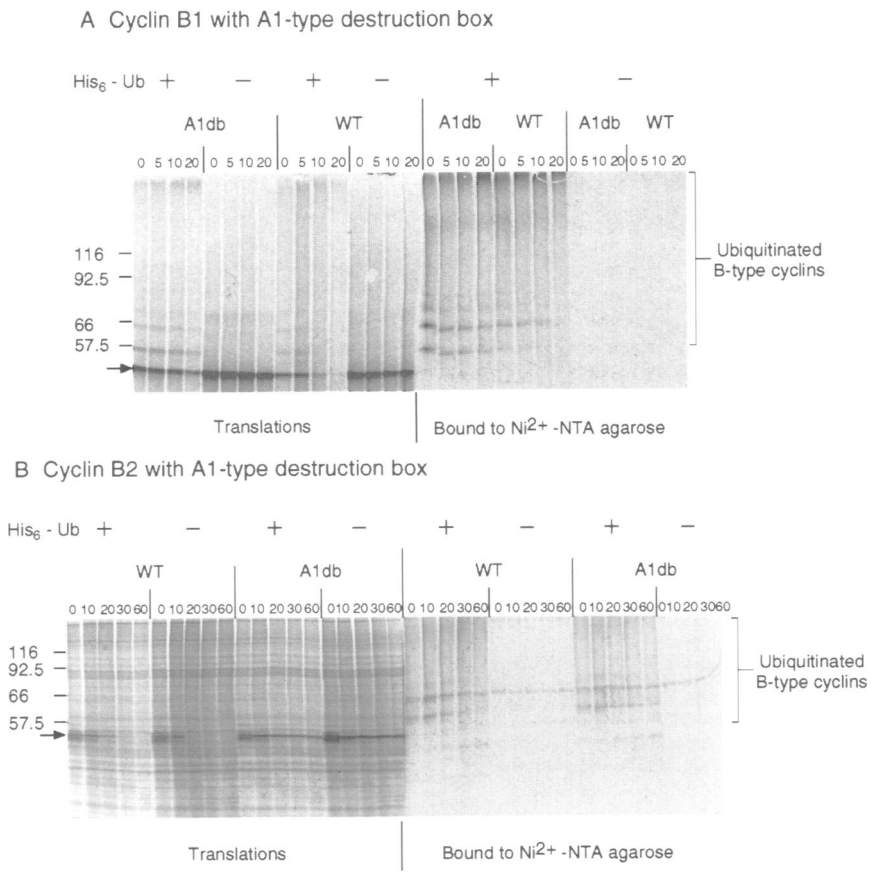

Fig. 7. Indestructible cyclins are still ubiquitinated. (**A**) Ubiquitination of the indestructible cyclin destruction box chimera (A1db)B1 in comparison with wild-type cyclin B1. The arrow indicates the unmodified cyclins. Left lanes: destruction of [^{35}S]methionine-labelled (A1db)B1 and B1 translated in the presence (+) or absence (−) of H_6-ubiquitin. Right lanes: affinity chromatography with Ni^{2+}-NTA–agarose. (**B**) Ubiquitination of the indestructible cyclin destruction box chimera (A1db)B2 in comparison with wild-type cyclin B2. In order to see the destruction of the wild-type cyclin B2 the amount of reticulocyte lysate was reduced to 20%. Left lanes: affinity chromatography of [^{35}S]methionine-labelled (A1db)B2 and B2 translated in the presence (+) or absence (−) of H_6-ubiquitin. Right lanes: affinity chromatography with Ni^{2+}-NTA–agarose.

the wild-type cyclin A1 was destroyed in the presence or absence of H_6-Ub, whereas <10% of the cyclin A1–B2 chimera was lost during the time-course. When H_6-Ub was added, however, recovery of wild-type cyclin A on Ni^{2+}-NTA–agarose showed a ladder of [^{35}S]methionine-labelled proteins with a spacing of ~7 kDa between each band as well as a higher-molecular weight smear. The intensity of these bands was reduced but not completely absent in the case of the A1–B2 chimera. Notably, the higher-molecular weight bands slowly increased during the incubation after addition of $CaCl_2$, as though ubiquitination could take place, but not proteolysis of the ubiquitinated adducts. No labelled proteins were retained by Ni^{2+}-NTA–agarose in the absence of added H_6-Ub.

