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Cell-Cycle Dependent Dynamic Change of 26S Proteasome Distribution in Tobacco BY-2 Cells

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The 26S proteasome is known to play pivotal roles in cell-cycle progression in various eukaryotic cells; however, little is known about its role in higher plants. Here we report that the subcellular distribution of the 26S proteasome is dynamically changed in a cell-cycle dependent manner in tobacco BY-2 cells as determined by immunostaining with anti-Rpn10 (a regulatory PA700 subunit) and anti-20S catalytic proteasome antibodies. The 26S proteasome was found to localize not only in nuclear envelopes and mitotic spindles but also in preprophase bands (PPBs) and phragmoplasts appearing in G_2 and M phases, respectively. MG132, a proteasome inhibitor, exclusively caused cell-cycle arrest not only at the metaphase but also the early stage of PPB formation at the G_2 phase and the collapse of the phragmoplast, which seems to be closely related to proteasome distribution in the cells.

Keywords: Cell cycle — Microtubule — Proteasome — Rpn10 — Subcellular distribution — Tobacco BY-2 cells.

Abbreviations: MTs, microtubules; PPB, preprophase band.

Introduction

The 26S proteasome with a molecular mass of ~2,500 kDa is a highly organized protein-degrading machinery that catalyzes the ATP-dependent degradation of ubiquitinated proteins (Coux et al. 1996, Baumeister et al. 1998, Hershko and Ciechanover 1998, Rechsteiner 1998). It is composed of a core proteinase, known as 20S proteasome, and a pair of symmetrically disposed PA700 regulatory particles (also known as the 19S complex) (Tanaka 1998, DeMartino and Slaughter 1999, Ferrell et al. 2000). PA700 is attached to both ends of the central 20S proteasome in opposite orientation to form the active 26S proteasome in an ATP-dependent manner (DeMartino and Slaughter 1999, Voges et al. 1999). The 20S proteasome, which acts as the proteolytic core of the 26S proteasome, is a barrel-like particle formed by the axial stacking of four rings composed of two outer α-rings and two inner β-rings, being associated in the order of αββα (reviewed in Bochtler et al. 1999). The catalytic β-type subunits are located in a chamber formed by the centers of the abutting β rings, and the α-type subunits form a physical barrier that prevents substrates from reaching active sites. The regulator, PA700, consists of two subcomplexes known as "base" and "lid", which, in the 26S proteasome, correspond to the portions of PA700 that are proximal and distal, respectively, to the 20S proteasome (Glickman et al. 1998). The base is composed of six ATPases and three regulatory components named Rpn1, Rpn2, and Rpn10, while the lid contains multiple non-ATPase subunits (Glickman et al. 1998). The base complex, which is thought to bind to the outer α-ring of the central 20S proteasome in an ATP-dependent manner, is likely to be involved in the opening of the gate of the α-ring for entry of the protein substrate.

Currently there is increasing evidence that various cell-cycle regulatory proteins, such as cyclins and CDK (cyclin-dependent kinase) inhibitors, are degraded rapidly and timely through the ubiquitin/proteasome pathway in specific cell-cycle stages, particularly the G_1/S transition and the M phase, of mammalian and yeast cells (King et al. 1996, Elledge and Harper 1998, Deshaies 1999, Zachariae and Nasmyth 1999). However, in higher plants, little is known about the involvement of the ubiquitin/proteasome pathway in the degradation of regulatory proteins involved in cell-cycle progression, except for a recent report on the degradation of cyclins A and B by the 26S proteasome (Genschik et al. 1998, Criqui et al. 2000).

The 26S proteasome was reported to be localized in the nucleus as well as in the cytoplasm in certain higher eukaryotic cells, such as ascidian eggs (Kawahara and Yokosawa 1992)

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and rat ovarian granulosa cells (Amsterdam et al. 1993); in particular, it found to be localized in nuclear envelopes and mitotic spindles at the interphase and the M phase, respectively, in these cells. A similar predominant localization of the 26S proteasome in the nucleus was also found in yeast (Enenkel et al. 1998, Wilkinson et al. 1998, Russell et al. 1999). However, to our knowledge, there is no previous report on the association between the subcellular distribution of the 26S proteasome and cell cycle in plants. However, a recent report indicates that the 26S proteasome is present in the nuclei at the interphase in higher plants (Kwok et al. 1999). Since major cell-cycle regulatory factors, such as cyclins and CDK inhibitors, are also conserved in higher plants, as in other mammalian and yeast cells, the events in cell-cycle progression in higher plants is thought to resemble those in other eukaryotic cells. However, it is still unclear whether or not the 26S proteasome plays an essential role in cell-cycle progression in plant cells. The mechanisms of the cell cycle in higher plants differ from those in mammals in various aspects; for example, the absence of a centrosome and cell plate formation for cytoplasmic division, implying the existence of plant-specific aspects in the cell cycle.

Taking into consideration the above findings, it is interesting to study the details of proteasome distribution in higher plant cells with special attention to the cell-cycle traverse. We used tobacco BY-2 cells, which have been established to be suitable for cell-cycle analysis in plants (Nagata et al. 1992) to resolve this problem. We show that the 26S proteasome is localized both in nuclear envelopes and mitotic spindles and also in plant-specific microtubule (MT) configurations: the PPB and the phragmoplast. We also report that MG132, a selective inhibitor of the proteasome (Rock et al. 1994), caused cell-cycle arrest not only at the metaphase but also at the early stage of PPB formation at the G_2 phase, and that the phragmoplast, which is expected to collapse at the G_1 phase, remained unchanged in cells committed to nuclear division. These results suggest a close relationship between 26S proteasome localization and the formation of PPB and the collapse of phragmoplast, and imply the necessity of proteolysis via the ubiquitin/proteasome pathway at certain stages of these cells. To our knowledge, this is the first report on the localization of the 26S proteasome in PPB and the phragmoplast in higher plant cells.

