A MICROTUBULE SLIDING SYSTEM INVOLVED IN THE ORGANIZATION OF PHRAGMOPLASTS

TETSUHIRO ASADA
DEPARTMENT OF BIOLOGY, FUCULTU OF SCIENCE
OSAKA UNIVERSITY
MAY 1994

A MICROTUBULE SLIDING SYSTEM INVOLVED IN THE ORGANIZATION OF PHRAGMOPLASTS

TETSUHIRO ASADA
DEPARTMENT OF BIOLOGY, FACULTY OF SCIENCE
OSAKA UNIVERSITY
MAY 1994

CONTENTS

abbreviations

AMPPNP, 5'-adenylylimidodiphosphate; **BSA**, bovine serum albumin; **DTT**, dithiothreitol; **EGTA**, ethylene glycol bis(2-aminoethyl-ether) tetraacetic acid; **ER**, endoplasmic reticulum; **GMPPNP**, 5'-guanylylimidodiphosphate; **PBS**, phosphate-buffered saline; **PCR**, polymerase chain reaction; **PIPES**, piperazine-*N,N'*-bis(ethanesulfonic acid); **PMSF**, phenylmethylsulfonyl fluoride; **PVP-40**, polyvinylpyrrolidone (Mr 40000); **SDS-PAGE**, sodium dodecylsulfate-polyacrylamide gel electrophoresis; **TAME**, p-tosyl-arginine methyl ester; **TCA**, trichloroacetic acid.

SUMMARY

In higher plant cells, cytokinesis is achieved by cell-plate formation mediated by the phragmoplast. The phragmoplast consists of two oppositely oriented sets of microtubules with their plus ends interdigitating at the plane of cell-plate formation. Such an arrangement of phragmoplast microtubules is thought to be essential for the accumulation of vesicles that form the cell plate. I have attempted to reveal the molecular mechanism that involved in the organization of the phragmoplast microtubule arrays.

The cell cycle of tobacco BY-2 cells was synchronized and cells in anaphase and telophase were permeabilized with a glycerol solution, and the glycerinated cells were used as a model system to characterize the incorporation of exogenously applied tubulin in phragmoplasts (chapter I). When the glycerinated cells were incubated with fluorescein-labeled tubulin and GTP, a fluorescent band appeared at the equatorial region of the phragmoplast, and the width of the band increased with incubation time. When the cells that had incorporated fluorescent tubulin at the equatorial region of the phragmoplast were further incubated with unlabeled tubulin and GTP, the fluorescent band at the equatorial region was split into two, suggesting that microtubules in the phragmoplast were translocated towards their minus ends concomitantly with tubulin polymerization at their plus ends that were located at the equatorial region of the phragmoplast. The translocation was induced effectively by GTP and less effectively by ATP, and was inhibited by the unhydrolysable nucleotide analogues GMPPNP and AMPPNP. These results suggest that the microtubule translocation in the phragmoplast is driven by a motor protein which causes minus-end headed movement of microtubules at the expense of energy generated by hydrolysis of GTP or ATP. The microtubule slidng system characterized in this study seems to be involved in the formation of the functional array of phragmoplast microtubules.

An attempt was made to identify proteins with the ability to translocate microtubules in an extract from phragmoplasts isolated from tobacco BY-2 cells (chapter II). Homogenization of isolated phragmoplasts in a solution that contain MgATP, MgGTP and a high

concentration of NaCl resulted in the release from phragmoplasts of factors with ATPase- and GTPase-activity that were stimulated by microtubules. A protein fraction with microtubule-dependent ATPase- and GTPase-activity caused minus-end-headed gliding of microtubules in the presence of ATP or GTP. Factors with the microtubule-translocating activity cosedimented with microtubules and were dissociated from sedimented microtubules by an addition of ATP or GTP. After cosedimentation and dissociation procedures, a 125-kDa polypeptide and a 120-kDa polypeptide were recovered in a fraction that supported minus-end-headed gliding of microtubules. The rate of microtubule gliding was 1.28 μm/min in the presence of ATP and 0.50 μm/min in the presence of GTP. These polypeptides that support slow minus-end-headed movements of microtubules are candidates for the motor proteins that cause translocation of phragmoplast microtubules.

