Radial Microtubule Organization by Histone H1 on Nuclei of Cultured Tobacco BY-2 Cells*

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In acenontio lar higher plant cells, the surface of the nucleus acts as a microtubule-organizing center, substituting for the centrosome. However, the protein factors responsible for this microtubule organization are unknown. The nuclear surfaces of cultured tobacco BY-2 cells possess particles that generate microtubules. We attempted to isolate the proteins in these particles to determine their role in microtubule organization. When incubated with plant or mammalian tubulin, some, but not all, of the isolated nuclei generated abundant microtubules radially from their surfaces. The substance to induce the formation of radial microtubules was confirmed by SDS-PAGE to be a protein with apparent molecular mass of 38 kDa. Partial analysis of the amino acid sequences of the peptide fragments suggested it was a histone H1-related protein. Cloning and cDNA sequence analysis confirmed this and revealed that when the recombinant protein was incubated with tubulin, it could organize microtubules as well as the 38-kDa protein. Histone H1 and tubulin formed complexes immediately, even on ice, and then clusters of these structures were formed. These clusters generated radial microtubules. This microtubule-organizing property was confined to histone H1; all other core histones failed to act as organizers. On immunoblot analysis, rabbit antibodies raised against the 38-kDa protein cross-reacted with histone H1 proteins from tobacco BY-2 cells. These antibodies virtually abolished the ability of the nucleus to organize radial microtubules. Indirect immunofluorescence showed that the antigen was distributed at the nuclear plasm and particularly at nuclear periphery independently from DNA.

Understanding the function of microtubule-organizing centers (MTOCs)3 is essential to our understanding of the mechanisms of division, growth, and differentiation, especially in higher plant cells. Higher plant cells differ fundamentally from animal or lower plant cells in that they lack the typical centrosomes, basal bodies, and spindle pole bodies that contain the materials to act as MTOCs. Nevertheless, in the cells of higher plants, dynamic changes in the microtubule (MT) structures still occur throughout the cell cycle, and MTOCs could still play important roles in the cell, determining suitable positions and polarities for MT arrays and regulating the dissolution of MTs. Higher plant cells might possess a different type of MTOC whose nature depends on the structural type of the MT (1), such as cortical, preprophase band, spindle, and phragmoplast. These structural differences might be correlated with the specific functions of the plant MTs, including participation in the regulation of new cellulose microfibril orientation (cortical MTs) (2–4), determination of the division plane (preprophase band MTs) (5), chromosome segregation (spindle MTs), and cell plate formation (phragmoplast MTs) (6, 7). MT organizing ability in higher plant cells is considered to be spread over the nuclear surface instead of at the centrosomes (8). In fact, Lambert (9, 10) demonstrated by electron microscopy that MTs are connected to the nuclear envelope in Hemanthus endosperm cells and suggested that the site of MT nucleation is associated with the nuclear envelope. It has been argued that the nuclear envelope offers an MTOC for interphase radial MTs, preprophase band MTs, and spindle MTs (11–14).

Some biochemical approaches have provided limited evidence of the potential role of protein factor(s) as candidates for MT-organizing particles (MTOPs) on nuclei. Isolated nuclei and their fragmented particles from cultured tobacco BY-2 cells were shown to have the ability to nucleate MTs (15). A similar result was obtained using nuclei from cultured maize cells (16) and from other plant cells (17, 18). Stoppin et al. (19) suggested the presence of a 100-kDa antigen that cross-reacts with monoclonal antibody against a 180-kDa protein in the celf thymus centrosome. MT nucleation from the surface of tobacco nuclei has been stimulated by adding MT-associated proteins (MAPs) and the presence of Spc98 homologue, which has been considered to interact with γ-tubulin in Saccharomyces cerevisiae, has been reported (20).

In cultured tobacco BY-2 cells, a 49-kDa protein, which was immunologically related to sea urchin centrosomal 51-kDa protein and later identified as EF-1α, was reported to localize around the nuclei in early G1 phase (21–23). γ-Tubulin, which monio)-1-propanesulfonate; DAPI, 4,6-diamidino-2-phenylinole; Ni-NTA, nickel-nitrilotriacetic acid.
appears to be specifically localized to MTOCs such as the centrosome of animal cells (24) and the spindle pole body of fungi (25, 26), is also an important candidate for MTOC in higher plant cells. However, because higher plant cells lack a centrosome and spindle pole body, the localization and function of γ-tubulin in these cells is still unclear. Antibodies raised against γ-tubulin revealed its characteristic localization along cortical MTs in a cell cycle-dependent manner (27). The same authors also identified several γ-tubulin genes, including two γ-tubulin genes of Arabidopsis, which showed 70% amino acid identity with the γ-tubulins of animal and fungi (28). γ-Tubulin was hypothesized to be engaged in the organization of cortical MTs located just inside the plasma membrane (27, 29).

Our immunofluorescence observation of cultured tobacco BY-2 cells using antibodies raised against tubulin revealed that more than half of the cells cultured for 5 days had nuclei with radially assembled MTs as shown in Fig. 1. This result prompted the need to identify the active protein factor(s) responsible for the assembly of radial MTs around the nucleus. In the present report, we describe a protein of ~38-kDa that has the ability to form aster-like structures in a characteristic manner when it is incubated with tubulin. We regard this active protein to be a constituent of the MTOPs of the nuclear surface in higher plants.