Results

Characterization of anti-Rpn10 and anti-20S proteasome antibodies

To investigate the subcellular distribution of 26S proteasome, we immunostained tobacco BY-2 cells with antibodies against 20S proteasome and Rpn10. These antibodies were previously raised against spinach 20S proteasome (Ozaki et al. 1992) and rice Rpn10, a subunit of the PA700 regulatory complex (Yanagawa et al. 1998). To examine the specificity of these antibodies, BY-2 cell extracts were tested by immunoblot analyses. The anti-Rpn10 antibody reacted with a single band with a molecular mass of approximately 56 kDa (Fig. 1A, lane 1). The anti-20S proteasome antibody reacted with at least four protein bands with sizes of approximately 29–31 kDa (Fig. 1B, lane 1), implying that both antibodies are specific and sufficient for immunological analysis. No band was observed for both pre-immune sera under the present conditions (see lane 2 of Fig. 1A, B).

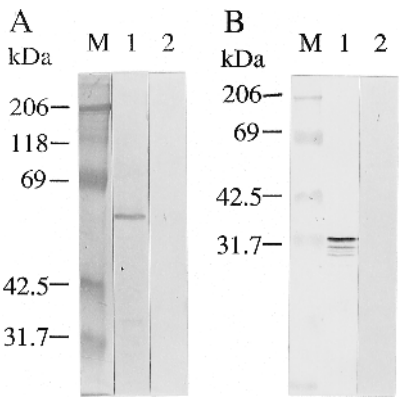

Fig. 1 Immunoblot analysis of tobacco BY-2 cells using antibodies against Rpn10 (A) and 20S proteasome (B). Crude extracts (20 μg of protein) from BY-2 cells were subjected to SDS-PAGE and followed by immunoblotting. Lanes 1 and 2 correspond to antisera and preimmune sera, respectively. M indicates molecular mass marker.

Two distinct forms of Rpn10 in tobacco BY-2 cells

Rpn10 was first identified as a recognition subunit for polyubiquitinated substrates and assigned various names such as human S5a (Deveraux et al. 1994), *Drosophila* μ-54 (Haracska and Udvardy 1995), *Arabidopsis* Mbp1 (van Nocker et al. 1996a), and yeast Mcb1 (van Nocker et al. 1996b, Fu et al. 1999) or Sun1 (Kominami et al. 1997). Finley et al. (1998) recommended that this subunit should be called Rpn10. We use this terminology in this report. It was previously reported that yeast Rpn10 is present not only in association with PA700 and/or 26S proteasome but also in the free form (van Nocker et al. 1996b, Takeuchi et al. 1999). On the other hand, one of the authors (K.T.) found recently that it is present in the 26S proteasome-bound form in mammalian cells, not in the free form (Tanahashi et al. 2000). Therefore, it is important to determine whether or not Rpn10 is present in two distinct forms in plant cells.

In this regard, crude extracts from BY-2 cells were analyzed by glycerol density gradient centrifugation. As previously reported for various cells including those of the budding yeast, spinach leaves and rats (Kominami et al. 1995, Fujinami et al. 1994, Tanahashi et al. 2000), sedimentation patterns of 20S and 26S proteasomes can be monitored by measuring their peptide-hydrolyzing activity using succinyl-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7 amide (Suc-LLVY-MCA) as the sub-

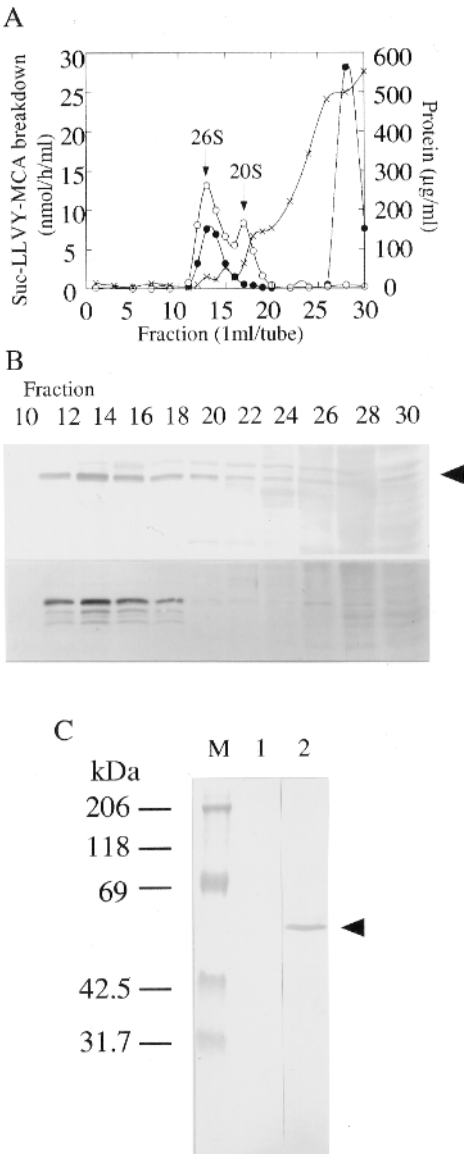

Fig. 2 Sedimentation velocity analyses of Rpn10 and 20S proteasome from tobacco BY-2 cells. Crude extracts (2 mg of protein) from BY-2 cells were fractionated by glycerol density gradient centrifugation (10–40% glycerol from fraction 30 to fraction 1). (A) Chymotrypsin-like activity of proteasomes. After fractionation, aliquots (10 μl) of individual fractions were used to assay the hydrolytic activity of 20S and 26S proteasomes using Suc-LLVY-MCA with (open circles) or without (solid circles) 0.02% SDS and protein (crosses). Fractions where purified 20S and 26S proteasomes are eluted are indicated by the arrows. (B) Immunoblot analysis. Proteins in 100 μl of each fraction were precipitated with acetone, subjected to SDS-PAGE and stained with antibodies against Rpn10 (upper) or 20S proteasome (lower). Numbers correspond to fraction numbers in (A). Arrowhead indicates the band of Rpn10. (C) Evidence for the existence of free-form Rpn10 in BY-2 cell extracts. Antiserum against Rpn10 was pretreated with CNBr-activated Sepharose resin coupled with recombinant histidine-tagged Rpn10 produced in *E. coli* (lane 1) or only Sepharose resin (lane 2). The resulting serum was used for immunoblotting using proteins in 100 μl of fraction 30. M indicates molecular mass marker. Arrowhead indicates the band corresponding to Rpn10.

