Cell Cycle-Dependent Proteolysis in Plants: Identification of the Destruction Box Pathway and Metaphase Arrest Produced by the Proteasome Inhibitor MG132

Pascal Genschik, Marie Claire Criqui, Yves Parmentier, Aude Derevier, and Jacqueline Fleck
Institut de Biologie Moléculaire des Plantes du CNRS, 12 rue du Général Zimmer, 67084 Strasbourg Cédex, France

It is widely assumed that mitotic cyclins are rapidly degraded during anaphase, leading to the inactivation of the cell cycle-dependent protein kinase Cdc2 and allowing exit from mitosis. The proteolysis of mitotic cyclins is ubiquitin/26S proteasome mediated and requires the presence of the destruction box motif at the N terminus of the proteins. As a first attempt to study cyclin proteolysis during the plant cell cycle, we investigated the stability of fusion proteins in which the N-terminal domains of an A-type and a B-type tobacco mitotic cyclin were fused in frame with the chloramphenicol acetyltransferase (CAT) reporter gene and constitutively expressed in transformed tobacco BY2 cells. For both cyclin types, the N-terminal domains led the chimeric cyclin–CAT fusion proteins to oscillate in a cell cycle-specific manner. Mutations within the destruction box abolished cell cycle-specific proteolysis. Although both fusion proteins were degraded after metaphase, cyclin A–CAT proteolysis was turned off during S phase, whereas that of cyclin B–CAT was turned off only during the late G2 phase. Thus, we demonstrated that mitotic cyclins in plants are subjected to post-translational control (e.g., proteolysis). Moreover, we showed that the proteasome inhibitor MG132 blocks BY2 cells during metaphase in a reversible way. During this mitotic arrest, both cyclin–CAT fusion proteins remained stable.

INTRODUCTION

The cell division cycle is a sequential process that permits the replication of the genome, the segregation of chromosomes to two daughter nuclei, and finally cytokinesis. Progression through the cell cycle involves many proteins, which are regulated both at the transcriptional and post-translational levels. Today, it has become clear that the timed destruction of key proteins plays an essential role in cell cycle progression. The first protein shown to be subjected to cell cycle-specific proteolysis was cyclin B (Evans et al., 1983). For many other proteins, cell cycle-specific proteolysis has been reported: among them are the G1 cyclins and the mitotic cyclins, the cyclin-dependent kinase inhibitors (CKIs), proteins involved in sister chromatid separation, and spindle components (reviewed in Peter and Herskowitz, 1994; Murray, 1995; King et al., 1996; Hoyt, 1997). The degradation of cell cycle regulator proteins is not just a way to break down a protein whose function is achieved at a specific step of the cell cycle, but it is also a way to directly control the cell cycle. Thus, the highly regulated proteolysis of CKIs allows DNA replication to begin, and degradation of proteins involved in sister chromatid separation is required at the onset of anaphase.

Proteins subjected to degradation are marked with ubiquitin tags and subsequently are targeted to the degradative action of the 26S proteasome. The ubiquitin/26S proteasome proteolytic pathway is highly conserved in eukaryotes and is involved in many other important cellular functions aside from cell cycle progression (reviewed in Hochstrasser, 1995). Degradation via this pathway is a two-step process: the protein is first tagged by the covalent attachment of ubiquitin; subsequently, it is degraded by a multicatalytic protease complex called the 26S proteasome. Conjugation of ubiquitin to the protein involves a cascade of three enzymes: E1, E2, and E3. The E1 (ubiquitin-activating) enzyme forms a high-energy bond with ubiquitin, which is then transesterified to a ubiquitin-conjugating enzyme (E2). The transfer of ubiquitin to the target protein substrate usually requires ubiquitin ligase activity (E3).

Proteolysis during mitosis requires specific E3 activity located on a large complex called the APC or cyclosome (reviewed in King et al., 1996; Townsley and Ruderman, 1998). At the onset of and during anaphase, several key proteins are degraded, for example, mitotic cyclins (reviewed in Murray, 1995), the Saccharomyces cerevisiae anaphase inhibitor Pds1p (Cohen-Fix et al., 1996), the Schizosaccharomyces pombe anaphase inhibitor Cut2p (Funabiki et al., 1996), the cohesion protein Scc1p (Michaelis et al., 1997), and a protein associated with the mitotic spindle Ase1p (Juang et al., 1997). Although the proteolysis of all these proteins is mediated by the APC complex, the timing of their respective degradation differs, indicating a further element in the
complexity of the degradation machinery. In vertebrates, cyclin A is degraded during metaphase, whereas cyclin B is degraded later during mitosis (Minsull et al., 1990; Whitfield et al., 1990; Hunt et al., 1992); in budding yeast, Pds1p proteolysis precedes that of cyclin Clb2p (Cohen-Fix et al., 1996). Thus, to complete the degradation of an APC substrate at the appropriate time, other proteins are involved in the recognition of the substrates and the regulation of the proteolytic machinery.