**EXPERIMENTAL PROCEDURES**

**Preparation of Nuclei**—Tobacco BY-2 cells were cultured at 27 °C in the dark with shaking in Linsmaier and Skoog medium and subcultured at intervals of 7 days. Cells cultured for 5 days were treated with a wall lysis medium to produce protoplasts. The lysis medium consisted of a 0.45 M solution of mannitol (pH 5.5) that contained 1% Cellulase Onozuka RS (Yakult Hon-sya, Tokyo, Japan) and 0.1% Pectolyase Y23 (Kikkoman Seisun, Tokyo, Japan). After incubation for 1.5 h at 27 °C, the protoplasts were sedimented by centrifugation for 5 min in 0.5% to lyse the plasma membrane. We tried various concentrations of Nonidet P-40 from 0.1 to 0.5% and found that almost 0.5% to lyse the plasma membrane. We regarded 0.5%, and at any concentration of 0.5% to lyse the plasma membrane. We regarded this active protein to be a constituent of the MTOPs of the nuclear surface in higher plants.

**Preparation of MAP-Free Tubulin and Labeling with Rhodamine and Fluorescein Isothiocyanate (FITC)**—Tubulin from porcine brain was isolated with buffer B (PIPES, 1 mM EGTA, 0.5 mM MgSO4, pH 6.9) by two cycles of temperature-dependent polymerization and depolymerization in the presence of 1 mM GTP according to Shelanski et al. (30). Then the tubulin was purified by column chromatography on DEAE-Sephal. After being washed with modified buffer B (10 mM PIPES, 1 mM EGTA, 0.5 mM MgSO4) containing 0.3 M NaCl, the tubulin was eluted from the column with above modified buffer B containing 0.8 M NaCl. The eluate was dialyzed overnight against buffer C (100 mM PIPES, 0.5 mM dithiothreitol, 1 mM EDTA, 33% glycerol, 0.1 mM GTP, 0.5 mM MgSO4, and 80 mM NaCl, pH 6.9) at 4 °C. Then, after the addition of GTP to 1 mM, the cycle of temperature-dependent polymerization and depolymerization was repeated twice. The resulting MAP-free tubulin preparation was stored in liquid nitrogen.

Rhodamine-labeled tubulin was prepared according to Hyman et al. (31). After polymerization of MAP-free tubulin in buffer C, the preparation was laid on buffer C containing 60% glycerol (instead of 33%) as a cushion and centrifuged for 30 min at 154,000 × g at 36 °C. The precipitated MTs were suspended to a concentration of 50 mg/ml in a PIPES buffer similar to buffer C, except with pH 8.0. Carboxytetramethylrhodamine succinimidyl ester (Molecular Probes Inc. Eugene, OR) was dissolved in Me2SO at 100 mM and then added to 1/10 volume of the MT preparation under vortexing. After 10 min, the reaction mixture was laid on the cushion buffer with 60% glycerol and centrifuged for 30 min at 154,000 × g. The resulting precipitate of MTs was depolymerized at 0 °C after suspension in buffer C. The rhodamine-labeled tubulin was purified further by two cycles of temperature-dependent polymerization and depolymerization and then stored in liquid nitrogen.

FITC-labeled tubulin was prepared by a procedure similar to that used for the preparation of rhodamine-labeled tubulin, with the substitution of FITC. Because of the low tubulin content in plant cells, the usage of plant tubulin in this investigation was limited; therefore, we employed tubulin from porcine brain, mainly. To confirm the identical property of plant tubulin with animal tubulin, we also conducted the isolation of plant tubulin. Tubulin from tobacco cells was prepared by the method of Mizuno et al. (32). Five hundred grams of suspension-cultured tobacco cells was extracted in 1000 ml of extraction buffer D (50 mM MES, 0.5 mM MgSO4, 1 mM dithiothreitol, 0.2 mM p-ABSF, 25 μg/ml leupeptin, 25 μg/ml pepstatin A, pH 6.5) with an ultrasonic disruptor (model UD-201; Tomy Seiko Co. Ltd., Tokyo, Japan). The homogenate was centrifuged for 1 h at 100,000 × g at 4 °C. From the supernatant, tubulin was isolated and purified by affinity chromatography on ethyl N-phenylcarbamate-Sepharose and ion exchange resin chromatography on DEAE-Sephal (GE Healthcare). Isolated plant tubulin was conjugated with carboxytetramethyl-rhodamine succinimidyl ester (Molecular Probes Inc.) as above and introduced under “Assay for MTOC” as porcine brain tubulin as follows.

**Assay for MTOC**—To detect MT organizing activity, a mixture of rhodamine-labeled tubulin and unlabeled tubulin (ratio 1:4), at the total concentration of 0.4 mg/ml, which is known
never to induce spontaneous polymerization, was added to the preparation of isolated nuclei, the solubilized protein preparation from the nuclei, or the recombinant protein preparation. After incubation in buffer B with 1 mM GTP and 10% Me₂SO at 27 °C for 30 min, the reaction mixture was observed under Fluoview laser confocal microscope (Olympus Corporation, Tokyo, Japan) to detect the formation of radial MTs from the surface of nuclei and the formation of “aster-like structures” or their clusters.

Preparation and Sources of Antibodies—A protein band corresponding to an active protein with an apparent molecular mass of 38 kDa by SDS-PAGE was cut after brief staining with an aqueous solution of Coomassie Brilliant Blue and then homogenized in liquid nitrogen. A rabbit was immunized three times with a mixture of Gerbu adjuvant 100 (Gerbu Biotechnik GmbH, Gaibling, Germany) and gel homogenate that contained ~80 µg of the 38-kDa protein for 4-week interval each. Serum was obtained from blood and centrifuged at 10,000 × g for 30 min at 4 °C. From the supernatants, the IgG fraction was purified using protein A-Sepharose (Amersham Biosciences) and used for immunoblotting analysis and indirect immunofluorescence studies.