strate (Fig. 2A). Note that the latent 20S proteasome was detected by the assay at a low concentration of SDS functioning as an artificial activator. As shown in Fig. 2B, proteins immunoreactive against anti-20S proteasome antibody sedimented in parallel with 26S proteasome activity in the absence of SDS with a small shoulder in fractions containing 20S proteasome (lower panel), while the distribution of the band corresponding to Rpn10 with a molecular mass of approximately 56 kDa (fractions 12–20) was apparently consistent with the distribution of 26S and 20S proteasomes (lower panel). Interestingly the same reactive band was also observed in slow-sedimenting fractions up to fraction 30 (upper panel), which clearly differed from the behavior of these proteasomes.

However, in slow-sedimenting fractions 28–30, the anti-Rpn10 antibody reacted with the 56-kDa protein and non-specifically with many other proteins because of the presence of large amounts of proteins in these fractions; thus, it is unclear whether or not the observed 56-kDa band corresponds to Rpn10. To distinguish the anti Rpn10 antibody-reactive protein from other proteins, the antiserum against Rpn10 was pretreated with a large amount of recombinant Rpn10 coupled with Sepharose resin, and the resulting serum was used again for immunoblotting with samples of fraction 30. As shown in Fig. 2C, the 56-kDa band was weakly observed for serum prepared by this treatment (lane 1), but not for serum pretreated with Sepharose resin without coupling to Rpn10 (lane 2). This strongly indicates that Rpn10 is present in the free form in small amounts in plant BY-2 cells although most Rpn10 proteins are associated with PA700 and/or 26S proteasome.

Levels of Rpn10 and 20S proteasome remain constant during cell-cycle progression in tobacco BY-2 cells

Next, we examined whether or not the levels of Rpn10 and 20S proteasome change during cell-cycle progression in BY-2 cells. The cells were synchronized using aphidicolin as described in “Materials and Methods”. When aphidicolin was removed, the cell cycle, which was arrested at the early S phase, proceeded rapidly into the S phase and was followed by the M phase about 8 h later (Fig. 3A). No appreciable changes in the levels of the bands reactive to anti-Rpn10 and anti-20S proteasome antibodies were observed throughout the cell cycle (Fig. 3B). Subsequently, we measured the levels of Rpn10 and 20S proteasome in the M phase, because of accumulating evidence showing the importance of ubiquitin/proteasome-dependent proteolysis during the M phase transition (King et al. 1996, Elledge and Harper 1998, Zachariae and Nasmyth 1999). In this regard, the cells were highly synchronized by treatment with aphidicolin and propyzamide thereby allowing examination of the levels of immunoreactive proteins during mitosis according to the procedure reported by Hasezawa and Nagata (1991) (Fig. 3C). No detectable changes in these levels were also observed between prophase and G_1 phase using both antibodies (Fig. 3D).

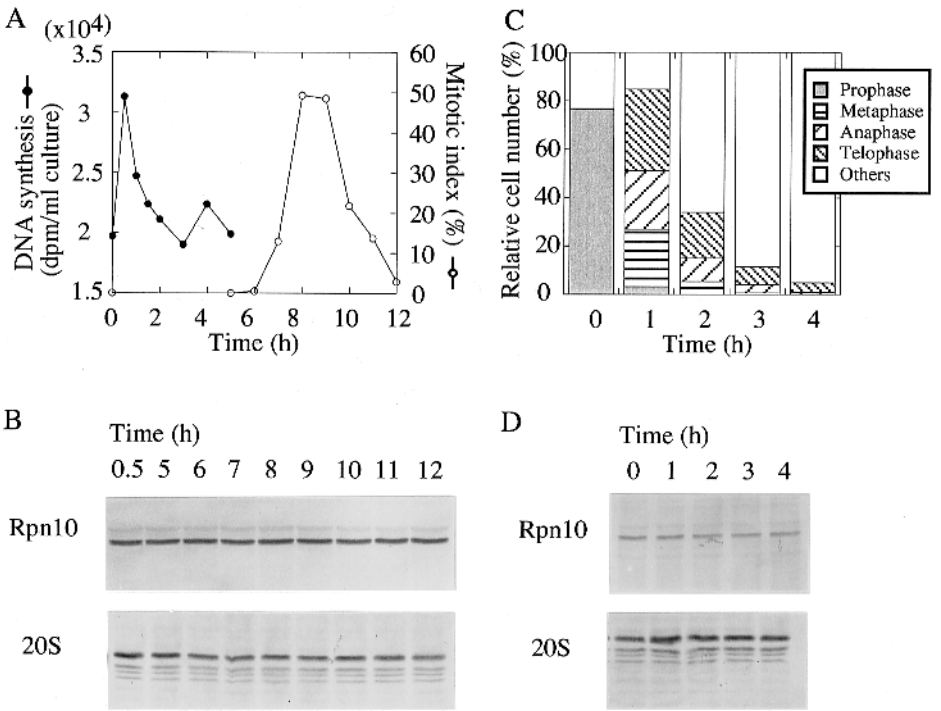

Fig. 3 Levels of Rpn10 and 20S proteasome during cell cycle and mitotic (M) phase progression in tobacco BY-2 cells. (A) After the removal of aphidicolin as described in "Materials and Methods", cell-cycle progression was monitored by [^{3}H]thymidine incorporation (solid circles) and mitotic index determination (open circles). (B) Crude extracts (20 μg of protein) in (A) were subjected to SDS-PAGE followed by immunoblot analysis using antibodies against Rpn10 or 20S proteasome. Numbers correspond to various times in (A). (C) After the removal of propyzamide as described in "Materials and Methods", the progression from M phase to G_1 phase was monitored by counting the number of cells and calculating the percentage of cells at each stage of the M phase. Cells in the prophase have prophase-type nuclei but not mitotic spindles in this case. Others indicate cells that are not in the phase of mitosis. (D) Crude extracts (20 μg of protein) in (C) were subjected to SDS-PAGE followed by immunoblot analysis using antibodies against Rpn10 or 20S proteasome. Numbers correspond to various times in (C).