The only structural motif identified in APC substrates is a nine-amino acid sequence called the destruction box (D box) motif in the N-terminal domain of the targeted proteins. This motif seems to be critical for the stability of the proteins because mutations in or removal of the box stabilizes cyclins A and B in cell-free systems (Glotzer et al., 1991; Luca et al., 1991; Lorca et al., 1992a; Holloway et al., 1993; van der Velden and Lohka, 1993; Brandeis and Hunt, 1996), in transfected and stably transformed vertebrate cells (Gallant and Nigg, 1992; Brandeis and Hunt, 1996), in frog oocytes (Murray et al., 1989; Luca et al., 1991), in Drosophila embryos (Rimmington et al., 1994; Sigrist et al., 1995), in budding yeast (Surana et al., 1993; Amon et al., 1994), and in fission yeast (Yamanoe et al., 1996). Furthermore, replacement of the D box sequences of Xenopus B1 and B2 cyclins by the cyclin A1 D box sequence stabilizes the proteins in the frog egg extracts, indicating that D box signals may not be interchangeable (Klotzbücher et al., 1996). Deletion of the N-terminal domain of Cut2p, which contains two sequences similar to the cyclin D box, stabilizes the protein, and it was shown that both sequences are required for proteolysis during anaphase (Funabiki et al., 1996). Degradation of Pds1p, the S. cerevisiae ortholog of Cut2p, also requires an intact D box (Cohen-Fix et al., 1996). Finally, the Ase1 protein contains five sequences similar to the D box motif, and mutation of one of them stabilizes the protein (Juang et al., 1997).

No data on proteolysis during the cell cycle have been reported thus far for plants. Significantly, however, a putative D box is conserved in all mitotic plant cyclins (reviewed in Plesse et al., 1998; Renaudin et al., 1998). Moreover, by using indirect immunofluorescence analysis, it was shown recently that the level of maize cyclin 1b1 declines dramatically during anaphase in root tip cells (Meuw et al., 1997), supporting the notion of B-type cyclin destruction during mitosis.

As a first step to study cyclin proteolysis in plants, we investigated the stability of fusion proteins in which the N-terminal domains of tobacco mitotic cyclins were fused in frame with the chloramphenicol acetyltransferase (CAT) reporter gene. We decided to use two mitotic cyclins: the A-type cyclin (CycA105; Reichheld et al., 1996) and the B-type cyclin (NTCYC1; Qin et al., 1995). Cyclin NTCYC1 belongs to the CycB1 group and was renamed Nicta;CycB1;1, and cyclin CycA105 belongs to the CycA3 group and was renamed Nicta;CycA3;1 according to the classification of Renaudin et al. (1998). The two cyclins are expressed at different times during the cell cycle. A-type cyclin mRNAs accumulate from S phase until the end of the G2 phase, whereas B-type cyclin mRNAs were found to accumulate exclusively from late G1 to early mitosis (Setiady et al., 1995; Qin et al., 1996; Reichheld et al., 1996). The N-terminal domains of both cyclins were fused to a reporter gene under the control of the cauliflower mosaic virus (CaMV) 35S constitutive promoter, and stably transformed tobacco BY2 cell lines were established. We demonstrate that the levels of the chimeric proteins oscillate during the cell cycle and that this oscillation is dependent on an intact D box. Furthermore, we used the proteasome inhibitor carbobenzoxy-leucinyl-leucinyl-leucinal (MG132) and show that it induces cell cycle arrest in tobacco BY2 cells during metaphase and inhibition of the proteolysis of chimeric cyclin-CAT fusion proteins.

RESULTS

Establishing Stably Transformed Tobacco BY2 Cell Lines Constitutively Expressing the Chimeric Cyclin-CAT Fusion Proteins

To study mitotic cyclin protein oscillations during the cell cycle, we fused the N terminus of an A-type cyclin (Nicta; CycA3;1) and a B-type cyclin (Nicta;CycB1;1) to the bacterial CAT reporter enzyme (constructs CycA105-CAT and CycB-CAT; Figure 1A). Both cyclins carry the D box motif (Rxx-Lxx[L/I]xN) in their N-terminal domain (where R and L residues are highly conserved and x stands for any amino acid). The dependence of reporter protein turnover on the intact D box was also investigated. In constructs CycA105mutDbox-CAT and CycBmutDbox-CAT (Figure 1A), we mutated the two highly conserved amino acids of the D box motifs from RxxLxx(L/I)xN to GxxVxx(L/I)xN. In vertebrates, it has been shown that these two mutations abolish the mitotic instability of cyclin B1 (Brandeis and Hunt, 1996). The chimeric genes were cloned into a binary vector under the control of the constitutive CaMV 35S promoter rather than the endogenous cyclin promoters to avoid transcriptional regulation of the constructs during the cell cycle. The constructs were introduced into tobacco BY2 cells by Agrobacterium-mediated transformation.