We next compared the ubiquitination of wild-type cyclin B1 and the (A1db)B1 construct in which the destruction box of cyclin B1 was substituted by the A1 type destruction box. Figure 7A shows that both constructs showed a ladder of poly-ubiquitinated bands when the translation products were recovered with Ni^{2+}-NTA–agarose. The high molecular weight smear of the indestructible (A1db)B1 increased during the time-course, whereas in the case of wild-type cyclin B1, the discrete bands corresponding to 1–3 ubiquitin adducts declined as proteolysis proceeded. The negative control of the cyclin constructs translated in the absence of H_6-Ub did not reveal any labelled proteins after affinity chromatography. These data suggest a somewhat surprising conclusion that the indestructible constructs can be ubiquitinated, but are not degraded. It is difficult to compare the rates of ubiquitination, however, particularly since the wild-type cyclins (and their ubiquitinated forms) are very unstable. Figure 7B shows the result of the ubiquitination assay with (A1db)B2, the most stable of the altered destruction box constructs. It was also able to form ubiquitinated adducts.

The H_6-Ub assay was also used with various indestructible mutants of cyclin A1, and gave very similar results. In general we found that the ubiquitination of destructible and indestructible versions of cyclin A was more similar than might have been expected from the results with the sea urchin cyclin B–protein A fusion construct, further suggesting that ubiquitination alone may not be sufficient for cyclin proteolysis. For example, Figure 8 shows an experiment using a cyclin A destruction box mutant (RTVL→ATVA), which is the equivalent mutation to the 13-66pA R42A construct. Total translation and destruction are shown on the left side of the autoradiogram and the recovery of H_6-Ub-bound cyclins is shown on the right. Although the translation efficiency of the destruction box mutant was lower then with the wild-type cyclin A1, higher-molecular weight forms covalently linked to H_6-Ub could easily be detected.

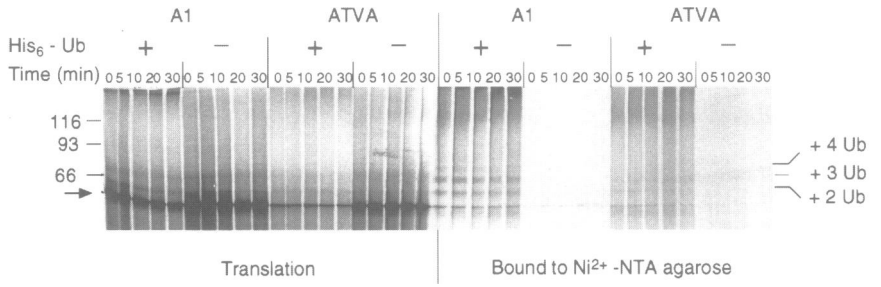

Fig. 8. Ubiquitination of the indestructible cyclin destruction box mutant (ATVA) in comparison with wild-type cyclin A1. Left lanes: destruction of [^{35}S]methionine-labelled cyclin A-ATVA and wild-type cyclin A1 translated in the presence (+) or absence (–) of H_6-ubiquitin. $CaCl_2$ was added at time point zero. Right lanes: affinity chromatography with Ni^{2+}-NTA–agarose.

