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Rapid Communication

Loss of Microtubules in the Interphase Cells of Onion (*Allium cepa* L.) Root Tips from the Cell Cortex and Their Appearance in the Cytoplasm after Treatment with Cycloheximide[1]

Yoshinobu Mineyuki*, Hidetoshi Iida, and Yasuhiro Anraku

Department of Biological Science, Faculty of Science, Hiroshima University, Kagamiyama 1-3-1, Higashi-Hiroshima 724, Japan (Y.M.); and Division of Cell Proliferation, National Institute for Basic Biology, 38 Nishigonaka, Myodaijicho, Okazaki 444, Japan (H.I., Y.A.)

As part of a project to investigate the mechanism of cortical microtubule (MT) alignment, we examined the effects of cycloheximide (CHM) on cortical MTs in the root tip cells of *Allium cepa* L. Results show that although a preprophase band of MTs remained in the cell cortex, interphase MTs disappeared from the cortical cytoplasm and then appeared concomitantly in the inner cytoplasm when the rate of de novo protein synthesis was reduced with CHM (11–360 μM for 2 h).

The interphase arrays of cortical MTs in higher plant cells coordinate with the deposition of cellulose microfibrils, and therefore, they are thought to be somehow involved in the organized deposition of cellulose microfibrils. Thus, by providing important spatial information to the cell wall they are thought to play an important role in the regulation of the orientation of cell elongation (Williamson, 1991). Using EM, Ledbetter and Porter (1964) reported electron-dense cross-bridges between MTs and the PM as early as 1964. Many have speculated this electron-dense material to be MT/PM-bridging proteins; however, there are no biochemical reports in support of this hypothesis. We reasoned that if MT/PM-bridging proteins exist, they might have a shorter half-life than the tubulin dimer. If this hypothesis is correct, then general protein synthetic inhibitors (such as CHM) would selectively deplete these bridging proteins relative to tubulin. This depletion of the protein would then cause a change in MT arrays. To test this hypothesis, the effect of protein synthesis inhibition caused by CHM on the cortical MT organization was examined.

MATERIALS AND METHODS

Plant Materials and Incubation Conditions

Onion (*Allium cepa* L. cv Highgold Nigou; Sakata Seed Co., Yokohama, Japan) seedlings were grown in the dark at 25°C for 4 d on a filter paper moistened with distilled water. For the CHM treatment, 4-d-old seedlings were transferred to a small plastic container (87 mm wide × 57 mm deep × 19 mm high; Iuchi-Seieido Co., Osaka, Japan) with 3 mL of CHM solution. Following the 2-h incubation, the distal 2-mm portions of the root tips were dissected for examination. CHM was purchased from Sigma and a 1-mg mL^{-1} stock solution was diluted in distilled water and kept at −20°C.

Immunocytochemistry

The fluorescence localization of MTs was carried out according to published procedures (Mineyuki et al., 1991) using a mouse monoclonal anti-β-tubulin antibody (Amersham Japan Co., Tokyo, Japan) as the primary antibody and a fluorescein isothiocyanate-linked sheep anti-mouse IgG antibody [$F(ab')_2$ fragment; Sigma] as the secondary antibody. Hoechst 33258 (10 mg L^{-1}) was included in the mounting medium to identify the interphase cells.

Samples were observed using a conventional fluorescence microscope (HPD-X2; Nikon, Tokyo, Japan). A confocal laser scanning microscope (MRC-500; Japan Bio Rad Co. Ltd., Tokyo, Japan) equipped with a Nikon X microscope and an objective of ×60 (numerical aperture 1.4) was used to observe the three-dimensional distribution of MTs.