For all constructs, two to three independently transformed cell lines were established and propagated in liquid medium. After several subcultures, the transformant growth curves were indistinguishable from that of the untransformed BY2 cell culture (shown in Figure 1B with one transgenic cell line per construct), and CAT mRNAs accumulated to the same level in asynchronously growing cell cultures (shown in Figure 1C with one of the CAT control transgenic cell lines). The accumulation level of CAT fusion proteins in the different transgenic cell lines showed a broad range of variation (5 to 2000 pg per 50 µg of total protein; data not shown). For example, the CAT fusion protein levels determined at mid-log growth phase from three different CycB-CAT transgenic
cell lines (CycB–CAT;10oct, CycB–CAT;22sept, and CycB–CAT;24oct) were 4, 30, and 400 pg per 50 μg of total protein, respectively. Nevertheless, despite this variation, the growth curves of all transgenic lines were comparable with those of the wild-type BY2 cell culture, indicating that the expression of the chimeric protein is not toxic and does not disturb the cell cycle.

After synchronization of the CAT control transgenic cell culture by a 24-hr treatment with aphidicolin (an inhibitor of DNA polymerase), mRNA accumulation under the control of the CaMV 35S promoter was constant throughout the cell cycle, as shown by RNA gel blot analysis (Figure 2B). Rehybridization of the same blot with the histone H4 probe and the mitotic cyclin B probe, respectively, showed the characteristic S and M phase mRNA accumulation patterns. Recently, Ito et al. (1998) also reported the constitutive expression of the firefly luciferase gene under the control of the CaMV 35S promoter throughout the BY2 cell cycle. All synchronized cell cultures were analyzed by RNA gel blotting and always showed the same constitutive expression of the chimeric constructs throughout the cell cycle (data not shown). Total protein was extracted from synchronized CAT control cultures, and the CAT protein level was determined. As expected, no significant changes of the CAT protein level were observed during the cell cycle (Figure 2A).

**Proteolysis of the Chimeric Cyclin–CAT Proteins throughout the Cell Cycle**

In a synchronized CycB–CAT cell culture, the chimeric protein started to accumulate during the G2 phase of the cell cycle and reached its maximum level 1 hr earlier than the peak in the mitotic index (Figure 3). At this time, many of the cells passed metaphase. The decrease in protein level paralleled the decline in the mitotic index and both stayed very low at the G1-to-S transition (S’ indicates the second DNA replication phase). The difference in chimeric protein levels between mitosis and interphase was ~10-fold. For CycA105–CAT constructs, the chimeric protein levels decreased similarly at the end of mitosis. However, the level of CycA105–CAT protein was always higher during S phase (directly after aphidicolin release) when compared with the level during G1 phase (13 to 16 hr) and was high during G2. In addition,
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The accumulation pattern during the cell cycle is different. For constructs CycBmutDbox-CAT and CycA105mutDbox-CAT, mutations inside the D box motif clearly abolished the cell cycle-specific oscillations of the fusion proteins (Figure 3). To address the problem of reproducibility, we performed the synchronization experiments with at least two independently transformed cell lines per construct. For constructs CycB-CAT and CycA105-CAT, we used three independently transformed cell lines. For a given construct, we observed that the oscillation patterns during the cell cycle of the ectopically expressed fusion protein were identical in high- or low-expressing transgenic cell cultures (data not shown).

To confirm that the proteolysis of CycA105-CAT fusion protein is already turned off during S phase, we followed the accumulation of the CycA105-CAT protein from 14 hr after aphidicolin release until 25 hr (roughly from G_{1} to the second mitosis; Figure 4A). In this synchronization experiment, the mitotic indexes for the first and second mitotic peak were 40% (data not shown) and 20% at 9 and 25 hr, respectively, after aphidicolin release. The level of CycA105-CAT protein during the first mitosis reached a value of 22 pg per 50 μg of total protein (data not shown). Under these conditions, the level of CycA105-CAT protein stayed low during G_{1} phase and started to accumulate during mid-S phase. In contrast to the cyclin A construct, the CycB-CAT fusion protein was present at low levels in a similar experiment, indicating that proteolysis of the B-type cyclin is maintained during DNA replication (data not shown). These results indicate that proteolysis of the A-type cyclin-CAT chimeric protein is turned off during S phase, which is earlier than for the cyclin B construct.

Unfortunately, we could not determine exactly when during mitosis the cyclin fusion protein proteolysis is turned on. From different synchronization experiments, we found that degradation of both CycA105-CAT and CycB-CAT fusion proteins only started when anaphase figures appeared (data not shown). Furthermore, we checked the stability of the chimeric proteins in metaphase-arrested cells. The two CycA105-CAT and CycB-CAT cultures were first synchronized with aphidicolin and then treated with the antitubulin drug oryzalin during early mitosis (when ≤5 to 10% of prophases could be counted) and reached a metaphase arrest of ~40% (Figure 4B). Under these conditions, after 4 hr of culture in the presence of oryzalin, >95% of the mitotic cells were in metaphase (the remainder being in prophase). Thus, we could show that in both cultures, the fusion proteins accumulated and were stable in the metaphase-blocked cells. Similar results were obtained when we used the antitubulin drug propyzamide (data not shown). From these observations, we can only conclude that cyclin fusion protein degradation is a postmetaphase event occurring during anaphase.
The Proteasome Inhibitor MG132 Induces Metaphase Arrest in Tobacco BY2 Cell Cultures