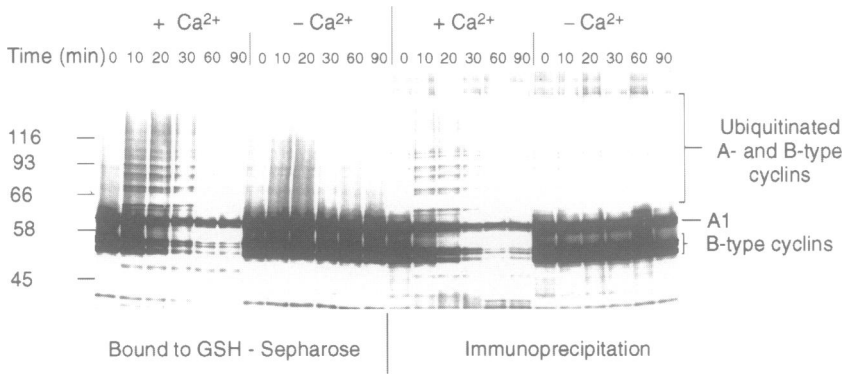

Fig. 9. Ubiquitinated cyclin is still bound to $p34^{cdc2}$. Cyclin A1 mRNA and endogenous B-type cyclins were translated in untreated *Xenopus* egg extract in the presence of [^{35}S]methionine. Bacterially expressed GST–cdc2 was added to half the reactions during translation (left 12 lanes). Cyclin degradation was induced by the addition of $CaCl_2$ as indicated. Samples were taken at the indicated times. The cyclins that were bound to $p34^{cdc2}$ were recovered with GSH–Sepharose when GST–cdc2 was present, or by immunoprecipitation with A17 anti-$p34^{cdc2}$ antibody. Left lanes: affinity chromatography with GSH–Sepharose. Right lanes: immunoprecipitation with anti-$p34^{cdc2}$ antibodies.

Ubiquitination does not dissociate cyclins from $p34^{cdc2}$

The foregoing results suggested an imperfect correlation between ubiquitination and cyclin destruction. On the other hand, the experiment shown in Figure 9 confirmed that there is a large increase in the rate of formation of ubiquitinated forms of cyclins after addition of $CaCl_2$ to the CSF extracts. This can be detected after long exposures of destruction assays in which labelled cyclins bound to $p34^{cdc2}$ were recovered either by immunoprecipitation with anti-$p34^{cdc2}$ antibodies (Figure 9, right-hand panels) or when translation took place in the presence of added GST–$p34^{cdc2}$, and the products recovered with affinity chromatography on GSH–Sepharose (Figure 9, left-hand panels). A ladder of ubiquitinated cyclins were bound to $p34^{cdc2}$ after $CaCl_2$ was added, disappearing as the cyclins were destroyed. Figure 9 shows that these discrete ubiquitinated forms of the cyclins were not present in the controls without added $CaCl_2$. It is intriguing that the ubiquitinated derivatives of cyclins remained bound to $p34^{cdc2}$, raising the question of how and when the stable cyclin–cdc2 dimers are dissociated, at what point the cyclin is degraded, and how the $p34^{cdc2}$ avoids modification and destruction, considering its tight association with the cyclin (Kobayashi *et al.*, 1994).

Discussion

In this paper, we show that B-type cyclins with the N-terminus of cyclin A1 are largely resistant to the normal destruction of mitotic cyclins that occurs when $CaCl_2$ is added to *Xenopus* egg extracts. By contrast, the domain swap constructs with the N-terminus of B-type cyclins grafted onto cyclin A1 were degraded with normal kinetics. The rate of degradation of the chimera A1–B1 was ~50% of normal, and the chimeric cyclin A1–B2 was essentially stable. When we changed only the three or four residues in the destruction box of cyclin B1 and B2 to generate the A-type 'destruction box', the results were even more striking. Cyclin B1 with the A1-type destruction box [(A1db)B1] was highly stabilized and cyclin (A1db)B2 was completely stable when compared with the wild-type cyclins. We previously showed that mutant cyclins A1 and B2 that are unable to bind to $p34^{cdc2}$ are indestructible (Stewart *et al.*, 1994), but all the cyclin chimeras reported in this paper can bind to $p34^{cdc2}$ and activate similar levels of H1 kinase when compared with the wild-type cyclins, suggesting that the mutations we introduced do not induce general misfolding of the cyclins. In any case, it is generally the case that misfolded proteins are better substrates for intracellular proteolysis than their correctly folded forms. Some of the cyclin chimeras were stable for at least 90 minutes, whereas the wild-type cyclins were rapidly degraded.