Subcellular localization of Rpn10 and 20S proteasome during cell cycle

As mentioned above, Rpn10 is present in two distinct forms in tobacco BY-2 cells: one is mostly bound with the 26S proteasome, and the other exists in the free form in the soluble fraction. Immunoblotting after glycerol density gradient centrifugation analysis (Fig. 2) also revealed the presence of free forms of 20S proteasome and PA700, which are not assembled in the 26S proteasome, in BY-2 cells. Accordingly, we consider that costaining with both anti-Rpn10 and anti-20S proteasome antibodies reveal the location of 26S proteasome, although it is uncertain whether or not free forms of PA700 and 20S proteasomes are distributed differently from 26S proteasome.

It is noteworthy that higher plant cells contain unique subcellular structures that are special configurations of MTs called preprophase bands (PPBs) (see arrows in Fig. 4A, B, Fig. 5A, D) and phragmoplasts (see arrows in Fig. 4I, J, arrowheads in Fig. 5M, P), both of which are lacking in other eukaryotic cells, such as yeast and mammalian cells. PPB is a ring structure in the equatorial region. It forms a wide ring around the nucleus at the late G_2 phase and contracts gradually to form a mature girdle-shaped narrow ring. It disappears gradually when the cells enter the M phase. On the other hand, the phragmoplast, which forms the cell plate, is ring-like in shape in the equatorial region that gradually enlarges from the anaphase to the telophase and then disappears at the G_1 phase. It is formed in the same plane as PPB (for a review, see Lambert and Lloyd 1994). We focused on these structures because they change dynamically during the cell cycle, appearing in specific cell-cycle stages.

Considering costaining with both anti-Rpn10 and anti-20S proteasome antibodies shows the localization of 26S proteasome, the 26S proteasome was found to be localized in nuclear envelopes of the cells throughout the interphase (Fig. 4K–O). In the M phase, however, the 26S proteasome appeared to move to the mitotic spindles (Fig. 4C–H) and overlapped partially with the MTs (Fig. 5G–L). Interestingly, the 26S proteasome was localized in PPBs (Fig. 4A, B, Fig. 5A–F) and the phragmoplasts (Fig. 4I, J, Fig. 5M–R). The 26S proteasome was present from wide PPBs to narrow ones and overlapped partially with the MTs in them. The 26S proteasome was also observed from small phragmoplasts to large ones. It appeared in parallel with the MTs and overlapped partially with the MTs in them. The fluorescence intensities in the case of using the anti-Rpn10 antibody were stronger than those in the case of