Partial amino acid sequence of the 125 kDa microtubule-motor-polypeptide was determined (chapter III). The sequence of a tryptic peptide of the 125-kDa polypeptide included a consensus sequence of the kinesin superfamily, EXYXXXXXDLL, indicating that the 125-kDa polypeptide is a polypeptide related to kinesin heavy chain. A part of cDNA encoding the 125-kDa kinesin-related polypeptide was amplified by polymerase chain reaction using two primers derived from two tryptic peptides of the 125-kDa polypeptide. The partial primary sequence predicted from the amplified cDNA was significantly similar to kinesin like proteins involved in the formation of the mitotic spindle in fungal cells or a frog egg. A peptide antibody specific to the 125-kDa polypeptide was raised and used to examine the localisation of 125-kDa polypeptide. The immunofluoresce indicated that the polypeptide was associated with microtubules in the preprophase band, the mitotic spindle, and the phragmoplast. Western blot analysis indicated that the amount of the 125-kDa polypeptide changed dramatically as the cell cycle progressed. The amount increased with the progression from S to G2 phase and rapidly decrease after the mitotic phase. These result suggests that the 125-kDa kinesin-related polypeptide plays an important role in several events in the plant cell division.

GENERAL INTRODUCTION

The microtubule is a fundamental cytoskeletal element, that is found in most of eukaryotic cells and involved in various cellular processes, including cell morphogenesis, spatial distribution of organelles, and cell motility. The microtubule-mediated events depend on the proper distribution and arrangement of microtubules. Therefore, to understand the basis of microtubule functions, it is important to clarify the mechanism that organizes microtubule arrays in the cell.

The mitotic spindle, an organized array of microtubules responsible for chromosome segregation, is an appropriate model to study the mechanism of the microtubule organization, and many informations about its organization mechanism have been obtained to date. It is well known that centrosomes in animal cells or spindle pole bodies in fungal cells generate microtubules and participate in the formation of bipolar spindles. Because these microtubule-organizing centers always catch the "minus" ends of microtubules, they can form the polarized array of microtubules (Kimble and Kuriyama, 1992). The presence in cells of microtubule-organizing centers is essential for the formation of the spindle, but only the presence of them does not allow cells to form the spindle. Recent mutant analysis of fungal cells indicates that activities of microtubule motor proteins are also required for the spindle formation (Endow and Titus, 1992).

Higher plant cells do not have centrosome, but they construct several functional arrays of microtubules in addition to the mitotic spindle. One of them is the phragmoplast that mediates a plant-cell-specific cytokinesis by mediating the cell plate formation. At the late stage of mitosis, microtubules between separating anaphase chromosomes increase in number to form the phragmoplast (Zhang et al., 1990). The phragmoplast consists of two oppositely directed sets of microtubules with their "plus" ends interdigitating on the equatorial plane (Euteneur and McIntosh, 1980; Hepler and Jackson, 1968), where a number of Golgi-derived vesicles accumulate and fuse to form the cell plate (Hepler, 1982). The oppositely directed microtubule array in the phragmoplast is

believed to be essential for the accumulation of vesicles that form the cell plate (Bajer et al. 1987). In contrast to spindle- and cytoplasmic-microtubules in animal cells with their fragil "minus" ends blocked by centrosomes, phragmoplast microtubules are arranged with their "minus" ends unblocked. Such an arrangement of phragmoplast microtubules raises a question of how microtubules in the phragmoplast are formed, arranged and maintained. Without any direct observation of microtubule assembly in the phragmoplast, we would not be able to expect to provide any answer to this question.

Thus, I newly developed an in vitro system which enable us to observe the assembly of phragmoplast microtubules and, by employing this newly developed system, I demonstrated that a microtubule sliding system was involved in the organization of the phragmoplast (Chapter I). I, therefor, isolated a candidate for the motor protein responsible for the microtubule sliding in the phragmoplast (Chapter II) and determined partial primary sequence of the candidate and examined its subcellular localization (Chapter III).