In Vivo Labeling and Extraction of Proteins

Ten onion seedlings were incubated in a plastic container with 3 mL of distilled water in the absence or presence of CHM. After 1.5 h of incubation, [^{35}S]Met (>1000 Ci $mmol^{-1}$; obtained from American Radiolabeled Chemicals Inc., St. Louis, MO) was added to the solution such that the final radioactivity of the solution was 25 μCi mL^{-1}. After 30 min of labeling with [^{35}S]Met, seedlings were rinsed with 1 mM nonradioactive Met, and 2-mm lengths of root tips were excised. The 10 pieces of root tip were placed in an Eppendorf

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* Corresponding author; fax 81-824-24-0734.

Abbreviations: CHM, cycloheximide; MT, microtubule; PM, plasma membrane.

tube containing a known amount of glass beads. The tube was weighed to calculate the weight of the root tips. The root tips were homogenized in 50 μL of SDS gel loading sample buffer (Laemmli, 1970) without 2-mercaptoethanol, glycerol, and bromphenol blue with a pellet pestle. The tube with the homogenate was placed in a heat block at 100°C for 3 min and then centrifuged for 3 min in an Eppendorf microfuge at 10,000 rpm.

The supernatant (100 μL) was put into a tube containing 2 mL of 6% TCA and 1 mM nonradioactive Met. It was kept on ice for at least 20 min. The mixture was filtrated using a GF/C glass filter (Whatman, Maidstone, UK). The filter was washed five times with 5 mL of 6% TCA containing 1 mM nonradioactive Met and then was washed twice with 99.5% ethanol. The filter was dried under a lamp, and the radioactivity associated with the filter was counted in 5 mL of Ready Protein (Beckman Japan, Tokyo, Japan) scintillant. The protein content was determined by the method described by Lowry et al. (1951) using BSA as a standard.

RESULTS

The interphase cells of 4-d-old onion root tips that were treated with various concentrations of CHM for 2 h were examined using anti-tubulin immunocytochemistry. The spatial distribution of MTs was examined after the reconstruction of stereo-pair images using confocal laser scan microscopy (Fig. 1). In cells without CHM, the majority of interphase cells had a transverse cortical array of MTs, and few MTs were seen in the cytoplasm or perinuclear region (designated A type; Fig. 1A). On the contrary, the number of MTs in the cytoplasm increased in cells treated with 3.6 μM CHM (designated B type; Fig. 1B), at which concentration protein synthesis was reduced to 75%. Very few MTs were in the cell cortex, and many MTs ran randomly in the cytoplasm when cells were treated with higher concentrations of CHM (36 and 360 μM) for 2 h (designated C type; Fig. 1C). The frequencies of these three types of MT arrays in root-tip cells treated with the various concentrations of CHM for 2 h are shown in Figure 2B. The effects of various concentrations of CHM on the incorporation rate of [^{35}S]Met into root-tip proteins were further examined in the same conditions (Fig. 2A). The more concentrated the CHM, the lower the incorporation rate of [^{35}S]Met into root-tip proteins. More than 90% inhibition was achieved when the concentration of CHM was 11 μM. At this concentration, cells with A-type MTs (Fig. 1A) were not seen, and the population of cells with B- or C-type MTs increased. Significantly, MTs in preprophase bands were observed in the cell cortex (Fig. 1D), although most of interphase cells were observed to have C-type MTs in the presence of 36 or 360 μM CHM.