Taken together, the results presented above indicate that plant mitotic cyclins are degraded by the D box pathway. Because the final stage in degradation of D box–containing proteins involves the 26S proteasome, we decided to test the effect of MG132, which is a very efficient proteasome inhibitor in mammalian cell cultures (Rock et al., 1994). MG132 has no effect on wild-type yeast cells because they are impermeant to this agent; experimentation requires the use of mutant strains with enhanced permeability to the drug (Lee and Goldberg, 1996). Here, we demonstrate that MG132 can be used in plant cell suspension cultures at a final concentration of 100 μM. The concentration effect and cytotoxicity of the drug for plant cells will be described elsewhere (M.C. Criqui, Y. Parmentier, A.C. Schmit, and P. Genschik, manuscript in preparation).

To examine the mitotic effect of MG132 on plant cells, BY2 cells were first synchronized with aphidicolin, and the drug was added during G₂ phase (at 4.5 hr after aphidicolin release, as soon as the first prophase was observed) or early mitosis (at 6.5 hr after aphidicolin release, when ~10% of mitotic cells, mostly in prophase, were observed). As a control, cells were subcultured in the presence of DMSO alone; no effect on mitotic progression could be detected (Figure 5A). In the presence of MG132, the BY2 cells could clearly enter mitosis and progress through prophase, but

Figure 3. Accumulation Patterns of the Cyclin–CAT Fusion Proteins during the Cell Cycle.

The progression through the cell cycle was monitored by ³H-thymidine incorporation and mitotic index determination. The curves presented in this figure were obtained with cell lines CycB–CAT;24oct (CycB–CAT), CycBmutDbox–CAT;10oct (CycBmutDbox–CAT), CycA105–CAT;22sept (CycA105–CAT), and CycA105mutDbox–CAT;24oct (CycA105mutDbox–CAT). Symbols are as given for Figure 2.
they were arrested at metaphase with fully condensed chromosomes congregating at the metaphase plate (Figures 5B and 6A). If MG132 was added at 6.5 hr of culture, almost 50% of the cells could be blocked in metaphase. Immunofluorescent staining of the microtubules in drug-treated cells showed that metaphase spindles and the interphase microtubules appeared to be normal (Figures 6E and 6F) compared with the untreated control BY2 cells (Figures 6C and 6D).

We also assayed histone H1 kinase activity in protein extracts from control and MG132-treated cells. Protein samples from untreated and drug-treated cells (at 8.5 and 13.5 hr after aphidicolin release; indicated by an asterisk in Figure 5B) were extracted by using a p13suc1-Sepharose matrix, which is commonly used to isolate cyclin-dependent kinase (CDK)-cyclin complexes (Labbé et al., 1991). The activity of the bound kinases was monitored by the in vitro phosphorylation of the histone H1 protein, which was used as a substrate (Figure 5C). In contrast to untreated cells, in which H1 kinase activity decreased at 13.5 hr of culture, activity remained elevated in the blocked cells. H1 kinase activity was already higher at 8.5 hr in the MG132-treated cells, in which the percentage of cells in prophase and metaphase was higher than in the control.

The addition of MG132 later during mitosis, at a time when most of the cells had passed metaphase, showed aberrant anaphase activity and also cells with very enlarged nuclei, which often underwent amitotic divisions (Figure 6B), indicating that 26S-dependent proteolysis is also involved in other steps during mitosis and not just the metaphase-to-anaphase transition. The effect of the drug on those cells is currently under investigation. We also used another peptide aldehyde, carbobenzoxyl-leucinyl-leucinyl-norvalinal (MG115), and found that in BY2 cell cultures, the drug had the same effect, although MG132 was slightly more effective (data not shown).

MG132 Stabilizes the Chimeric Cyclin–CAT Fusion Proteins

We examined the effect of the proteasome inhibitor on chimeric cyclin–CAT protein stability. CycB–CAT and CycA105-
CAT transgenic cell cultures were first blocked with aphidicolin. In early mitosis after aphidicolin release (when ~5 to 10% of prophases were observed), half of the cultures were treated with MG132. Mitotic indexes and CAT protein levels were determined at 1-hr intervals in cell cultures either treated or not with MG132 (Figure 7A). As reported above, the transition from prophase to metaphase was not affected by the drug, and the treated cells accumulated during metaphase. For both cultures, the cyclin-CAT proteins accumulated and remained stable during drug treatment. However, the level of the cyclin-CAT fusion protein was always lower in the MG132-treated cells than in the controls. RNA gel blot analysis indicated that the drug has an effect on transcription or RNA stability because the cyclin-CAT mRNA level was

![Graph A](image1)

**Figure 5.** Metaphase Arrest in MG132-Treated Synchronized BY2 Cells.

(A) The mitotic index throughout the cell cycle in aphidicolin-synchronized BY2 cells in the presence (+) and absence (−) of 0.2% DMSO. Arrows indicate the times after aphidicolin release at which MG132 was added (4.5 hr [1] or 6.5 hr [2]).