CHAPTER I.
MICROTUBULE TRANSLOCATION IN PHRAGMOPLASTS OF CULTURED TOBACCO CELLS

INTRODUCTION

Microtubules change their arrays according to the progression of cell cycle. In plant cells, they assme four different kinds of arrays, namely, cortical microtubules ,the prophase band of microtubules, the mitotic spindle, and the phragmoplast. The changes in the array during the cell cycle strongly suggest the occurrence of changes in the nature and the distribution of microtubule organizing centers. Thus, the studies on the microtubule organizing center are indispensable for the full understanding of the mechanism which regulate the function of microtubuler apparatus. But, nothing has been clarified of the microtubule-organizing center in plant cells. Since, the phragmoplast is a plant cell specific cytokinetic apparatus, I chose the phragmoplast as a model system to study the mechanism which regulate microtubule arrays in plant cells. The phragmoplast has attracted the attention of many cell biologists. Inoue (1968) observed the birefringence of the phragmoplast "fiber" of Haemanthus endosperm cells under a polarization microscope and he examined the effect of the ultraviolet microbeam irradiation on the phragmoplast "fiber". He found that the birefringence disapeared if the equatorial region of the phragmoplast was irradiated, suggesting that the equatorial region of the phragmoplast is involved in formation or meintenance of phragmoplast "fiber", namely, phragmoplast microtubules. To study the role of the equatorial region of the phragmoplast, I used tobacco BY-2 cells with phragmoplasts, whose plasma membrane had been permeabilized with a glycerol solution, namely glycerinated tobacco BY-2 cells. I examined how exogenously applied tubulin were incorporated in the phragmoplast of glycerinated tobacco cells.

MATERIALS AND METHODS

Culture and synchronization of the cell cycle of tobacco BY-2 cells

Tobacco BY-2 cells (Nicotiana tabacum 'Bright Yellow 2') were cultured in modified Linsmaier and Skoog's medium (LS medium) supplemented with 3% (w/v) sucrose, 370 mg/ml KH_2PO_4, 1.0 mg/ml thiamin hydrochloride and 0.2 mg/ml 2,4-dichlorophenoxyacetic acid (Nagata et al., 1981). The cell cycle of BY-2 cells was synchronized by treatments with aphidicolin and propyzamide (Kakimoto and Shibaoka, 1988).

Preparation of glycerinated cells and fluorescent tubulin

BY-2 cells, approximately 80% of which were in anaphase or telophase, were treated with cell-wall digesting enzymes [1% (w/v) cellulase Onozuka RS (Yakult Pharmaceutical Co. Ltd., Takarazuka, Hyogo), and 0.1% (w/v) pectolyase Y-23 (Seishin pharmacentical Co. Ltd., Tokyo, Japan) in 0.4M mannitol, pH 5.5] for 5 min at room temperature and then washed twice with 0.5M mannitol solution. To permeabilize the plasma membrane, cells were suspended in 50 mM Pipes buffer (pH 7.0) containing 60% glycerol, 1 mM $MgCl_2$, 5 mM EGTA and 1 mM DTT for 10 min at 4-10°C, and then 0.5-2 hr on ice. The permeabilized cells were reffered to as glycerinated cells.

Tubulin was prepared from bovine brains by a two-cycle assembly and disassembly procedure that was followed by purification on a phosphocellulose column (Shelanski, 1973). DTAF (dichlorotriazonilamino fluorescein)-labeled tubulin was prepared as described by Keith et al. (1981). Tubulin and DTAF-tubulin was resolved in PME-buffer at 8-10 mg/ml and stored at -70°C until used.

Assay for tubulin incorporation and microtubule translocation in glycerinated cells

Glycerinated cells were washed twice with PME-buffer (75 mM Pipes, pH 7.0 containing 1 mM $MgCl_2$ and 1 mM EGTA) and suspended in PME-buffer containing 5 μM taxol, 4 mM GTP and 15% glycerol. DTAF-