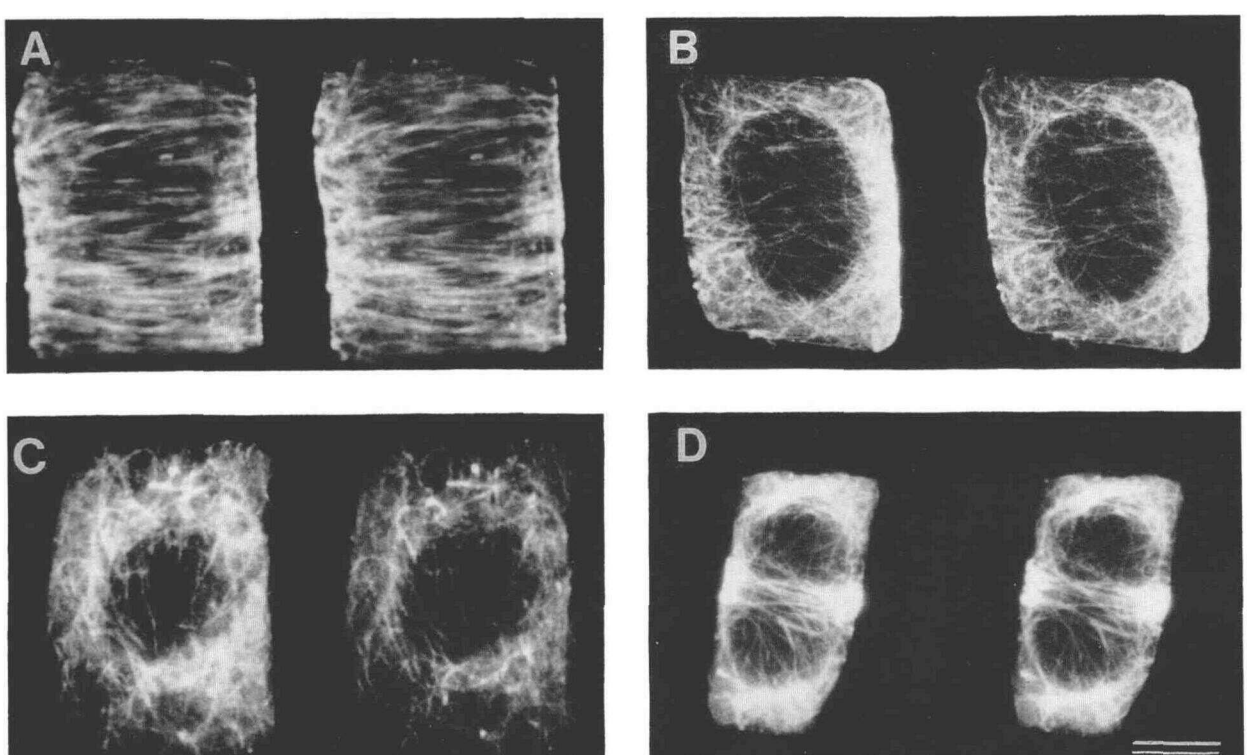

Figure 1. Stereo-pair images of interphase MT localization in onion root tip cells. Root tips were treated with 3.6 μM (B) and 36 μM (C and D) CHM for 2 h. The cell depicted in A is from a root tip without CHM treatment. Note that MTs are in the cortex of the cell in A, but they are in the cytoplasm in the cell in C. D, Cell with a preprophase band in the presence of CHM. Bar = 10 μm.