(B) Percentage of mitotic cells (prophase, metaphase, anaphase, and telophase) in untreated and MG132-treated BY2 cell cultures. The asterisks indicate the times chosen to assay histone H1 kinase activity. The 1 and 2 indicate the times at which MG132 was added (see [A]).

(C) Histone H1 kinase assays were carried out with p13 suc1–Sepharose—bound protein fractions from untreated (−) and drug-treated (+) cell cultures. Histone H1 loading was controlled by Coomassie Brilliant Blue R 250 staining (data not shown). Phosphorylated histone H1 was visualized by autoradiography and estimated by direct Cerenkov counting of the labeled bands.

(D) Percentage of mitotic cells after MG132 release.
already lower 3 hr after the drug treatment (Figure 7B). Furthermore, we obtained a similar mRNA accumulation pattern when we used the constitutively expressed Arabidopsis EF-Tu elongation factor as a probe (data not shown).

Nevertheless, MG132 does not affect the level of all mRNAs equally, because inhibition of the 26S proteasome by this drug induces accumulation of the polyubiquitin Ubi.U4 mRNA. Recently, an increase of heat shock hsp70 mRNAs was also found in HepG2 cells treated with different proteasome inhibitors (Zhou et al., 1996), and induction of heat shock proteins after drug treatment was reported both in mammalian and yeast cells (Bush et al., 1997; Lee and Goldberg, 1998).

However, the polyubiquitin Ubi.U4 gene is not readily heat shock inducible (Genschik et al., 1994a), and it is not clear whether the signaling that activates this gene uses the same pathway as that which activates the hsp genes. Nonetheless, our results indicate that a defect in proteolysis by the 26S proteasome can lead to the activation of at least one component of this proteolytic pathway (e.g., a polyubiquitin gene).

**DISCUSSION**

In recent years, the molecular characterization of plant genes involved in cell cycle control (e.g., CDKs, cyclins, CKIs, and retinoblastoma protein homologs) suggests that the basic mechanisms controlling the cell cycle are also highly conserved in plants. However, until now, the function of proteolysis during plant cell cycle progression has not been addressed. In this study, we used two approaches: (1) we analyzed the stability of mitotic cyclin–CAT fusion proteins in synchronized BY2 cell cultures; and (2) we studied the effect of the proteasome inhibitor MG132 on mitosis.

Previously, it was shown that the highly conserved D box motif RxxLxxIxxN in the N terminus of mitotic cyclins and other APC substrates is important for their degradation (see the Introduction). In this study, we showed the function of two different tobacco mitotic cyclin N-terminal domains in cell cycle–dependent proteolysis. In the constructs tested, the cyclin core regions were replaced by the CAT reporter protein, thereby preventing the chimeric protein from interacting with CDK proteins. For the Xenopus cyclins A and B2, it was previously shown that mutations affecting the binding of CDK stabilize these cyclins, even if the D box is intact (Stewart et al., 1994; van der Velden and Lohka, 1994), indicating that proteolysis of those cyclins requires their binding to CDK1. Nevertheless, this interaction is not required for Xenopus cyclin B1 and sea urchin cyclin B (Glotzer et al., 1991; Holloway et al., 1993; Stewart et al., 1994; van der Velden and Lohka, 1994). Moreover, fusion of the 105 N-terminal residues of mouse cyclin B1 to the CAT reporter enzyme led to the degradation of chimeric proteins at the end of mitosis in stably transformed NIH3T3 cells, as occurred with the endogenous B-type cyclin (Brandeis and Hunt, 1996). Curiously, a similar experiment in which the 86 N-terminal residues of mouse cyclin B2 were fused to the CAT reporter enzyme gave the same results (Brandeis and Hunt, 1996). Our data clearly indicate that all of the information necessary for cell cycle–specific breakdown for both tobacco A-type and B-type cyclins is located in the N-terminal domains of the proteins and that interaction with a CDK partner is not required for degradation of the chimeric proteins. In addition, the degradation of the chimeric proteins is dependent on an intact D box. Thus, we demonstrate that in plants as well as in animals, a proteolytic pathway is activated at the end of metaphase and that this pathway recognizes the highly conserved D box motif.

The accumulation pattern of the CycB–CAT fusion protein during the BY2 cell cycle is similar to the expression pattern of the tobacco B-type cyclin genes (Setiady et al., 1995; Qin et al., 1996; Reichheld et al., 1996; results not shown), despite the constitutive expression of the chimeric construct. Thus, if the chimeric fusion protein reflects the endogenous tobacco B-type cyclin level, as is the case for the mouse B1 cyclin (see above), then the protein accumulates during late G2 phase and is degraded after metaphase. The window of stability of the CycA105–CAT fusion protein is wider than...
that for the B-type cyclin. During S phase, the chimeric protein level started to increase again and was high throughout the G2 phase. The endogenous mRNAs of this cyclin also accumulate during the S and G2 phases (Reichheld et al., 1996). Thus, the patterns of protein stability for both cyclins during the cell cycle are consistent with the expression patterns of their respective genes.