tubulin or unlabeled tubulin was added to 20-50 μl of the cell suspension and incubated at 30°C. The density of cells during incubation was adjusted to ~100 /μl. In the experiments in which cells incubated with DTAF-tubulin were further incubated with unlabeled tubulin, the cell suspension with DTAF-tubulin was mixed with 10 volume of PME-buffer containing 10 μM taxol, and the cells were collected by centrifugation and incubated with unlabeled tubulin as in the incubation with DTAF-tubulin. Tubulin polymerization did not require taxol, but polymerized microtubules did not withstand procedures necessary for changing the incubation medium in the absence of taxol. In the experiment of which results are shown in Figure 2a, glycerinated cells with phragmoplasts were incubated first with 0.5 mg/ml DTAF-tubulin together with 4 mM GTP for 20 min and then with an appropriate concentration of unlabeled tubulin together with 4 mM GTP. In the experiment of Figure 2b, glycerinated cells were incubated with 0.5 mg/ml DTAF-tubulin together with 4 mM GTP for 20 min 0.5 mg/ml unlabeled tubulin together with 3 mM GTP and 1 mM GMPPNP for 30 min at 30°C. The cells were washed three times with 4 mM GTP or 4 mM ATP in 75 mM Pipes buffer, pH 7.0, containing 10 μM taxol, 4 mM MgCl2, 1 mM EGTA and 1 mM DTT. At appropriate times, the cells were sampled on a glass slide and fixed with cooled methanol at -15°C. The cells were observed under an Olympus BHS-RFK fluorescence microscope.

Immunofluorescence microscopy

Glycerinated cells were fixed for 40 min in PME buffer that contained 3.7% formaldehyde and then washed three times with PBS. Fixed samples were suspended in PBS-BSA (PBS that contained 1% bovine serum albumin) and to the suspension was added mouse monoclonal antibodies against chick brain a-tubulin (Amersham, Buckinghamshire, U.K.) and the suspension with the antibodies was incubated for 30 min at room temprature. The samples were washed three times with PBS-BSA and then incubated for 30 min with FITC-labeled rabbit antibodies against mouse IgG (ICN Immunobiologicals, Lisle, U.S.A.) in PBS-BSA.The cells were observed under an Olympus BHS-RFK fluorescence microscope.

tubulin or unlabeled tubulin was added to 20-50 μl of the cell suspension and incubated at 30°C. The density of cells during incubation was adjusted to ~100 /μl. In the experiments in which cells incubated with DTAF-tubulin were further incubated with unlabeled tubulin, the cell suspension with DTAF-tubulin was mixed with 10 volume of PME-buffer containing 10 μM taxol, and the cells were collected by centrifugation and incubated with unlabeled tubulin as in the incubation with DTAF-tubulin. Tubulin polymerization did not require taxol, but polymerized microtubules did not withstand procedures necessary for changing the incubation medium in the absence of taxol. In the experiment of which results are shown in Figure 2a, glycerinated cells with phragmoplasts were incubated first with 0.5 mg/ml DTAF-tubulin together with 4 mM GTP for 20 min and then with an appropriate concentration of unlabeled tubulin together with 4 mM GTP. In the experiment of Figure 2b, glycerinated cells were incubated with 0.5 mg/ml DTAF-tubulin together with 4 mM GTP for 20 min 0.5 mg/ml unlabeled tubulin together with 3 mM GTP and 1 mM GMPPNP for 30 min at 30°C. The cells were washed three times with 4 mM GTP or 4 mM ATP in 75 mM Pipes buffer, pH 7.0, containing 10 μM taxol, 4 mM MgCl2, 1 mM EGTA and 1 mM DTT. At appropriate times, the cells were sampled on a glass slide and fixed with cooled methanol at -15°C. The cells were observed under an Olympus BHS-RFK fluorescence microscope.

Immunofluorescence microscopy

Glycerinated cells were fixed for 40 min in PME buffer that contained 3.7% formaldehyde and then washed three times with PBS. Fixed samples were suspended in PBS-BSA (PBS that contained 1% bovine serum albumin) and to the suspension was added mouse monoclonal antibodies against chick brain a-tubulin (Amersham, Buckinghamshire, U.K.) and the suspension with the antibodies was incubated for 30 min at room temprature. The samples were washed three times with PBS-BSA and then incubated for 30 min with FITC-labeled rabbit antibodies against mouse IgG (ICN Immunobiologicals, Lisle, U.S.A.) in PBS-BSA.The cells were observed under an Olympus BHS-RFK fluorescence microscope.