Monoclonal antibodies against α- and β-tubulin were purchased from Amersham Biosciences. Monoclonal antibodies against the sea urchin centrosomal 51-kDa protein was a kind gift of Prof. Hasezawa, S. (Graduate School of Biological Science, Tokyo University), and the monoclonal antibody raised against the N-terminal region (from the N terminus to the amino acid residue 111) of γ-tubulin from Aspergillus nidulans was provided by Dr. T. Horio (School of Medicine, The University of Tokushima, Tokushima, Japan).

Extraction of Histones, Two-dimensional Polyacrylamide Gel Electrophoresis—Histones were extracted with 0.4 N H₂SO₄ from isolated nuclei or whole cells cultured for 5 days. After centrifugation of the mixture at 10,000 × g for 15 min, the proteins in the supernatant were precipitated with 20% trichloroacetic acid by centrifugation at 10,000 × g. The resulting precipitate was washed by suspending in cold acetone to remove trichloroacetic acid and centrifuged at 10,000 × g for 5 min. The protein precipitate was washed repeatedly until the entire removal of trichloroacetic acid from preparation monitoring by pH test paper after dissolving in small volume of water. The proteins were resolved by SDS-PAGE or two-dimensional PAGE. The first dimension electrophoresis was performed in an acetic acid-urea-Triton gel that contained 15% acrylamide, 1 N acetic acid, 8 M urea, and 0.5% (w/w) Triton X-100. The second was performed in a 12.5% acrylamide gel that contained SDS. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250.

Immunoblotting Analysis—After electrophoresis on SDS-PAGE on a 12.5% gel by the procedure of Laemmli (33) or two-dimensional PAGE on acetic acid-urea-Triton X-100/SDS, the proteins were transferred onto a nitrocellulose filter. Each nitrocellulose filter was then incubated in 1% bovine serum albumin in phosphate-buffered saline (PBS, pH 7.4) for 1 h at 36 °C. Separate filters were then incubated with mouse monoclonal antibody or rabbit polyclonal antibodies for 1 h at 36 °C and then washed five times, for 5 min each time, with PBS containing 0.05% Tween 20 (PBS-T). Peroxidase-conjugated antibodies raised in goats against mouse IgG (H + L) (Bio-Rad) or against rabbit IgG (H + L) (Bio-Rad) were diluted 1:1000 in 1% bovine serum albumin/PBS and incubated with the appropriate nitrocellulose filters for 1 h at 36 °C. After five washes with PBS-T, the protein bands were visualized by incubation of the nitrocellulose filters with the peroxidase substrate 4-chloro-1-naphthol (0.05%) and hydrogen peroxide (0.03%).

Indirect Immunofluorescence Studies—Tobacco BY-2 cells cultured for 5 days were fixed at room temperature for 1 h with 3.7% formaldehyde in 50 mM phosphate buffer (pH 7.5), which contained 3 mM EGTA and 5 µM phenylmethylsulfonyl fluoride. The cells were treated for 10 min with a mixture of 1% Cellulase Onozuka RS and 0.1% Pectolyase Y-23 in 0.45 M mannitol to render the cell wall permeable to antibodies. After being washed in 0.1% Nonidet P-40 for 15 min, the cells were incubated with 1% bovine serum albumin for 15 min and then dispersed on a glass slide that had previously been coated with polylysine. A mixture of monoclonal antibody raised against α-tubulin from chick brain (at a dilution of 1:500; Amersham Biosciences) and rabbit antibodies raised against the 38-kDa protein from the nuclei of tobacco BY-2 cells (at a dilution of 1:500) was applied for 1 h at 36 °C for indirect immunofluorescence double-staining of cells. A mixture of FITC-conjugated goat anti-mouse IgG (H + L) (at a dilution of 1:1000, Organon Teknika, West Chester, PA) and rhodamine-conjugated goat anti-rabbit IgG (H + L) (at a dilution of 1:1000, Organon Teknika) was then applied to well washed cells, and the cells were incubated for 1 h at 36 °C. Finally, 0.2 µg/ml of 4,6-diamidino-2-phenylindole (DAPI) was applied for the observation of DNA. For the determination of 38-kDa protein in aster-like structures, the fixed preparation was applied on pollysine-coated coverslips and was treated with a mixture of monoclonal antibody raised against α-tubulin from chick brain (at a dilution of 1:500; Amersham Biosciences) and rabbit antibodies raised against the 38-kDa protein from the nuclei of tobacco BY-2 cells (at a dilution of 1:500) as above. However, as a second antibodies, a mixture of Texas Red-conjugated sheep anti-mouse IgG (H + L) (at a dilution of 1:1000, GE Healthcare) and FITC-conjugated goat anti-rabbit IgG (H + L) (at a dilution of 1:1000, Organon Teknika) was applied and incubated for 1 h at 36 °C. The nuclei were isolated from synchronized S/G2 phase tobacco BY-2 cells as described in a previous report (35) in the presence of 0.5% Nonidet P-40. The isolated nuclei were fixed in 0.1% glutaraldehyde in acetone-methanol (1:1, v/v) at −20 °C for 1 h and were then rehydrated in 50 mM PIPES buffer, pH 6.9, and attached to a pollysine-coated coverslip. After being washed and blocked with 5% skim milk in 50 mM PIPES buffer (pH 6.9) for 1 h, the nuclei were incubated with affinity-purified polyclonal antibody against tobacco histone H1 (1:250 to 1:500) for 1 h, then washed, and incubated for 1 h with rhodamine-conjugated goat anti-rabbit IgG (1:350; Molecular Probes), and finally they were treated for 5 min with 0.2 µg/ml solution of DAPI. The preparations were observed after being embedded in a glycerol solution containing 0.01% n-propyl gallate under a Delta Vision System (Applied Precision, Mercer Island, WA) or Fluoview laser confocal microscope.
Determination of Partial Amino Acid Sequence of 38-kDa Protein—The purified 38-kDa protein was transferred to a polyvinylidine difluoride membrane filter (Bio-Rad) after separation by SDS-PAGE. After brief staining with 0.1% Ponceau S, the band corresponding to the 38-kDa protein was cut, and peptide fragmentation was performed according to the method of Iwamatsu (34) by digestion with Achromobacter Protease I (Wako Fine Chemicals, Tokyo, Japan). Each peptide fragment was separated by liquid chromatography on a C18 column (ODS Column; Wako), and the amino acid sequence of the N terminus then was analyzed by protein sequencer (model 473A; Applied Biosystems).