These results lead to a conclusion that differs significantly from those originally obtained by Glotzer *et al.* (1991), who wrote that '...the N terminus of cyclin is not only necessary but also sufficient for M-phase-specific degradation'. While this may be true for sea urchin cyclin B, it is clearly not so for the endogenous cyclins found

in the frog egg. Similar discrepancies were noted by Van der Velden and Lohka (1994), who showed that although the detached N-terminus of cyclin B2 was not degraded, addition of this construct to egg extracts could inhibit the destruction of endogenous cyclins, implying that it was able to compete with the endogenous cyclins for some essential component of the degradation machinery. Others have also found that quite large portions of cyclins are required to mediate cell cycle-specific degradation; Amon *et al.* (1994) had to use almost full-length Clb2 fused to β-galactosidase in order to produce a chimera that was degraded with similar kinetics to the cyclin itself. We favour the idea that the correct presentation of the destruction box normally requires the cyclin to be bound to $p34^{cdc2}$, and that there may be some interaction between the destruction box and the cyclin itself. Unfortunately, the recent structure of cyclin A in complex with CDK2 lacks the N-terminal 170 residues of cyclin A (Jeffrey *et al.*, 1995), so the shape and disposition of the destruction box is still unknown.

Thus, although there seems to be a pathway for mitotic cyclin destruction that depends on the conserved sequence in the destruction box, different signals are required for each cyclin that finally lead to proteolysis. A variety of degradation signals would not only guarantee cyclins to be recognized as a substrate for proteolysis, but also allow a more precise timing of their destruction. For some reason, these signals are not interchangeable.

Cyclin ubiquitination

Glotzer *et al.* (1991) were the first to find that the N-terminus of cyclin B is a target for the ubiquitin degradation pathway. They showed that a fusion protein consisting of residues 13–90 of sea urchin cyclin B fused to staphylococcal protein A, which undergoes cell cycle-regulated destruction, was ubiquitinated. In contrast, a 'destruction box' mutant of this fusion protein, which is indestructible, showed very much less poly-ubiquitination. It is thought that the destruction box provides the recognition site for a cyclin-specific ubiquitin ligase which covalently links ubiquitin to one or more lysine residues within the cyclin N-terminus (although it is not yet known if some ubiquitination sites are more important than others). These results, together with those of Hershko *et al.* (1991), suggest that cyclin ubiquitination is necessary for degradation and that the destruction box is required for this process (Glotzer *et al.*, 1991). Thus, cyclins that cannot be ubiquitinated cannot be degraded. By implication, indestructible cyclins would probably not be ubiquitinated. We wanted to test this prediction, and to see if cyclin chimeras that failed to be destroyed did so because they were not ubiquitinated. The assay we used differed from that of Glotzer *et al.* in several respects. They used interphase extracts of frog eggs in which cyclin destruction was constitutively induced by addition of recombinant Δ90 sea urchin cyclin B, and used bacterially synthesized model substrates that were labelled by iodination *in vitro*. By contrast, we used CSF-arrested egg extracts that were transiently induced to destroy cyclins by addition of 0.4 mM Ca^{2+}, and our substrates were produced by cell-free translation *in situ*. We detected ubiquitinated intermediates by addition of bacterially produced N-terminally His_6-tagged ubiquitin, which could be recovered from reactions using Ni^{2+} affinity chromatography. We were able to validate our assay system by obtaining essentially identical results when using the original constructs of Glotzer *et al.* Conversely, none of four stable proteins we tested ($p33^{cdk2}$, $p34^{cdc2}$, GST or maltose-binding protein) formed detectable adducts in this assay. These results give us confidence that this assay can provide a valid test for the ubiquitination of the indestructible cyclin chimeras. We found that the stabilized and the indestructible cyclin chimeras showed the same characteristic higher-molecular weight polypeptides when compared with the wild-type cyclins, but the pattern of ubiquitinated intermediates was somewhat different from that of wild-type cyclins. The ubiquitinated forms in the indestructible mutants tended to accumulate during the reaction, whereas the higher-molecular weight derivatives of the wild-type cyclins decreased, due to their destruction. In addition, the wild-type cyclins tended to show higher levels of discrete high-molecular weight bands. It is difficult to compare the level of ubiquitination between the stable chimeras and the wild-type cyclins, partly because of the extreme heterogeneity of the adducts, and also because the translation efficiency of different cyclin constructs varied considerably. Nevertheless, taking all these considerations into account we can say that the cyclin chimeras are ubiquitinated, even though they are not degraded. We also found that highly ubiquitinated forms of cyclins were still associated with $p34^{cdc2}$, showing that ubiquitination *per se* is not what dissociates cyclins from their kinase partners. Thus, it would appear that while ubiquitination is an important part of the cyclin destruction process, other factors must play a role.