In Xenopus extracts, it has been shown that human cyclin B—but not cyclin A—is able to turn on the proteolytic machinery (Lorca et al., 1992b). This activation is most probably controlled by the phosphorylation of APC and is not directly induced by cyclin B-Cdc2; however, at least one downstream kinase is required for phosphorylation (reviewed in Townsley and Ruderman, 1998). Recently, evidence was provided that the mouse Polo-like kinase activates APC by phosphorylation after the kinase itself is activated during early mitosis by cyclin B-Cdc2 (Kotani et al., 1998). The rapid increase in accumulation of the CycB–CAT fusion protein just before the D box pathway is activated suggests that this may also be the case for plants.

Even if the stability of the plant mitotic cyclins during the cell cycle is similar to that in the animal model, there are at least two important differences. (1) The tobacco cyclin B fusion protein accumulates only late during G2 phase, and the window of stability is very sharp, whereas animal mitotic B-type cyclins are already starting to accumulate during S phase (Pines and Hunter, 1989; Jackman et al., 1995; Brandeis and Hunt, 1996). Meanwhile, the CycA105–CAT protein has already accumulated during S phase, at a time when CycB–CAT degradation occurs. The differences in stability between both fusion proteins during S phase and early G2 phase could be explained by the fact that the CycA105–CAT construct may carry information at its N terminus that protects the chimeric protein from degradation. Alternately, the CycB–CAT fusion protein may be a much better substrate for proteolysis than is the CycA105–CAT protein at a time when the proteolytic machinery is not fully active. (2) In animal cells, degradation of cyclin A occurs during metaphase, which is earlier than for cyclin B. Cyclin B is degraded during anaphase, and the spindle assembly checkpoint cannot prevent its destruction (Minshull et al., 1990; Whitfield et al., 1990; Hunt et al., 1992). In BY2 cells, however, the degradation of both cyclin A and B fusion proteins starts only after metaphase, and their proteolysis was not detected in

Figure 7. Accumulation Patterns of the Cyclin–CAT Protein and mRNAs during the Cell Cycle in Untreated and MG132-Treated CycB–CAT and CycA105–CAT Cell Cultures.

(A) Progression throughout the cell cycle was monitored by mitotic index determination (open diamonds) in untreated (−MG132) and drug-treated (+MG132) cell cultures. The fusion protein level is indicated by solid squares. The arrows indicate the times at which 100 μM of MG132 was added. In the treated cultures, cells arrested exclusively in metaphase. In these experiments, we used cell lines CycB–CAT;10oct (CycB–CAT) and CycA105–CAT;10oct (CycA105–CAT).
Connection of the cell cycle to the proteasome

The proteasome is a multienzyme complex responsible for the degradation of ubiquitinated proteins. It is known to play a role in regulating cell cycle progression, particularly during mitosis. The presence of the proteasome in both animal and yeast cells indicates its importance in these organisms. When the drug propyzamide was added to plant cells, it led to mitotic arrest at metaphase with highly condensed chromosomes. For both cyclin A– and cyclin B–CAT chimeric proteins, the drug led to stabilization of cyclin B1. This suggests that the proteasome is involved in the degradation of certain cyclins during the metaphase-to-anaphase transition.

The proteasome is a proteolytic machinery that is responsible for the degradation of ubiquitinated proteins. It is composed of a 26S proteasome that contains a 20S core particle and regulatory subunits. The 20S particle is a cylindrical structure composed of 14 subunits arranged in a 4 × 4 × 2 array. The regulatory subunits are responsible for the specificity of the proteolytic activity.

The proteasome is involved in the degradation of certain cyclins during the metaphase-to-anaphase transition. This is because the proteasome is responsible for the degradation of cyclins, which are important for cell cycle progression. The degradation of cyclins is necessary for their proper localization and function during the cell cycle. The proteasome is a crucial component of this process, and its inhibition can lead to mitotic arrest.

METHODS

Procedures

Unless stated otherwise, all procedures for manipulating DNA and RNA were conducted according to Sambrook et al. (1989) and Ausubel et al. (1994).

Proteasome Inhibitors

Carbobenzoxyl-leucinyl-leucinyl-norvalinal (MG115) and carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) were provided by PEPTIDES International, Inc. (Louisville, KY). The drugs were dissolved in DMSO and were never kept for >1 month at −20°C.