Sequence Analysis of cDNA—Poly(A)+ RNA was isolated from tobacco BY-2 cells with RNA extraction kits and mRNA purification kits (GE Healthcare). A Lambda-ZAP II (Stratagene) cDNA library was constructed from the poly(A)+ RNA. The library of 9 × 10^5 plaques was screened by the rabbit antibodies raised against the 38-kDa protein, using a picoBlue immunoscreening Kit (Stratagene). DNA sequencing was performed with a Thermo sequenase primer cycle sequencing kit (GE Healthcare) and a model LIC-4200L DNA sequencer (LicoR, Lincoln, NE).

Expression of Recombinant 38-kDa Protein in Escherichia coli—The 38-kDa protein was expressed as a fusion protein using the pET-32 vector (Novagen, Madison, WI), which provides an N-terminal TRX and His tag. Genes encoding the 38-kDa protein were amplified by reverse transcription-PCR for poly(A)+ RNA isolated from tobacco as above. The PCR primers GAGATGGCGACTGAAGAAACCA and TTACTTCCTCCCCTTCTAGC were used. After the PCR product had been subcloned, it was cloned into pET32 vector and the transformation of E. coli cells was performed. After the cells had grown to an A_600nm of 0.6, they were induced by the addition of isopropyl-β-D-thiogalactoside to 1.5 mM and then further cultured for 4 h at 37 °C. The cells were then precipitated by centrifugation for 5 min at 10,000 × g and extracted with lysis buffer (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl, 0.05% Tween 20, pH 8.0). After each sample had been centrifuged for 10 min at 15,000 × g, the supernatant was mixed with ½ volume of a 50% slurry of Ni-NTA resin (Qiagen) and stirred at room temperature for 45 min before being loaded into a column. The column was washed with 10 volumes of lysis buffer and then with 10 volumes of washing buffer (lysis buffer adjusted to pH 6.3 with 1 N HCl). Fusion protein was eluted with elution buffer (lysis buffer adjusted to pH 4.5 with 1 N HCl) and then dialyzed against 50 mM PIPES buffer (pH 6.9) to remove urea. The dia-lyzed preparation of recombinant 38-kDa protein was centrifuged at 100,000 × g for 30 min at 4 °C to remove aggregations, and then its ability to form aster-like structures was determined as noted under "Assay for MTOC."

RESULTS

Microtubules Protruding from the Surfaces of Isolated Nuclei or Nuclear Fragments—More than 50% of the tobacco BY-2 cells cultured for 5 days were incubated with MAP-free tubulin in polymerization buffer (buffer B with 1 mM GTP and 10% Me_2SO, pH 6.9) at 27 °C for 30 min, radial MT assembly occurred on the surfaces of the nuclei (Fig. 1B, C, and D) or absence (A) of 0.5% Nonidet P-40 was observed after incubation in buffer B with 0.4 mg/ml MAP free rhodamine-conjugated tubulin, 1 mM GTP, and 10% Me_2SO at 27 °C for 15 min. Observation was performed by Delta Vision System. Bar, 10 μm.

FIGURE 1. Generation of radial MTs from the nuclear surface of tobacco BY-2 cells and isolated nuclei. Radial MTs were observed by indirect immunofluorescent staining using monoclonal antibody raised against tubulin and FITC-labeled second antibody (A). Radial MT generation from nuclei that were isolated in the presence (B and C) or absence (D) of 0.5% Nonidet P-40 was observed after incubation in buffer B with 0.4 mg/ml MAP free rhodamine-conjugated tubulin, 1 mM GTP, and 10% Me_2SO at 27 °C for 15 min. Observation was performed by Delta Vision System. Bar, 10 μm.

In addition to nuclei that possessed radially generated MTs around their surfaces, we often observed aster-like structures when isolated nuclei were incubated with rhodamine-conjugated tubulin in polymerization buffer B with 1 mM GTP and 10% Me_2SO. During incubation, some factor(s) responsible for the function of MTOPs on the surfaces of nuclei might be released.