Somewhat similar conclusions to these were very recently reported by Mahaffey *et al.* (1995), who found that *Xenopus* cyclin B2 formed ubiquitin conjugates in CSF-arrested frog egg extracts in which the protein is perfectly stable. An interesting general problem is of how and when the intrinsically stable kinase-binding domain of the cyclins is dissociated from its partner, to which it is extremely tightly bound. This is particularly hard to understand in view of the high stability of cyclins that lack their N-terminal domains. Does the destruction box-dependent ubiquitination make the cyclin a substrate for a highly processive protease, presumably the 26S proteasome, which consumes the protein like a strand of noodle? Or is the protein first denatured or even cut by other (as yet unidentified) enzymes so that it drops its kinase partner and cannot cause damage by generating indestructible intermediates? Further work is required to delineate the pathway and its regulation. The results presented in this paper, together with our previous finding that only cyclins associated with $p34^{cdc2}$ were rapidly degraded, leads us to suspect that specific interaction between the destruction box and the cyclin/CDK complex is required for dissociation and degradation of the cyclin moiety.

Materials and methods

Cyclin constructs

The cyclin constructs were obtained by mutagenesis using the PCR, carried out essentially as described by Horton and Pease (1991). The constructs were subcloned into a pGEM vector. An *Nco*I site was

introduced at equivalent positions corresponding to proline residues in the N-terminal domains of cyclins A1 (P137), B1 (P97) and B2 (P93), leaving the amino acid sequence unchanged. For each cyclin we designed 5′ and 3′ primers encoding a single nucleotide exchange that resulted in a *Nco*I site. The resulting constructs were subcloned into pGEM vectors.

Cyclin chimeras were constructed by digesting constructs with *Nco*I and an appropriate restriction enzyme either at the 5′ or the 3′ end of the gene. The resulting fragment was then substituted by the N- or C-terminal fragment of another cyclin digested with the same enzymes. In some cases it was necessary to make intermediate subclones in pUC18.

Destruction box swap mutants

The cyclin destruction box chimeras were constructed by PCR mutagenesis as described above with specific oligonucleotides to convert the destruction box of cyclin B1 or B2 into the A1 type, or the cyclin A1 destruction box into the B1 type destruction box.

Oligonucleotides:

A1db in cycB1
5′ CCTCGTACAGTCTTGGGAGTCATTGGAGACAAG
3′ CTTGTCTCCAATGACTCCCAAGACTGTACGAGG

A1db in cycB2
5′ AGACGAACTGCTTTGGGAGTGATTGGCGACAAAGTG
3′ CACTTTGTCGCCAATCACTCCCAAAGCAGTTCGTCT

B1db in cycA1
5′ CAAAGAACTGCGTTGGGTGACATCGGTAACAATGAA
3′ TTCATTGTTACCGATGTCACCCAACGCAGTTCTTTG

The resulting constructs were subcloned into pGEM1. In all cases, the sequences were checked by DNA sequencing.

RNase treatment of CSF extract

The mobility of some of the cyclin chimeras is the same as their corresponding wild-type parent. In order to distinguish them from the translation products of wild-type cyclin mRNA, we routinely treated the CSF extract with RNase essentially as described by Murray (1991). RNase A was added to the extract at a final concentration of 1 μg/ml and incubated for 15 min at 10°C followed by the addition of RNA Guard (Pharmacia, Uppsala, Sweden) at a final concentration of 2000 U/ml and incubated for a further 10 min at 10°C. This RNase-treated extract was then used immediately for the translation of the RNA of interest.