Cyclin-Chloramphenicol Acetyltransferase Fusion Constructs

XbaI and SacI sites were introduced 5′ and 3′, respectively, of the chloramphenicol acetyltransferase (CAT) coding sequence from the Escherichia coli enzyme by polymerase chain reaction (PCR)-based site-directed mutagenesis. The PCR reaction was performed using oligonucleotide 1 (5′-CTAAGGAACTAGATGGAGAAGAAAAATCTC-3′) and oligonucleotide 2 (5′-GACACAAAGCCTCCCTAAAAAATTACG-3′) as the upstream and downstream primers, respectively, and plasmid pRT99-CAT as the template (Töpfer et al., 1988). The XbaI-SacI fragment was cloned into the pBluescript II SK+ (Stratagene, La Jolla, CA) vector. The same strategy was used to introduce BamHI and XbaI sites upstream and inside of, respectively, the coding regions of tobacco cyclin A (CycA105, renamed Nicta;CycA1;1; Reichheld et al., 1996) and tobacco cyclin B (NTCYC1, renamed Nicta;CycB1;1; Qin et al., 1995). For cyclin A, we used oligonucleotide 3 (5′-CAGCCGCGATGTTGGATTGAGT-3′) and oligonucleotide 4 (5′-CGTCTGCTCGTACAGTACAGTCACATC-3′); for cyclin B, we used oligonucleotide 5 (5′-CAAGAAGATCCTTGCCATGTTGAGT-3′) and oligonucleotide 6 (5′-ACTTCTGCTTCAATCGATGAC-3′) as upstream and downstream primers, respectively.

The PCR reaction led to the amplification of the coding region covering the first 137 amino acids of cyclin A and the first 134 amino acids of cyclin B, roughly from Met (at position +1) to the cyclin box. Both PCR fragments were cloned into the pBluescript II SK+ vector and designated pSKCycA105 and pSKCycB, respectively. The cyclin BamHI-XbaI fragments were subcloned into pSKCAT, leading to constructs pSKCycA105-CAT and pSKCycB-CAT, in which the cyclin coding regions were in frame with the CAT coding region. In constructs pSKCycA105mutDbox and pSKCycBmutDbox, we mutated high H1 kinase activity and stabilization of cyclin B1 (Sherwood et al., 1993). However, the metaphase arrest of the drug in both mammalian and plant cells cannot be ascribed to cyclin B stabilization because sister chromatid separation can proceed while mitotic CDK is active (Holloway et al., 1993; Surana et al., 1993). Thus, the metaphase arrest provoked by MG132 in BY2 cells suggests strongly the existence of a plant protein whose degradation by the proteasome at the metaphase-to-anaphase transition is absolutely required for sister chromatid separation.

It is well established that the degradation of the cell cycle regulatory proteins carrying the D box is carried out by the 26S proteasome in both animal and yeast cells. Thus, we tested the effects of the tripeptide proteasome inhibitor MG132 on mitosis. When the drug was added late in the G2 phase, the BY2 cells were still able to break down the nuclear envelope, set up the mitotic spindles, and congress the highly condensed chromosomes to the metaphase plate. For both cyclin A- and cyclin B-CAT chimeric proteins, the drug led to their stabilization. In mammalian cells, the same drug led also to mitotic arrest at metaphase with
two highly conserved amino acids in the destruction box (D box) motif from RxxLxx(L/I)xN to GxxVxx(L/I)xN by PCR using oligonucleotide 7 (5′-ACTGAAAGGTGTCCTGTGGAG-3′) and oligonucleotide 8 (5′-AGTGGATACCATTTGGGAAGG-3′) for cyclin A and oligonucleotide 9 (5′-AGAAATGAGCCTGTCCTGTGGAGAC-3′) and oligonucleotide 10 (5′-CCTCCTGCTTGGCTATTCT-3′), respectively, for cyclin B. All PCR constructs were sequenced on both strands. The BamHI-Sacl DNA fragments from each of the five constructs (pSKCAT, pSKCycA105-CAT, pSKCycA105mutDbx-CAT, pSKCycB-CAT, and pSKCycBmutDbx-CAT) were subcloned into the binary pBI121.1 vector (Clontech, Palo Alto, CA), replacing the β-glucuronidase reporter gene.

**BY2 Cell Culture, Transformation, and Synchronization**

A rapidly growing suspension culture of tobacco BY2 cells (Nicotiana tabacum cv Bright Yellow 2) was maintained by weekly dilution (1:5:100) of cells into fresh medium modified according to Nagata et al. (1992) and cultured at 27°C and 130 rpm in the dark. The plasmids described above were introduced by electroporation into the disarmed Agrobacterium tumefaciens strain LBA4404. BY2 cells were stably transformed as follows. Four milliliters of a 3-day-old BY2 culture was cocultivated with 100 μL of an Agrobacterium culture in Petri dishes in the dark for 2 days at 27°C. Cells were washed three times by centrifugation and were grown either in 40 mL of fresh medium modified according to Nagata et al. (1992), or plated on solid medium, both supplemented with carbenicillin (500 μg/mL) and kanamycin (100 μg/mL). When the cultures reached maximal density (after 1 week), 2 mL of the cultures was transferred to 80 mL of fresh medium containing carbenicillin and kanamycin. The cultures were then subjected to four or six rounds of subculturing until they reached the growth rate of the untransformed BY2 cell culture. When the transformants were plated on solid medium, 3 weeks of growth were required before calli could be recovered. Between 50 and 300 calli were pooled in liquid medium and processed as described above.