Purification of the Protein Factor Forming Aster-like Structures—Homogenization of the isolated nuclei in a glass homogenizer with the low-ionic-strength buffer E (10 mM PIPES, 1 mM dithiothreitol, 25 μg/ml leupeptin, 0.1 mM p-ABS, 1 μM pepstatin A, pH 6.9) at 4 °C did not release any active factors for the formation of aster-like structures from the nuclear material into the soluble fraction after centrifugation at 10,000 × g. Moreover, when the resulting precipitate was
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Purification of active protein factor inducing the formation of aster-like structures. A, purification process was monitored by SDS-PAGE. Lane M, marker of molecular mass; lane J, tobacco whole cell extract; lane 2, total extract of nuclei; lane 3, nuclear sap fraction; lane 4, extract of nuclei in hypotonic buffer (10 mM PIPES, 1 mM MgCl2, pH 6.9); lane 5, insoluble nuclear material in hypotonic buffer; lane 6, soluble nuclear fraction in buffer containing 0.5 mM NaCl; lane 7, insoluble nuclear material in buffer containing 0.5 mM NaCl; lane 8, soluble fraction after heat treatment of the preparation from lane 6; lane 9, insoluble material after heat treatment of the preparation from lane 6. The activity was monitored by observing the formation of aster-like structures under a laser confocal microscope, Fluoview, after incubation of each preparation with rhodamine-labeled tubulin in tubulin polymerization buffer B with 1 mM GTP and 10% Me2SO. The numbers on the lower panels correspond to the lane numbers of the above SDS-PAGE; none, corresponds to no addition of nuclear extract. The ability to form aster-like structures was detected in lanes 5, 6, and 8. Bar, 10 μm. B, protein preparation as shown in the lane 8 in A applied on gel filtration column (Sephadex G-200, 1.6 × 65 cm) for further purification, and the ability of proteins to form aster-like structures in every 35 fractions was determined. SDS-PAGE analysis of proteins in several fractions corresponds to each fraction number is shown. Activity was detected in every fractions that contained the 38-kDa protein. The numbers on the lower panels correspond to the elution numbers from the gel filtration column; none, corresponds to no addition of nuclear extract. Bar, 10 μm. C, preservation of activity of 38-kDa protein after purification on SDS-PAGE. The position of acrylamide gel corresponding to the migration of 38-kDa protein was cut and eluted electrophoretically. After precipitation with acetone (final to 80%) and following centrifugation at 10,000 × g for 5 min, the resulting precipitate was dissolved in 10 mM PIPES buffer (pH 6.9), and its ability to form aster-like structures was determined. Bar, 10 μm.

Confirmation of MT-organizing factor to be a protein by treatment with nucleases. A, MT organizing activity was detected in every preparations including 38-kDa protein. However, the fact that the activity of MT organization was retained even after serious conditions for proteins such as heat treatment and SDS-PAGE suggests the active factor(s) to be possibly nonproteinaceous substance. Then the purified preparation (initial) using gel filtration column including 38-kDa protein and the treatments with protease (trypsin, V8 protease) and nuclease (DNase, RNase) were used to determine the activity after heat treatment for 5 min in boiling water, and no addition of factor(s) (none) was also offered for assay. Bar, 10 μm. The SDS-PAGE analysis of 38-kDa protein and the products after trypsin and V8 protease treatment are also shown. B, SDS-PAGE analysis of complexes produced from 38-kDa protein and tubulin at low temperature. The supernatant (sup) and the sedimented complexes (ppt) after centrifugation at 10,000 × g for 15 min are shown. The sedimented complexes shows the presence of nearly equimolar quantities of both proteins.

FIGURE 2. Purification of active protein factor inducing the formation of aster-like structures. A, purification process was monitored by SDS-PAGE. Lane M, marker of molecular mass; lane J, tobacco whole cell extract; lane 2, total extract of nuclei; lane 3, nuclear sap fraction; lane 4, extract of nuclei in hypotonic buffer (10 mM PIPES, 1 mM MgCl2, pH 6.9); lane 5, insoluble nuclear material in hypotonic buffer; lane 6, soluble nuclear fraction in buffer containing 0.5 mM NaCl; lane 7, insoluble nuclear material in buffer containing 0.5 mM NaCl; lane 8, soluble fraction after heat treatment of the preparation from lane 6; lane 9, insoluble material after heat treatment of the preparation from lane 6. The activity was monitored by observing the formation of aster-like structures under a laser confocal microscope, Fluoview, after incubation of each preparation with rhodamine-labeled tubulin in tubulin polymerization buffer B with 1 mM GTP and 10% Me2SO. The numbers on the lower panels correspond to the lane numbers of the above SDS-PAGE; none, corresponds to no addition of nuclear extract. The ability to form aster-like structures was detected in lanes 5, 6, and 8. Bar, 10 μm. B, protein preparation as shown in the lane 8 in A applied on gel filtration column (Sephadex G-200, 1.6 × 65 cm) for further purification, and the ability of proteins to form aster-like structures in every 35 fractions was determined. SDS-PAGE analysis of proteins in several fractions corresponds to each fraction number is shown. Activity was detected in every fractions that contained the 38-kDa protein. The numbers on the lower panels correspond to the elution numbers from the gel filtration column; none, corresponds to no addition of nuclear extract. Bar, 10 μm. C, preservation of activity of 38-kDa protein after purification on SDS-PAGE. The position of acrylamide gel corresponding to the migration of 38-kDa protein was cut and eluted electrophoretically. After precipitation with acetone (final to 80%) and following centrifugation at 10,000 × g for 5 min, the resulting precipitate was dissolved in 10 mM PIPES buffer (pH 6.9), and its ability to form aster-like structures was determined. Bar, 10 μm.