Chemical reagents

ATP, GTP, AMPPNP, GMPPNP, and dichlorotriazonil-amino-fliorescein (DTAF) were obtained from Sigma Chemical Co.(St. Louis, USA)

RESULTS AND DISCUSSIONS

Tobacco BY-2 cells with phragmoplasts were treated with glycerin to permeabilize the plasma membrane and DTAF (dichlorotriazonilamino fluorescein)-labeled tubulin was introduced into the glycerinated cells, an array of phragmoplast microtubules was well preserved in the glycerinated cells, as judged by anti-tubulin immunofluorescence (Fig.1 f). As has been reported , there were no staining of the equatorial region where microtubules are interdigitating with their plus ends (Fig. 1 f).

A bright fluorescent band appeared at the equatorial region of the phragmoplast in the glycerinated cells incubated with DTAF-tubulin in the presence of GTP (Fig.1a), suggesting that DTAF-tubulin have polymerized onto the plus ends of pre-existing phragmoplast microtubules. Weak, broad fluorescence appeared at the distal portions of the phragmoplasts (Fig.1b,c), suggesting that polymerization of DTAF-tubulin have occurred at the minus ends (fig.4a). Interestingly, the width of fluorescent band at the equatorial plane increased (Fig.1c), and the distal, weak fluorescent regions moved away from the equatorial plane (Fig.1b,c), during the extended incubation. These observation have suggested a possibility that microtubules in the phragmoplast are translocated towards their minus ends concomitantly with tubulin polymerization onto their plus ends. The microtubule translocation in the phragmoplast was clearly demonstrated by the experiment cells like the one in Figure 1 b were further incubated with unlabeled tubulin. The fluorescent band at the equatorial region is split in two by a newly formed, unlabeled band and each of resultant pair of fluorescent bands is translocated away from the equatorial plane (Fig.1d,e and Fig.4b). Figure 2a shows that the unlabeled band appears after a short lag period, during which the interdigitating portions of fluorescent microtubules move apart, and increases its width with time. The rate of translocation seems to depend on the rate polymerization when the tubulin concentration is lower than 0.5 mg/ml. The separation of the fluorescent band dose not occur in the absence of unlabeled tubulin (Fig.2a). But

when the tubulin concentration is 0.5 mg/ml or higher, the rate of translocation seems to be limited by the activity of the microtubule-translocating system.

In the presence of GMPPNP or AMPPNP, phragmoplasts incorporate DTAF-tubulin at the equatorial region, but the changes in the fluorescent pattern with time were distinct from those observed in their absence. The distal, weak fluorescent regions were overlaid by the broadening fluorescent band at the equatorial region. The simple explanation of this observation is that GMPPNP or AMPPNP inhibit microtubule translocation without inhibiting tubulin polymerization. To visualize the effects of these drugs more clearly, the cells were incubated first with unlabeled tubulin in the presence of GMPPNP or AMPPNP and then with DTAF-tibulin in the presence of GMPPNP or AMPPNP. DTAF-tibulin was incorporated at the sites distant from the equatorial region (Fig. 3d, f), indicating that the sites of addition of tubulin were separated from the equatorial region during the first incubation (Fig. 4c). This seems to be the result of polymerization of unlabeled tubulin onto plus ends of microtubules, the translocation of which was inhibited by GMPPNP or AMPPNP. In the absence of these drugs, a bright fluorescence appeared at the equatorial region, and weak, broad fluorescence appeared distal to the broad unlabeled zones formed during the first incubation with unlabeled tubulin (Fig. 3b).

To examine whether microtubules that have polymerized in the presence unhydrolysable analogues of GTP or ATP can be translocated by GTP or ATP, glycerinated cells were incubated first with DTAF-tubulin in the absence of such an analogue and then with unlabeled tubulin in the presence of GMPPNP or AMPPNP. After washing out free tubulin and analogues, the cells were incubated with GTP or ATP. When GMPPNP was used for the inhibition of the microtubule translocation, the fluorescent band at the equatorial region that appeared during the initial incubation was split to two by an unlabeled band during incubation with GTP (Fig. 2b), giving a pattern like that shown in Figure 1d or e. The result indicate that GTP causes the translocation of microtubules that have polymerized during the incubation with GMPPNP (Fig. 4d). ATP also caused the separation of the fluorescent band, but was far less

effective than GTP (Fig. 2b). The experiment in which AMPPNP was used gave essentially the same results as those shown in Figure 2b: GTP was far more effective than ATP. The finding that the microtubule translocation is inhibited by GTP and is effectively induce by GTP strongly suggests that GTP is preferable substrate of the mechanochemical enzyme for the translocation of phragmoplast microtubules. We cannot at this stage explain why AMPPNP inhibits translocation in the presence of GTP.