Tobacco BY2 cells were synchronized according to Nagata et al. (1992). Briefly, transgenic and control cell suspensions at stationary phase (7 days old) were subcultured for 24 hr in a medium containing aphidicolin (3 μg/mL; Sigma), washed with 1 L of sucrose solution at 40 g/L, and then suspended in 100 mL of medium modified according to Nagata et al. (1992). DNA synthesis was determined by subculturing 1 mL from the cell suspensions in the presence of 1.5 μCi 3H-TTP (94 Ci/mmol; Amersham Corp.) for 30 min at 27°C on a rotary shaker.

For extended washing of the cells, DNA was extracted, and estimation of 3H-thymidine incorporation was carried out as described previously (Genschik et al., 1994b). For mitotic index determination, cells were stained with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Sigma) in the presence of 0.2% Triton X-100; interphase, prophase, metaphase, anaphase, and telophase figures were determined for at least 600 cells by using UV light microscopy. When analysis of late (i.e., postmitotic) stages was needed, 1.54 μg/mL of propyzamide (Sumitomo Chemical Co., Osaka, Japan) or 3.46 μg/mL of oryzalin was added before the preprophase stage, ~5 to 6 hr after aphidicolin release, maintained for 4 hr, and removed by extensive washes.

**RNA Extraction and Gel Blotting**

Total RNA from the cell cultures was isolated as described by Verwoerd et al. (1989). RNA (20 μg per lane) was separated on a formaldehyde-agarose gel, blotted onto Hybond N (Amersham Corp.) nylon membrane by capillary transfer using 20 × SSPE (1 × SSPE is 0.18 M NaCl, 0.01 M Na2HPO4, and 0.001 M Na2EDTA, pH 7.7), and UV cross-linked to the membrane. The integrity and the amount of RNA applied to each lane were verified by control hybridizations using a tomato 25S rRNA probe (Kiss et al., 1989). The histone H4 probe corresponds to the 196-bp restriction fragment AccI-Ddel of the coding region of the gene H4A748 (Chaboute et al., 1987). The probe specific for the polyubiquitin gene Ubi.4 corresponds to the 626-bp restriction fragment Sall-HindIII of gene Ubi.4 (Genschik et al., 1994a). The cyclin B probe corresponds to a Nicta;CycB1;1 cDNA (Qin et al., 1995). The CAT probe corresponds to the complete CAT coding sequence (Töpfer et al., 1988). The probes were labeled with [α-32P]dCTP (3000 Ci/mmol; Amersham Corp.) by the random priming method (Feinberg and Vogelstein, 1983). RNA gel blots were hybridized overnight at 42°C in 5 × SSPE, 50% formamide, 10% dextran sulfate, 1% SDS, and 50 μg of denatured salmon sperm DNA. The blots were subsequently washed in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS for 30 min at 42°C and in 0.2 × SSC and 0.1% SDS for 30 min at 42°C.

**Protein Extraction, CAT Immunoassay, and Histone H1 Kinase Activity**

BY2 cells were homogenized with a Dounce tissue grinder (Wheaton, Millville, NJ) in extraction buffer (25 mM Tris-HCl, pH 7.5, 15 mM MgCl2, 15 mM EGTA, 150 mM NaCl, 0.1% Tween-20, 1 mM DTT, and complete protease inhibitor cocktail mix (Boehringer Mannheim)) and centrifuged at 20,000g. The protein content was determined by using the Bio-Rad protein assay kit. CAT protein content was determined by using the CAT ELISA kit (Boehringer Mannheim). No cross-reacting protein was found in the extracts from nontransformed BY2 cell cultures. For histone H1 kinase activity, all extracts were prepared as described above in the presence of 5 mM NaF. One hundred micrograms of total protein was incubated with 50 μL of 25% (v/v) p13Suc1–Sepharose beads (kindly provided by László Bögre, Institut für Mikrobiologie und Genetik, Vienna, Austria) for 2 hr on a rotary shaker at 4°C. After incubation, the washing conditions of the beads and the kinase reaction were as published by Magyar et al. (1993).

**Immunofluorescence Analysis**

Immunofluorescence analysis was done according to Chang-jei and Sonobe (1993). Antibodies used were a mouse antibody raised against tubulin (Amersham Corp.) diluted 1:3000 and fluorescein isothiocyanate–conjugated goat antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) raised against mouse IgG diluted 1:250.

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REFERENCES


Cell Cycle–Dependent Proteolysis in Plants: Identification of the Destruction Box Pathway and Metaphase Arrest Produced by the Proteasome Inhibitor MG132
Pascal Genschik, Marie Claire Criqui, Yves Parmentier, Aude Derevier and Jacqueline Fleck
Plant Cell 1998;10;2063-2075
DOI 10.1105/tpc.10.12.2063

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