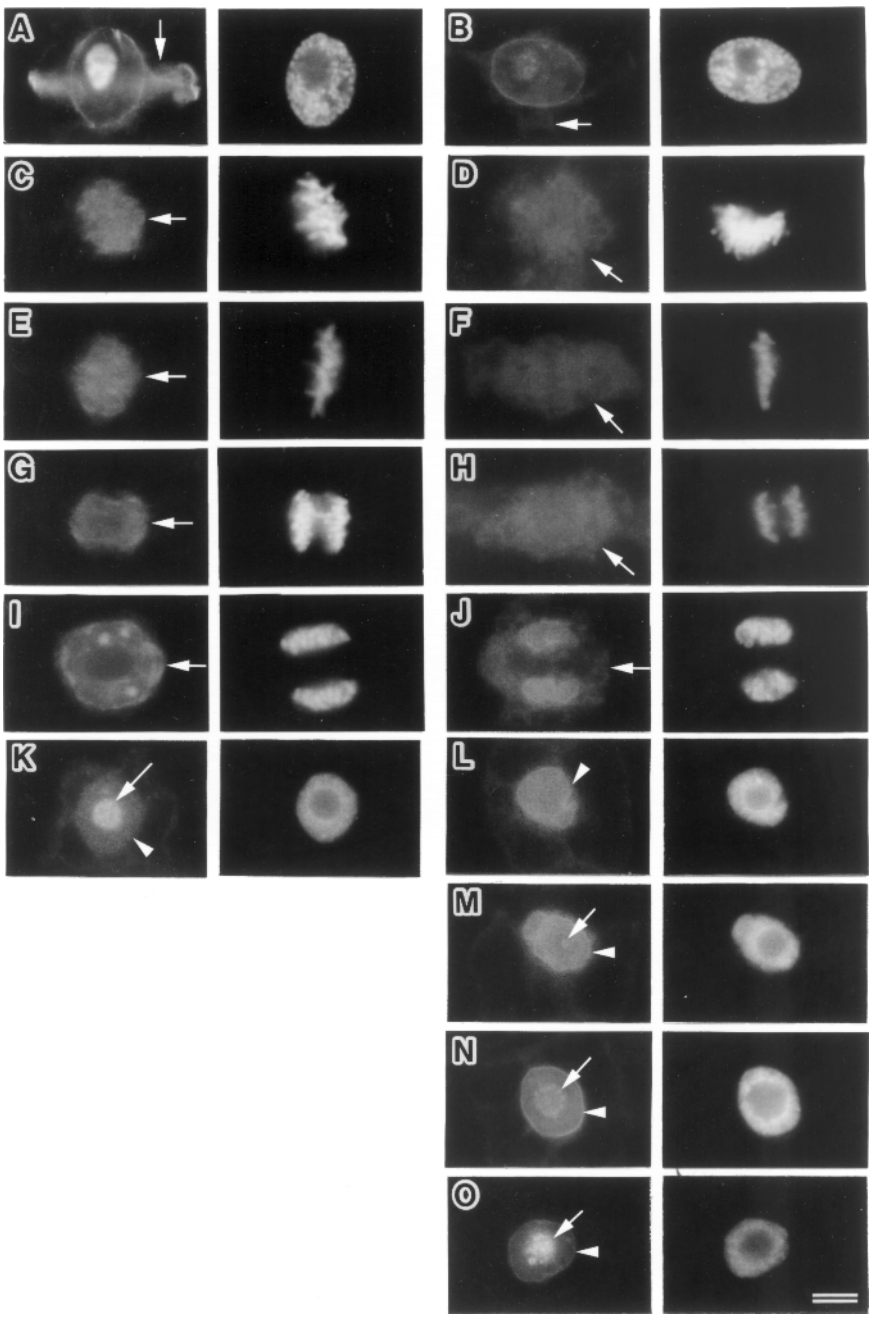

Fig. 4 Changes in the distribution of Rpn10 and 20S proteasome in tobacco BY-2 cells during cell-cycle progression. BY-2 cells were stained with antibodies against Rpn10 (A, C, E, G, I and K, left) and 20S proteasome (B, D, F, H, J, L, M, N and O, left) at each stage of the cell cycle; G_2/M phase (A, B), prometaphase (C, D), metaphase (E, F), anaphase (G, H), telophase (I, J), and interphase (K–O). Cells were also stained with DAPI (A–O, right). The scale bar is 10 μm. (A and B) Arrows indicate preprophase bands. (C–H) Arrows indicate mitotic spindles. (I and J) Arrows indicate phragmoplasts. (K–O) Arrows indicate the staining in nucleoli. Arrowheads indicate nuclear envelopes.

using the anti-20S proteasome antibody in PPBs and phragmoplasts.

Unique localization of Rpn10, differs from that of 20S proteasome

Unexpectedly, the nucleoli of cells at the interphase showed different patterns of staining with antibodies against Rpn10 and 20S proteasome. As shown in Fig. 4K, Rpn10 was always localized in the nucleoli during the interphase. In contrast, the anti-20S proteasome antibody stained the nucleoli of approximately 20% of the interphase cells (Fig. 4M, O), while 80% of that showed no staining (Fig. 4L). In the anti-20S proteasome antibody staining, 20S proteasome seemed to be

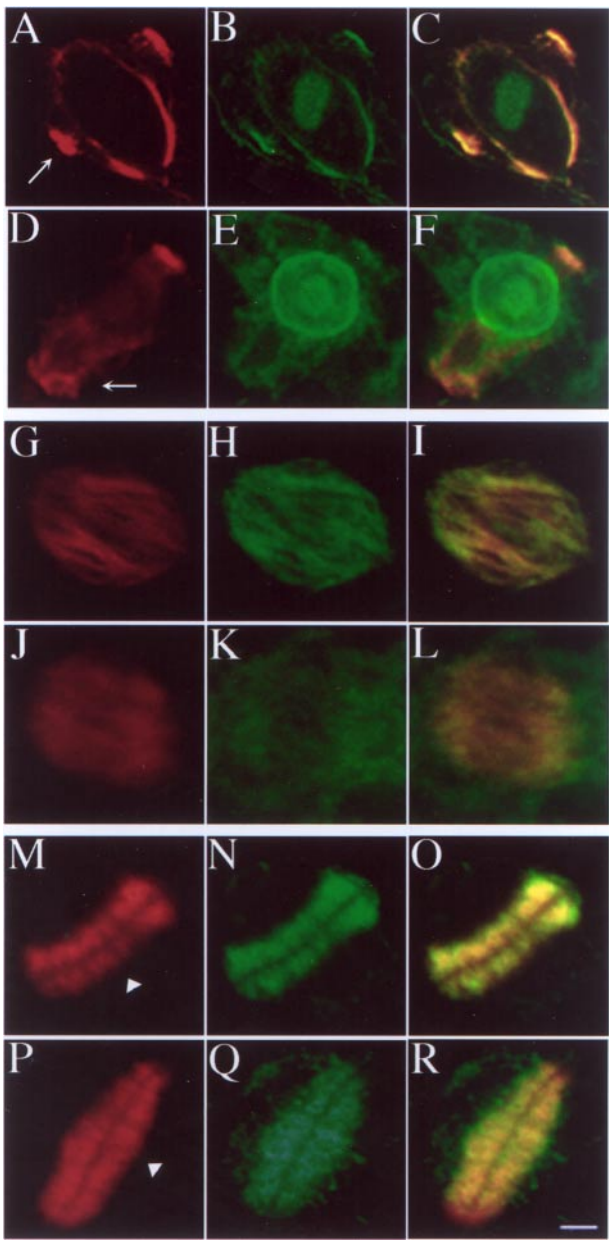

Fig. 5 Confocal laser scanning micrographs showing colocalization of 26S proteasome and MTs. BY-2 cells were stained with antibodies against Rpn10 (B, H and N) and 20S proteasome (E, K and Q). Cells were also stained with anti-tubulins antibodies (A, D, G, J, M and P). The images are merged (C, F, I, L, O and R). Proteasome subunits and MTs are shown in green and red, respectively. The scale bar is 5 μm. (A–F) Cells with PPBs at late G_2 phase are shown. Arrows indicate PPBs. (G–L) Mitotic spindles in cells at M phase. (M–R) Phragmoplasts at telophase indicated by arrowheads.

located in various areas of the nucleoli, such as its center (Fig. 4M), around it (Fig. 4N) or its entire region (Fig. 4O). However, such variations of staining patterns were independent of the phase of the cell cycle in BY-2 cells (data not shown).

Effect of MG132 on cell-cycle progression and proteasome distribution

It is well known that proteolysis mediated by the proteasome and its partner, ubiquitin, plays essential roles in cell-cycle progression (Elledge and Harper 1998, Zachariae and Nasmyth 1999, Deshaies 1999). Therefore, it was important to examine how MG132, a proteasome inhibitor, affects cell cycle and the localization of 26S proteasome in BY-2 cells. For synchronization, when aphidicolin was added to the media for 24 h as described in "Materials and Methods", most cells were arrested at the early S phase. When aphidicolin was removed, however, the cell cycle proceeded normally, following the order of S-G_2-M-G_1. The number of cells at each stage was determined and results are shown in Fig. 6A (left panel). MG132 or E64d, a cystein protease inhibitor, was added to the culture media at various times after the cell cycle was re-started and all cells were further cultured for 11 h after removal of aphidicolin. Then, cell-cycle parameters were analyzed (Fig. 6A, right panel).