Our results strongly suggest that the equatorial region of phragmoplasts is associated with a specific activity that couples microtubule polymerizations at the plus ends with microtubule translocation towards the minus ends. This microtubule translocation bears some resemblance to the sliding of microtubules in the isolated diatom spindle system (Masuda and Cande, 1987) and microtubule translocation in the microtubule-chromosome complex system (Mitchson and Kirshner, 1985), but with a different mechanochemical enzyme: the phragmoplast system seems to use GTP, whereas the other two use ATP. This implys that phragmoplast is associated with a unique microtubule motor protein which have not been previously identified.

The oppositely directed microtubule array in the phragmoplast seems to be essential for the accumulation of vesicles filled with cell-plate materials at the equatorial plane. The system of the microtubule translocation in the phragmoplast should be important in constructing such an array of phragmoplast microtubules.

FIGURES

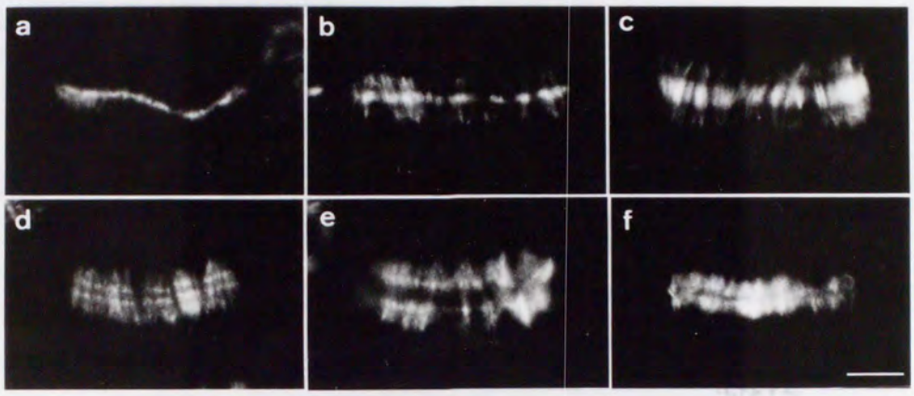


Fig. 1 Incorporation of exogeneous tubulin into the phragmoplast. a-c, Glycerinated cells with phragmoplasts incubated with 0.5 mg/ml DTAF-tubulin for a, 5 min, b, 20 min or c, 50 min. d,e, Cells like the one in Fig. 1b incubated further with 0.5 mg/ml unlabeled tubulin for d, 5 min or e, 20 min. f, Anti-tubulin immunofluorescence of a phragmoplast in a glycerinated cell. Scale bar, 10 μm.

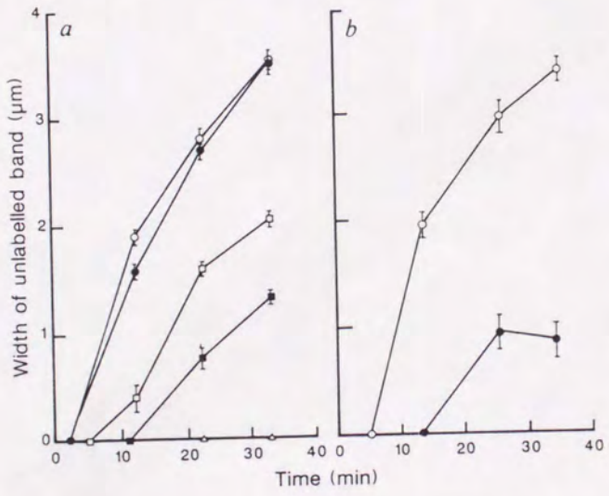


Fig. 2 The rate of microtubule translocation in the phragmoplast. a, Dependency of the rate of microtubule translocation in phragmoplasts on the concentration of exogeneous tubulin. Rates of translocation in the presence of 1 mg/ml (open circle), 0.5 mg/ml (closed circle), 0.25 mg/ml (open square), or 0.1 mg/ml (closed square) or in its absence. b, Dependency of the translocation of prepolymerized microtubules on nucleotide triphosphate. Rates of translocation in the presence of 4 mM MgGTP (open circle) or MgATP (closed circle) are given. For each measurement more than 20 phragmoplasts were examined. Vertical bar, standard error.