FIGURE 3. Confirmation of MT-organizing factor to be a protein by treatment with nucleases. A, MT organizing activity was detected in every preparations including 38-kDa protein. However, the fact that the activity of MT organization was retained even after serious conditions for proteins such as heat treatment and SDS-PAGE suggests the active factor(s) to be possibly nonproteinaceous substance. Then the purified preparation (initial) using gel filtration column including 38-kDa protein and the treatments with protease (trypsin, V8 protease) and nuclease (DNase, RNase) were used to determine the activity after heat treatment for 5 min in boiling water, and no addition of factor(s) (none) was also offered for assay. Bar, 10 μm. The SDS-PAGE analysis of 38-kDa protein and the products after trypsin and V8 protease treatment are also shown. B, SDS-PAGE analysis of complexes produced from 38-kDa protein and tubulin at low temperature. The supernatant (sup) and the sedimented complexes (ppt) after centrifugation at 10,000 × g for 15 min are shown. The sedimented complexes shows the presence of nearly equimolar quantities of both proteins.
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Inhibition of MT Organization by Antibodies Against 38-kDa Protein—To determine whether MT organization was a histone H1-dependent process, the nuclei were treated with antibodies raised against the 38-kDa protein. After adsorption on polylysine-coated coverslips, the incubation with rhodamine-labeled tubulin was performed. The generation of MTs was inhibited markedly after treatment with antibodies raised against 38-kDa protein, although preimmune serum did not induce any inhibitory effect (Fig. 4C). Immunofluorescence analyses indicated that the 38-kDa protein might act as a component of an MTOP in the aster-like structures, because this protein was always located at the center of the aster-like structures but not along the MTs (Fig. 4D).

Cross-reactivity of Antibodies Against 38-kDa Protein with Nuclear Histone H1—On immunoblotting, the antibodies raised against 38-kDa protein were confirmed to cross-react with 38-kDa protein in extract of whole nuclei. Lane 1, stained by Coomassie Brilliant Blue R-250. Lane 2, immunoblotting using antibodies raised against 38-kDa protein. B, two-dimensional PAGE analysis of basic proteins isolated from tobacco cells is shown as Coomassie Brilliant Blue R-250 staining (left) and immunoblotting (right). A black arrowhead indicates the typical position of histone H1 on two-dimensional PAGE. C, inhibition of radial MT generation by antibodies raised against 38-kDa protein was studied. The nuclei incubated without antibodies beforehand, generate radial MTs from the surface of them. The nuclei incubated in the added antibodies previously blocked radial MT generation. The nuclei incubated in the added preimmune serum beforehand generate radial MTs as well as the nuclei previously incubated without antibodies. Bar, 10 μm. D, the localization of 38-kDa protein in the aster-like structures is confirmed. MTs are shown by fluorescence of Texas Red (red). The localization of 38-kDa protein is shown by the staining of the FITC-confined region as shown by arrows at the center of aster-like structures (green). The merged immunofluorescence figure suggests that the localization of 38-kDa protein, or histone H1, was restricted to the centers of the aster-like structures, not along the total length of the MTs. Bar, 10 μm. E, recombinant 38-kDa protein with TRX-His tag was produced in E. coli cells. Most of the recombinant protein formed inclusion bodies and was, therefore, purified with affinity column of Ni-NTA resin in the presence of 8 m urea. After dialysis of the samples, SDS-PAGE (1) and immunoblotting analysis (2) were performed, and the ability of recombinant 38-kDa protein to form aster-like structures was examined. The recombinant histone H1 was confirmed to act as a MTOP, as did the isolated 38-kDa protein.

These complexes were then functioning as a nucleation and elongation factor in the generation of MTs to form aster-like structures.

Acid Sequences of N-terminal Regions of Peptide Fragments of 38-kDa Protein—The partial sequences of the N-terminal regions of four peptide fragments were determined as follows: fragment 1, TGSSQHAITK; fragment 2, YFEMIKDA; fragment 3, FIEDK; and fragment 4, AITK. The sequences of these four peptide fragments are identical to sequences in histone H1 of the tobacco plant (36).

Inhibition of MT Organization by Antibodies Against 38-kDa Protein—To determine whether MT organization was a histone H1-dependent process, the nuclei were treated with antibodies raised against the 38-kDa protein. After adsorption on polylysine-coated coverslips, the incubation with rhodamine-labeled tubulin was performed. The generation of MTs was inhibited markedly after treatment with antibodies raised against 38-kDa protein, although preimmune serum did not induce any inhibitory effect (Fig. 4C). Immunofluorescence analyses indicated that the 38-kDa protein might act as a component of an MTOP in the aster-like structures, because this protein was always located at the center of the aster-like structures but not along the MTs (Fig. 4D).

Cloning and Sequence Analysis of cDNA Encoding 38-kDa Protein—To identify the sequence of the 38-kDa protein, we screened a tobacco BY-2 cDNA expression library with antibodies raised against the 38-kDa protein. Several positive clones were selected, and their sequences were analyzed. Identical sequences were obtained among all these positive clones. The analyzed sequence of the 38-kDa protein was confirmed to consist of 279 amino acids, with an estimated molecular mass of 29,559 Da, including an nuclear localization signal consensus sequence, KKEKE (amino acids 38–42), and all of the above mentioned peptide sequences (TGSSQHAITK, YFEMIKDA, FIEDK, and AITK). Primary sequence analysis of the 38-kDa protein revealed that it was identical to a histone H1 variant from tobacco plant (NCBI protein identification code BAA88671). The difference between the apparent molecular mass of 38 kDa protein on SDS-PAGE and the estimated molecular mass from sequence analysis might be due to its strong basic property.