As shown in Fig. 6A–C, when MG132 was added to the culture media at 1 h after the removal of aphidicolin, most of the cells were arrested at the early stage of PPB formation, which remained as a wide bundle, and no cells with narrow mature PPBs were observed (see asterisks in Fig. 6C). The 26S proteasome stained with the antibodies against both Rpn10 and 20S proteasome was observed in wide bundles of PPBs (Fig. 6J–M). Moreover, as for the nucleoli, Rpn10 was found to be localized in all cells (Fig. 6K), but the 20S proteasome was found only in some cell populations (Fig. 6M and other data not shown). The observations at this stage resemble those of cells cultured in the absence of MG132, as described above.

On the other hand, cells were arrested at the metaphase when MG132 was added 5 h (approximately 42%) or 8 h (approximately 28%) after the removal of aphidicolin (Fig. 6A, D, E). This is consistent with the findings of Genschik et al. (1998). The 20S proteasome was localized in mitotic spindles in cells arrested at the metaphase by the treatment with MG132 (Fig. 6P, Q), which was similar to the localization of 20S proteasome observed at the metaphase in cells cultured in the absence of MG132. In contrast, Rpn10 was localized not only in mitotic spindles but also in chromosomes in cells arrested at the metaphase (Fig. 6N and see arrow of panel O).

When MG132 was added 8 h after the removal of aphidicolin, the collapse of phragmoplasts was inhibited in some cells (Fig. 6G, arrows), although the cells seemed to proceed to the G_1 phase without showing any abnormality, because their daughter nuclei appeared to have separated normally, based on staining with DAPI (Fig. 6F). The 26S proteasome was observed in the remaining phragmoplasts by staining with anti-Rpn10 and anti-20S proteasome antibodies (Fig. 6R–U).

No significant effects on cell cycle were observed when E64d was added to the cells 1, 5, and 8 h after the removal of aphidicolin (Fig. 6A, H, I, and other data not shown).