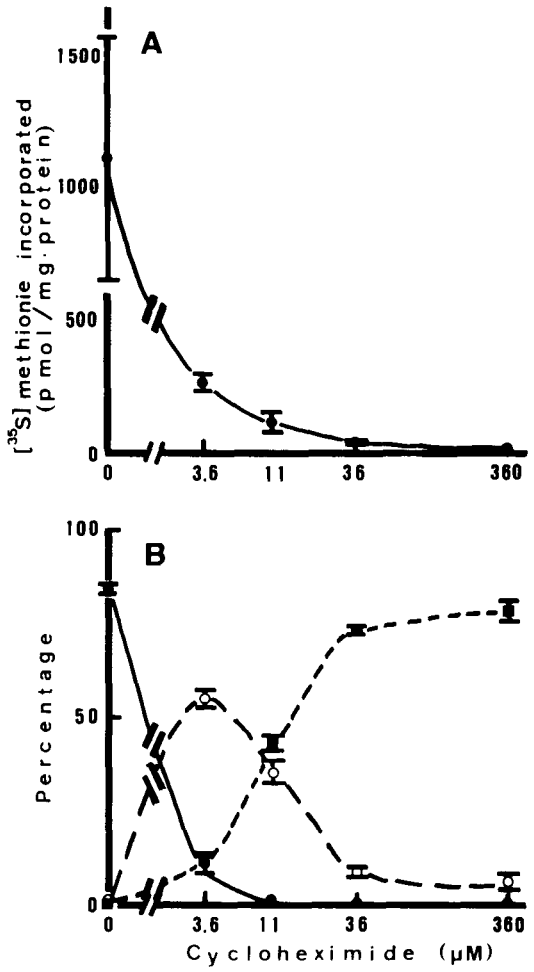

Figure 2. Effect of a 2-h treatment of onion root tips with various concentrations of CHM on the incorporation of [^{35}S]Met into protein (A) and on MT pattern (B). Onion seedlings (4 d old) were treated with various concentrations of CHM for 2 h, and 2-mm-long root tips were used for the experiments. Labeling with 25 μCi mL^{-1} [^{35}S]Met took place in the final 30 min of incubation. The percentage of cells with an interphase transverse cortical MT array with few cytoplasmic MTs (●; see Fig. 1A), of cells with interphase cytoplasmic MTs without cortical MTs (■; see Fig. 1C), and of cells at the intermediate stage (○; see Fig. 1B) were determined by a fluorescence microscope. The results are the means of two (A) or three (B) different experiments, and SE values are shown by bars.

DISCUSSION

The present observations show that the number of cytoplasmic MTs increases when protein synthesis is reduced with CHM. After 2 h of incubation in CHM there is a significant reduction in protein synthesis; however, the fact that MTs are still demonstrable following this treatment indicates that tubulin is relatively stable. The number of cortical MTs decreased when cytoplasmic MTs appeared (Fig. 1B). The B-type MT array may represent an intermediate type between A and C. This observation indicates that cytoplasmic MTs replace cortical MTs following CHM treatment.

The connection of MTs to PM by electron-dense bridges was reported by many authors using EM (Williamson, 1991), although specific molecules have yet to be identified. If some of the molecules that are involved in the connection of MTs to PM are unstable proteins, their amount per cell will decrease quickly when de novo protein synthesis is inhibited by CHM. The displacement of MTs from the cell cortex to the cytoplasm can take place simply because of the absence of components that are involved directly or indirectly in the connection of MTs to PM. Of course, we cannot deny the possibility that the CHM is inhibitory to some process other than the synthesis of these proteins (MacDonald and Ellis, 1969).

Two types of oriented cortical MTs are characteristic of higher plant cells. They are implicated in functions of particular importance to plant morphogenesis. One (the interphase cortical array) regulates the direction of cell expansion by controlling the orientation of the cellulose microfibril deposition (Williamson, 1991). Another (the preprophase band) determines division plane orientations (Gunning, 1982). Electron-dense materials are known to link MTs and PM, both in the interphase cortical MT array and in the preprophase band (Hardham and Gunning, 1978; Williamson, 1991). In our studies we found that MTs in the preprophase band remained in the cell cortex, yet interphase cortical MTs disappeared following 2 h of CHM treatment. This difference in the sensitivity to CHM between the interphase cortical MTs and the preprophase band of MTs suggests that some components involved in the connection of MTs to PM are different between the preprophase band and the interphase cortical MT array.

Plants respond to environmental cues by adjusting their pattern of growth. For example, shoots respond to light and grow toward a light source (phototropism). Phototropic and gravitropic bending are thought to be correlated with a change of the cortical MT orientation in the cell. For example, the reorientation of MTs in maize coleoptile occurs within 60 min after the induction of a phototropic response by blue light (Nick et al., 1990). If MTs stayed in a position for a long time, the cells would not be able to change their growth direction quickly. The rapid turnover of a PM/MT-bridging protein in the interphase cortical MT arrangement may be a mechanism for adjusting to this kind of quick growth response in plants.

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