Recombinant 38-kDa Protein in E. coli—We used SDS-PAGE and immunoblotting to determine the properties of recombinant TRX-His tagged 38-kDa protein purified in an affinity column with Ni-NTA resin. The ability of the protein to form aster-like structures was also confirmed after incubation with tubulin in polymerization buffer. Stable aster-like structures...
were observed (Fig. 4E), as in the case of 38-kDa protein purified from the tobacco nuclei, confirming that histone H1 acts as an organizer of MTs.

**Indirect Immunofluorescence Observation**—The very intensive staining was observed around nucleoli of those cells that had nuclei with no radial MTs generated around them (Fig. 5, A–D). In contrast, in the cells that generating radial MTs around their nuclei, the fluorescence was observed at the nuclear rim and nuclear plasm. Particularly, the staining of nuclear rim was intense and not overlapped with the staining of chromosomal DNA (Fig. 5, E–H). One of the most impressive observations was the pattern of staining of the spindles; no fluorescence was detected at the spindle pole region, but staining was detected at around the chromosomes in metaphase cells (Fig. 5, I–L). In the cells with phragmoplasts, the staining with anti-38-kDa protein revealed mainly at the chromosomes of daughter nuclei (Fig. 5, M–P). The localization of histone H1 in the isolated nuclei from synchronized cells in S/G2 phase was clearly shown. The intense fluorescence of nuclear rim was observed (Fig. 5, Q–S) as well as in the immunostaining of the cells in the corresponding stage of the cell cycle. The immunofluorescent staining in the nuclei that were isolated in the presence of detergent was identical to those isolated in the absence of detergent (35).

**DISCUSSION**

In cultured tobacco BY-2 cells, radial MTs are generated from the surfaces of the nuclei at an particular stage of the cell cycle. We can speculate that cells containing nuclei with radial MTs on their surfaces are in the S, G2, and early G1 phases (37, 38). Initially we believed that \(\gamma\)-tubulin would be the protein factor with the ability to organize MTs on the nuclei as in the centrosomes of animal cells (24, 39), the spindle pole bodies of fungi (25, 26), and plastid of bryophyte (41). Because \(\gamma\)-tubulin is widely accepted to be a minus-end-directed component of MTOCs, one would expect tubulin incorporation to occur preferentially at the distal end of MTs if \(\gamma\)-tubulin were a true organizer in the MTOPs on plant nuclei. However, in our previous (15) study we showed that tubulin incorporation occurred preferentially at the proximal ends of aster-like MTs. Nevertheless, this observation does not prove that the polarity of MTs is plus end-proximal. Recently our experiments

**FIGURE 5. Immunofluorescence observation with antibodies raised against 38-kDa protein.** In the cells containing nuclei with surface-associated radial MTs, the intensive fluorescence is detected at around the nucleoli but not at around the nuclear rim (A–D). In the cells containing nuclei with radial MTs emanating from the surfaces of them, the much intensive immunostaining is observed specifically at around the nuclear rim, independently of the chromosomal DNA staining (E–H). Insets, enlargement of the marked area stained with rhodamine (F) and DAPI (G) are merged. The independent immunostaining of histone H1 and DNA at nuclear rim is clearly shown. In the mitotic stage, the immunofluorescence is detectable at the position of the chromosomes but not at the spindle poles. Histone H1, therefore, might present mainly in association with chromosomes (I–L). In the cells with phragmoplasts, histone H1 might be associated mainly with chromosomes in daughter nuclei (M–P). The nuclei that were isolated from synchronized S/G2 phase tobacco BY-2 cells in the presence of 0.5\% Nonidet P-40 shows the intensive immunostaining at around nuclear rim (Q–S) as well as immunostaining in the cells containing nuclei with radial MTs around them. The staining with anti-tubulin, or microtubule staining is shown by green (A, E, I, and M); the staining with anti-38 kDa protein, or histone H1 staining is shown by red (B, F, J, N, and Q); the staining with DAPI or DNA staining is shown by blue (C, G, K, O, and R); the merged images (D, H, L, P, and S). Bars in D, H, L, and P, 10 \(\mu\)m; bar in S, 5 \(\mu\)m; bar in inset in G, 1 \(\mu\)m.
employing both caged fluorescent probes and bleaching assay and polarity-marked microtubules revealed that the polarity of MTs in aster-like structures is plus end-proximal (35). This indicates the functioning of some novel mechanism of MT organization on the nuclear surface. We, therefore, investigated a protein with an apparent molecular mass of 38 kDa on SDS-PAGE as a potential MTOP on the nuclei of tobacco BY-2 cells. Cloning of the cDNA encoding the 38-kDa protein and investigation of the recombinant protein confirmed that the 38-kDa protein was a major histone H1 variant of cultured tobacco BY-2 cells and acts as an MTOP on the nuclear surface in cooperation with tubulin. We assumed that these novel MTOP complexes or radial MTs in aster-like structures is plus end-proximal (35). This continuous incorporation of tubulin at the proximal end (35); therefore, the idea inevitably arose that the histone H1-tubulin complex might act to push out the elongating microtubule, progressively. A review by Kaczanowski and Jerzmanowski (42) suggests the interaction of histone H1 with tubulin. Moreover, Multigter et al. (43) reported that histone H1 stabilizes the microtubules of sea urchin flagella, and Mitchieux et al. (40) demonstrated the ability of histone H1 to interact with tubulin. Interesting papers as above encouraged us strongly to study the interaction between histone H1 and tubulin in higher plant cells.