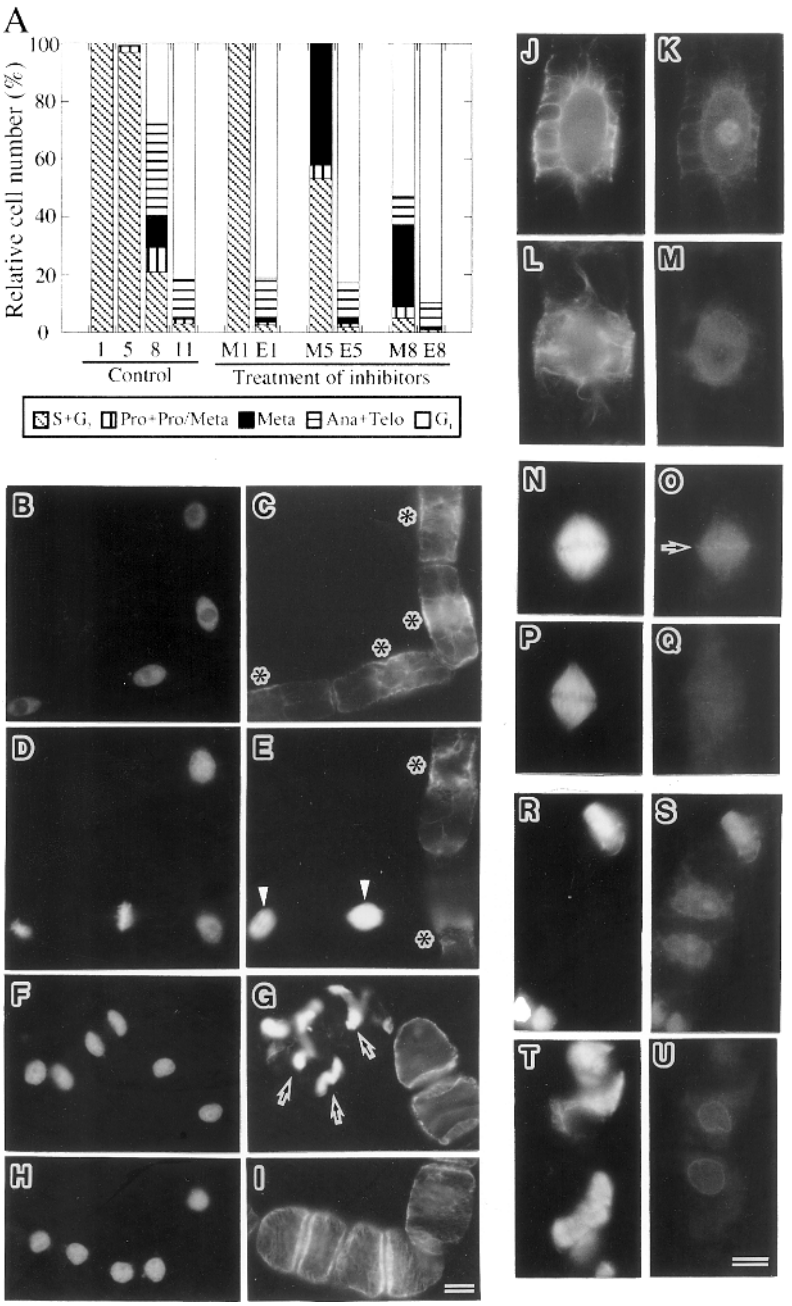

Fig. 6 Effect of MG132 on cell-cycle progression and proteasome distribution in tobacco BY-2 cells. (A) For synchronization, BY-2 cells were treated with aphidicolin. Then, after the removal of aphidicolin, cells were cultured for 11 h. 100 μM MG132 (M1, M5 and M8) or 100 μM E64d (E1, E5 and E8) was added to cells at 1 h (S phase), 5 h (G_2 phase), and 8 h (M phase). Population of cells at various cell-cycle stages at indicated times was analyzed as in Fig. 3. Cells were stained with DAPI and then the number of cells in each phase was counted. Cells to which reagents were not added were also examined at 1, 5, 8, and 11 h (control 1, 5, 8, 11). (B, C) Cells to which MG132 was added at 1 h after the removal of aphidicolin were further cultured for 11 h, and then stained with DAPI (B) and anti-tubulin antibodies (C). Asterisks indicate immature PPBs arrested at the early stage of their formation. (D, E) MG132 was added at 5 h, and the cells were collected at 11 h and then stained with DAPI (D) and anti-tubulin antibody (E). Asterisks indicate the immature PPBs arrested at the early stage of their formation. Arrowheads indicate the mitotic spindles arrested at the metaphase. (F, G) MG132 was added at 8 h, and the cells were collected at 11 h and then stained with DAPI (F) and anti-tubulin antibody (G). Arrows indicate that the phragmoplasts failed to collapse. (H, I) E64d was added at 1 h, and the cells were collected at 11 h and then stained with DAPI (H) and anti-tubulin antibody (I). The scale bar is 20 μm. (J–U) The cells, whose cell cycle was arrested at the G_2 phase (J–M) and the metaphase (N–Q), and in which collapse of the phragmoplast was inhibited (R–U) by the treatment with MG132 as described in (A), were stained with antibodies against Rpn10 (K, O and S), 20S proteasome (M, Q and U) and tubulins (J, L, N, P, R and T). Arrow indicates the staining of Rpn10 in the position of the chromosome. The scale bar is 10 μm.

Discussion

In the present study, we examined in detail the relationship between 26S proteasome and the cell cycle in tobacco BY-2 cells. The levels of 26S proteasome, which were measured by immunoblotting with antibodies against 20S proteasome and Rpn10, a PA700 subunit, remained unchanged throughout the cell cycle. However, by immunocytochemical analysis, we found that the subcellular distribution of 26S proteasome dynamically changed in a cell-cycle dependent manner in the synchronized culture of tobacco BY-2 cells. In particular, the 26S proteasome was detected in nuclear envelopes and mitotic spindles. We also found the localization of 26S proteasome in the PPB and the phragmoplast during cell-cycle progression in tobacco BY-2 cells, implying that the 26S proteasome is involved not only in the cell division at the M/G_1 phase but also in the determination of the plane for plant cell division at an earlier phase. The PPB is thought to determine the plane for cell division and to leave some information for future cell division, while the phragmoplast is responsible for the formation of a cell plate for cell division (Lambert and Lloyd 1994). Therefore, the present results indicate that the 26S proteasome may be responsible for the plant-specific system of cell division.

Wilkinson et al. (1998) reported that 26S proteasome was present as a spot in the middle of two daughter nuclei at the late M phase in yeast cells. Taken together, the common system of proteolysis involving the 26S proteasome may be present in the cell division planes of both plants and yeasts, although it is unclear whether or not the spot in yeast has the same functions as the phragmoplast in plants. In addition, as described in "Introduction", since the 26S proteasome is known to be localized in nuclear envelopes at the interphase and in mitotic spindles at the M phase in other higher eukaryotic cells, such as ascidian egg cells (Kawahara and Yokosawa 1992) and rat ovarian granulosa cells (Amsterdam et al. 1993), it is likely that the ubiquitin/proteasome pathway plays pivotal roles in cell-cycle progression common to a wide spectrum of eukaryotic cells.

Furthermore, we found that Rpn10 exists not only in the proteasome-bound form but also in the free form in tobacco cells, as reported in yeast (van Nocker et al. 1996b), but not in mammalian cells (Tanahashi et al. 2000). In this context, it is intriguing that Rpn10 is present in the nucleoli of cells at the interphase possibly in the free form. Alternatively, it is possible that Rpn10 is present in the nucleoli in the PA700-bound form. This possibility must be the subject of future research. Regarding the role of Rpn10 in the nucleoli, it may contribute to the recruitment of ubiquitinated substrates from the nucleoli to the nucleoplasm where the 26S proteasome exists in abundance. Alternatively, it is also likely that the 26S proteasome shuttles between the nucleoli and the nucleoplasm, although the mechanism underlying the movement of 26S proteasome into and out of these subnuclear compartments remains elusive at present.

Recently, nucleoli were found to have the ability to sequester Cdc14 (Shou et al. 1999, Visintin et al. 1999) and Mdm2 (Weber et al. 1999) in a cell-cycle-dependent manner, indicating that the nucleolus has additional function(s) related to cell-cycle control besides its well-known role in the biosynthesis of ribosomal RNA: this may be related to the spatial localization of Rpn10. Another possibility is that Rpn10 may play distinct roles quite different from those of 26S proteasome in the nucleoli, because there is accumulating evidence that Rpn10 possesses diverse functions independent of ubiquitin-chain binding for proteolysis, such as transcription (Anand et al. 1997, Aravind and Ponting 1998), DNA repair (Schauber et al. 1998, Lambertson et al. 1999) and development in plants (Fu et al. 1999, Girod et al. 1999).

Little is known about the role of 26S proteasome during cell-cycle progression in higher plants in spite of tremendous progress in research addressing the importance of an association between proteolysis, involving proteasome and ubiquitin, and the cell cycle in yeast and mammalian cells (Elledge and Harper 1998, Deshaies 1999, Zachariae and Nasmyth 1999). In this work, investigations of the cell cycle using MG132 revealed that the 26S proteasome participates at least in three stages of the cell cycle in higher plant BY-2 cells: the late G_2 phase, the metaphase and the M/G_1 phase. Firstly, treatment with MG132 caused cell-cycle arrest at the G_2 phase; in particular, the cells were conclusively arrested at the early stage of PPB formation, which may indicate the presence of 26S proteasome in PPBs. These findings indicate that the cells probably need 26S proteasome to degrade certain protein(s), required to traverse this cell-cycle stage, and that the 26S proteasome may be necessary for the contraction of PPBs for maturation rather than the initiation of their formation.