Cyclin destruction assay

Cyclin destruction assays were carried out as previously described by Kobayashi *et al.* (1992) with minor alterations. Most of the chimeric cyclin mRNAs were poorly translated in pure frog egg extract. We therefore routinely translated all our cyclin mRNAs in a mixture of RNase-treated CSF-arrested egg extract with 10% reticulocyte lysate. Typically, translation of 1 μg/ml of mRNA took place for 1–2 h in the presence of [^{35}S]methionine. The reaction was terminated by the addition of cycloheximide (100 μg/ml final concentration) to block further protein synthesis. A 2 μl aliquot of this translation reaction was mixed with 10 μl of fresh cycloheximide-treated CSF extract. Cyclin destruction was triggered by the addition of $CaCl_2$ to a final concentration of 0.5 mM. Samples were taken at intervals after adding the $CaCl_2$ and analysed by SDS–PAGE. The intensity of the labelled cyclin bands on the autoradiograph were quantified by scanning densitometry.

Affinity chromatography of egg and reticulocyte extracts on GSH–Sepharose

The mRNAs encoding the chimeric constructs and their corresponding wild-type cyclins were translated in a 1:1 mixture of nuclease-treated, CSF-arrested extract and rabbit reticulocyte lysate for 2 h at 23°C in the presence of 1 mCi/ml (final concentration) [^{35}S]methionine. During the translation bacterially expressed and purified GST–p34^{cdc2} (80 μg/ml final concentration) was present to facilitate the recovery of p34^{cdc2}-bound cyclin constructs by affinity chromatography on GSH–Sepharose (Pharmacia, Uppsala, Sweden). Translation was terminated by the addition of cycloheximide (100 μg/ml final concentration) and 5 μl were incubated with GSH–Sepharose diluted in bead buffer (10 mM Tris–HCl 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). Samples were rotated at 4°C for 1 h. The p34^{cdc2}–cyclin complexes bound to GSH–Sepharose were washed four times with bead buffer and transferred into a fresh tube. Samples were divided in two and one aliquot of the [^{35}S]methionine-labelled complexes was directly analysed by SDS–PAGE and autoradiography. The other portion of the beads was tested for H1 kinase activity as described below.

H1 kinase assay

To determine whether the cyclin chimeras formed active complexes with p34^{cdc2}, the protein complexes bound to GSH–Sepharose were assayed for H1 kinase activity in a buffer containing [γ-^{32}P]ATP (Amersham PB 10218, Amersham, UK) . The kinase buffer [5 mM Tris–HCl pH 7.5, 10 mM NaCl, 0.5 mM NaF, 0.1 mM DTT, 30 μM EDTA, 200 μg/ml histones, 15 mM $Mg(OAc)_2$ and 100 μM ATP] was added to the GSH–Sepharose beads and immediately incubated at 25°C for 10 min. The reaction was stopped by addition of 5× SDS sample buffer and reactions were analysed by SDS–PAGE and autoradiography.

Ubiquitination assays

Plasmids encoding mRNAs of interest were translated in a 1:1 mixture of RNase-treated, CSF-arrested egg extract and reticulocyte lysate in the presence of 0.5 mCi/ml [^{35}S]methionine. To this extract was added 1 mM ATP, 0.1 mM EGTA, 1 mM $MgCl_2$ and an ATP-regenerating system (10 mM creatine phosphate and 0.1 mg/ml creatine phosphokinase). Bacterially expressed and purified histidine-tagged ubiquitin (H_6-Ub) was added to a final concentration of 0.5 mg/ml. Translation was carried out for 30 min at 23°C, stopped with cycloheximide (0.1 mg/ml) and destruction was initiated by the addition of $CaCl_2$. In the control reactions, ubiquitin storage buffer was added in place of H_6-Ub. Aliquots were taken at intervals to analyse translation efficiency and destruction as well as for affinity chromatography with Ni^{2+}-NTA–agarose (Qiagen). To recover ubiquitin-linked proteins, reactions were stopped in a 50- to 100-fold volume of binding buffer (20 mM Tris–HCl pH 7.5, 0.5 M NaCl and 5 mM imidazole). 10 μl of Ni^{2+}-NTA–agarose beads were added and samples rotated for 30–60 min at 4°C. Samples were washed three times with buffer II (20 mM Tris–HCl pH 8.0, 0.5 M NaCl, 25 mM imidazole, pH 8.0, 0.5% Triton X-100 and 0.5% Tween-20) and once with binding buffer. To prevent non-specific binding, 1% milk powder was added to the first two washes. Ubiquitin-conjugated proteins were eluted from the resin by boiling in SDS sample buffer with 10 mM EDTA pH 8.0. Samples were analysed by SDS–PAGE and autoradiography.