**REFERENCES**

1. Wasteneys, G. O., and Zhenbiao, Y. (2004) Plant Physiol. 136, 3884–3891
2. Ledbetter, M. C., and Porter, K. R. (1963) J. Cell Biol. 19, 239–250
3. Shibaoka, H., and Nagai, R. (1994) Curr. Opin. Cell Biol. 6, 10–15
4. Giddings, T. H., and Staehelin, L. A. (1991) in The Cytoskeletal Basis of Plant Growth and Form (Lloyd, C. W., ed), pp. 229–292. Academic Press, London
5. Pickett-Heaps, J. D., and Northcote, D. H. (1966) J. Cell Sci. 1, 109–120
6. Gunning, B. E., and Wick, S. M. (1985) J. Cell Sci. 2, (suppl.) 157–179
7. Baskin, T. I., and Cande, W. Z. (1990) Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 277–315
8. Baluska, F., Volkmann, D., and Barlow, P. W. (1997) Int. Rev. Cytol. 175, 91–135
9. Lambert, A. M. (1980) Chromosoma 76, 295–308
10. Lambert, A. M. (1993) Curr. Opin. Cell Biol. 5, 116–122
11. Clayton, L., Black, C. M., and Lloyd, C. W. (1985) J. Cell Biol. 101, 319–324
12. Falconer, M. M., Donaldson, G., and Seagull, R. W. (1988) Protoplasma 144, 46–55
13. Flanders, D. J., Rawlins, D. J., Shaw, P. J., and Lloyd, C. W. (1990) Development 110, 897–904
14. Staiger, C. J., and Lloyd, C. W. (1991) Curr. Opin. Cell Biol. 3, 33–42
15. Mizuno, K. (1993) Protoplasma 173, 77–85
16. Stoppin-M. V., Vantard, M., Schmit, A. C., and Lambert, A. M. (1994) Plant Cell 6, 1099–1106
17. Schmit, A. C. (2002) Int. Rev. Cytol. 220, 257–289
18. Schmit, A. C., Endle, M. C., and Lambert, A. M. (1996) Chromosoma 104, 405–413
19. Stoppin, V., Lambert, A. M., and Vantard, M. (1996) Eur. J. Cell Biol. 69, 11–23
20. Erhardt, M., Stoppin-M., Campagne, S., Canaday, J., Munter, J., Fabian, T., Sauter, M., Muller, T., Peter, C., Lamber, A.-M., and Schmit, A.-C. (2002) J. Cell Sci. 115, 2423–2431
21. Hasezawa, S., and Nagata, T. (1993) Protoplasma 176, 64–74
22. Kumagai, F., Hasezawa, S., and Nagata, T. (1995) Bot. Acta 108, 1–7
23. Kumagai, F., Hasezawa, S., and Nagata, T. (1999) Eur. J. Cell Biol. 78, 109–116
24. Zhang, Y., Jung, M. K., and Oakley, B. R. (1991) Cell 65, 817–823
25. Oakley, B. R., Oakley, C. E., Yoon, Y., and Jung, M. K. (1990) Cell 61, 1289–1301
26. Horio, T., Uzawa, S., Jung, M. K., Oaldey, B. R., Tanaka, K., and Yanagida, M. (1991) J. Cell Sci. 99, 693–700
27. Liu, B., Yuvic, H. C., and Paleitz, B. A. (1993) J. Cell Sci. 104, 1217–1228
28. Liu, B., Joshi, H. C., Wilson, T. J., Silflow, C. D., Paleitz, B. A., and Snustad, D. P. (1994) Plant Cell 6, 303–314
29. Murata, T., Sonobe, S., Baskin, T. I., Hyodo, S., Hasezawa, S., Nagata, T.,
Nuclear Microtubule Organization by Histone H1

Horio, T., and Hasebe, M. (2005) Nat. Cell Biol. 7, 961–968
30. Shelanski, M. L., Gaskin, F., and Cantor, C. R. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 765–768
31. Hyman, A. A., Dreschel, D., Kellogg, D., Salser, S., Sawin, K., Steffen, P., Wordeman, L., and Schley, J. M. (1990) Methods Enzymol. 196, 478–485
32. Mizuno, K., Koyama, M., and Shibaoka, H. (1981) I. Biochem. (Tokyo) 89, 329–332
33. Laemmli, U. K. (1970) Nature 227, 680–685
34. Iwamatsu, A. (1991) Seikagaku 63, 139–143
35. Hotta, T., Haraguchi, T., and Mizuno, K. (2007) Cell Struct. Funct. 32, 79–87
36. Szekeres, M., Haizel, T., Adam, E., and Nagy, E. (1995) Plant Mol. Biol. 27, 597–605
37. Hasezawa, S., and Nagata, T. (1991) Bot. Acta 104, 206–211
38. Kumagai, F., Yoneda, A., Tomida, T., Sano, T., Nagata, T., and Hasezawa, S. (2001) Plant Cell Physiol. 42, 723–732
39. Stearns, T., Evans, L., and Kirschner, M. (1991) Cell 32, 577–581
40. Mithieux, G., Alquier, C., Roux, B., and Roussset, B. (1984) J. Biol. Chem. 259, 15523–15531
41. Shimamura, M., Brown, R. C., Lemmon, B. E., Akashi, T., Mizuno, K., Nishihara, N., Tomizawa, K., Yoshimoto, K., Deguchi, H., Hosoya, H., Horio, T., and Mineyuki, Y. (2004) Plant Cell 16, 45–59
42. Kaczanowski, S., and Jerzmanowski, A. (2001) J. Mol. Evol. 53, 19–30
43. Multigner, L., Gagnon, J., Van Dorselaer, A., and Job, D. (1992) Nature 360, 33–39