Secondly, regarding the effect of MG132 on the metaphase, our observations are essentially in agreement with those of Genschik et al. (1998) and Criqui et al. (2000), who reported the arrest of the cell cycle at the metaphase by the treatment with MG132. Interestingly, we also found that the Rpn10 protein is localized, independent of the presence of 20S proteasome, in chromosomes of cells arrested at the metaphase, providing additional evidence of the existence of Rpn10 independent of 20S proteasome in BY-2 cells, although its functions remain to be elucidated.

Finally, regarding the effect of MG132 on the M/G_1 phase, the phragmoplast failed to collapse in spite of the completion of nuclear division. Its failure to collapse together with the localization of 26S proteasome in the phragmoplast suggests that the 26S proteasome probably plays a certain role in the collapse of the phragmoplast rather than in its expansion. It also probably degrades target protein(s), which might be necessary to stabilize the structure of the phragmoplast. Moreover, the 26S proteasome was observed in the remaining phragmoplasts, implying that the disappearance of the phragmoplasts might result in the release of the 26S proteasome.

In conclusion, in the present study we reported that the

level of 26S proteasome remained unchanged throughout the cell cycle in tobacco BY-2 cells and its subcellular distribution dynamically changed in a cell-cycle-dependent manner. The 26S proteasome was localized not only in nuclear envelopes and mitotic spindles but also in PPBs and phragmoplasts that appeared at the late G_2 and M phases, respectively. These results strongly indicate that the 26S proteasome plays certain roles in cell-cycle progression by changing its subcellular distribution rather than its quantity. MG132 caused exclusively cell-cycle arrest not only at the metaphase but also at the early stage of PPB formation at the G_2 phase and the collapse of the phragmoplast, which seems to be closely related to proteasome distribution in cells. Intriguingly, Rpn10 was present in the nucleoli of all cells at the interphase, which was different from the heterogeneous distribution of 20S proteasome, indicating the specific role(s) played by Rpn10 in the nucleoli.

Materials and Methods

Reagents

The materials used were obtained from the following: Suc-LLVY-MCA (Peptide Institute, Minoh, Japan); carbobenzoxy-Leu-Leu-Leu-CHO (aldehyde) (MG132) (Peptide Institute, Minoh, Japan); E64d (Peptide Institute, Minoh, Japan); and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co., St. Louis, U.S.A.).

Immunological analysis

Crude extracts from tobacco BY-2 cells were prepared according to the method of Yanagawa et al. (1999). SDS-PAGE was carried out according to the method of Laemmli (1970) in 10% or 12.5% slab gel. The protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as the standard.

A polyclonal antibody against the spinach 20S proteasome was prepared as described previously (Ozaki et al. 1992). A polyclonal antibody against Rpn10 was raised in a rabbit using the purified histidine-tagged Rpn10 protein from rice, which was previously cloned (Yanagawa et al. 1998), as the antigen.

Immunoelectrophoretic blot analysis was carried out according to the method of Towbin et al. (1979). Anti-rabbit IgG conjugated with alkaline phosphatase (Promega, Madison, U.S.A.) was used as a secondary antibody with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates for alkaline phosphatase.

Synchronization of tobacco BY-2 cells

At weekly intervals, suspension cultures of a tobacco BY-2 cell line, derived from seedlings of *Nicotiana tabacum* L. cv. Bright Yellow 2, were diluted 95-fold with a modified Linsmaier and Skoog medium (Linsmaier and Skoog 1965), as described by Nagata et al. (1992). The cell suspension was agitated on a rotary shaker at 130 rpm at 27°C in the dark.

For synchronization, the cells were treated with 5 μg ml^{-1} aphidicolin for 24 h as previously described by Nagata et al. (1992). To specify the M phase by sequential synchronization, the cells were treated with 3 μM propyzamide at 5 h after the removal of aphidicolin as previously described by Hasezawa and Nagata (1991). DNA synthesis was determined by [^{3}H]thymidine incorporation as previously described (Amino et al. 1983). To determine the mitotic index, the cells were stained with DAPI and at least 400 cells were counted per phase by immunofluorescence microscopy (Axiophot FL; Carl Zeiss, Oberkochen, Germany).

Immunofluorescence microscopy

Immunostaining of tobacco BY-2 cells was carried out as described by Hasezawa and Nagata (1991). The cells were incubated for 1 h with the antibodies against Rpn10 or 20S proteasome, which were diluted before use at 1 : 200 and 1 : 40, respectively. The cells were then treated for 1 h with a FITC-conjugated antibody against rabbit IgG raised in a sheep (Sigma Chemical Co., St. Louis, U.S.A.), which was diluted at 1 : 10 as a secondary antibody. Note that no significant staining was detected using pre-immune sera or antisera immunodepleted with the purified antigens. The cells were also stained with a solution of 20 μg $liter^{-1}$ DAPI for 5 min.

For double staining with each antibody described above and the antibody against tubulins (1 : 1 mixture of mouse anti-α-tubulin and anti-β-tubulin, Amersham Co., Buckinghamshire, U.K.), two sets of primary and secondary antibodies were employed. To stain each antigen (Rpn10 or 20S proteasomes), the set of first and second antibodies was used as described above. To stain MTs, antibodies against tubulins diluted at 1 : 400 and a rhodamine-conjugated antibody against mouse IgG raised in a goat (Cappel Lab., Westchester, U.S.A.) diluted at 1 : 10 were employed.

The specimens were examined under a fluorescence microscope (Axiophot FL; Carl Zeiss, Oberkochen, Germany). Photomicrographs were recorded on a Super Prest film (Fuji Photo Film Co., Tokyo, Japan). The localizations of each antigen (Rpn10 or 20S proteasome) and MTs were examined under a fluorescence microscope (IX, Olympus Co., Ltd., Tokyo, Japan) equipped with a confocal laser scanning head and control systems (GB-200, Olympus).

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