In vivo labelling of bacterially expressed proteins

Escherichia coli BL21(DE3) containing the plasmid encoding the protein to be expressed were grown in 2× TY medium until the A_{600} reached 0.6–0.7. Bacteria were collected by centrifugation, washed with M9 minimal media and resuspended in M9 minimal media supplemented with essential amino acids minus methionine. The cells were incubated at 37°C for 45 min in the presence of 0.1 mM IPTG, at which time [^{35}S]methionine (10 μCi/ml) was added. Cells were harvested after incubation at 37°C for a further 15–30 min and lysed in the appropriate buffer according to the protein purification.

Preparation of His$_6$-ubiquitin

His$_6$-ubiquitin in pET3b was transformed into *E.coli* strain BL21(DE3) and 400 ml cultures were grown at 37°C to an A_{600} of ~0.8. Expression of the recombinant proteins was induced with 100 μM IPTG at 23°C for 12–16 h. The cells were harvested and lysed with buffer I (20 mM Tris–HCl pH 7.5, 0.5 M NaCl and 5 mM imidazole) supplemented with 1 mM PMSF, 5 mM benzamidine and 1 mg/ml lysozyme) at 4°C for 15 min. After sonication, insoluble material was pelleted at 18 000 *g* for 30 min, the supernatant filtered through a 0.45 μm filter (Millipore) and applied onto Ni^{2+}-NTA–agarose (Qiagen) equilibrated with buffer I. The matrix was washed with 5 column volumes of buffer I, followed by 10 column volumes of buffer II (20 mM Tris–HCl pH 8.0, 0.5 M NaCl, 25 mM imidazole, 0.5 % Triton X-100 and 0.5% Tween-20) and 5 column volumes with buffer I. The H_6-ubiquitin was eluted with 20 mM Tris–HCl pH 8.0, 0.5 M NaCl and 150 mM imidazole, dialysed against 20 mM Tris–HCl pH 8.0, 20 mM NaCl, 1 mM DTT and stored at –70°C

Expression and purification of GST and MBP

For purification of GST-tagged proteins, cells were lysed in a buffer containing 50 mM Tris–HCl pH 7.3, 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 0.5% Tween-20, 1 mM DTT, 1 mM PMSF, 5 mM benzamidine and 1 mg/ml lysozyme. The sonicated and clarified supernatant was applied onto GSH–Sepharose (Pharmacia) equilibrated with buffer A (50 mM Tris–HCl pH 8.0, 0.5 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% Triton X-100, 0.5% Tween-20, 5 mM DTT), washed twice with buffer B (20 mM Tris–HCl pH 8.0, 0.5 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA and 5 mM DTT) and eluted with 20 mM

Tris–HCl pH 9.0, 0.5 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 10 mM reduced GSH (Sigma).

Cells expressing MBP were lysed in 10 mM Na-phosphate, pH 7.0, 0.5 M NaCl, 0.25% Tween-20, 10 mM 2-mercaptoethanol, 10 mM EGTA, 10 mM EDTA containing 1 mg/ml lysozyme and applied onto Amylose resin (New England Biolabs), equilibrated with the same buffer. The column was washed once with column buffer (10 mM Na-phosphate, pH 7.0, 0.5 M NaCl, 1 mM Na-azide, 10 mM 2-mercaptoethanol, 1 mM EGTA) supplemented with 0.25% Tween-20 and once without 0.25% Tween-20. The protein was eluted with column buffer containing 10 mM maltose. Purified proteins were dialysed and stored as described above.

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