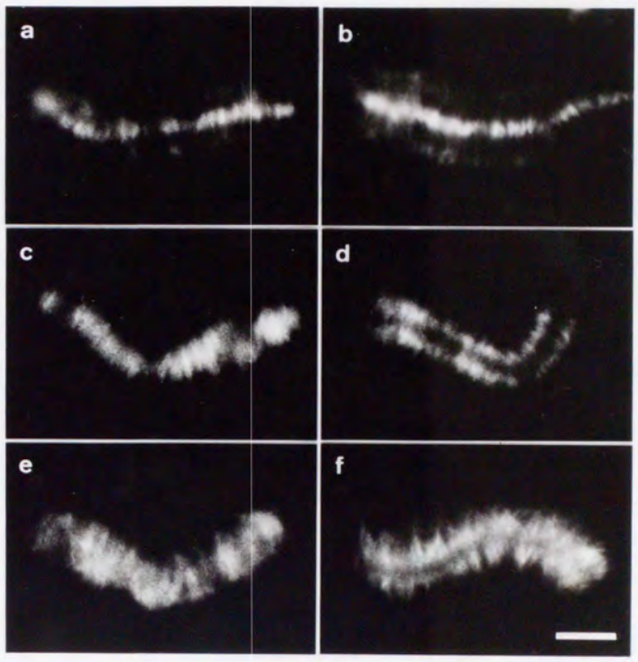


Fig. 3 Effects of GMPPMP and AMPPNP on incorporation of exogeneous tubulin into phragmoplasts. a, c, e, Glycerinated cells with phragmoplasts incubated with 0.5 mg/ml DTAF-tubulin for 45 min. The incubation was carried out in the presence of a, 4 mM GTP, c, 2 mM GTP plus 2 mM GMPPNP, or e, 4 mM GTP plus 0.1 mM AMPPMP. b, d, f, Glycerinated cells with phragmoplasts incubated with 0.5 mg/ml unlabeled tubulin for 30 min and then with 0.5 mg/ml DTAF-tubulin for 15 min. Both the first and second incubations were carried out in the presence of b, 4 mM GTP, d, 2 mM GTP plus 2 mM GMPPNP, or f, 4 mM GTP plus 0.1 mM AMPPNP. Scale bar, 10 μm.

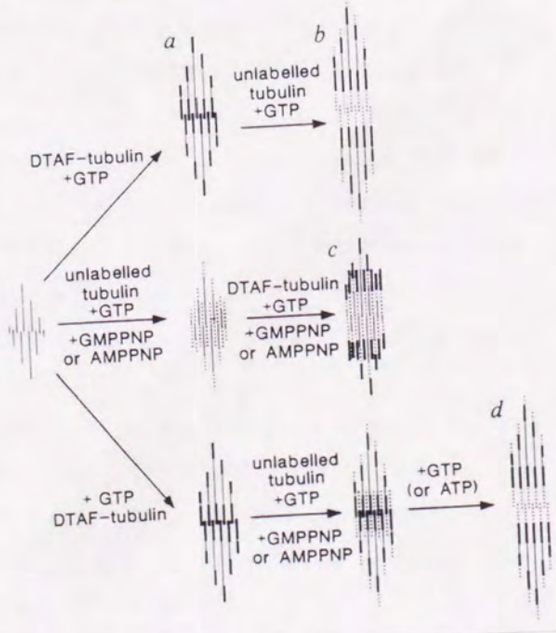


Fig. 4 Schematic illustration to explain fluorescence patterns shown in a, Fig. 1b,c; b, Fig. 1d, e; c, Fig. 3d, f; and d, Fig. 2b. Longitudinal cross-sectional views of one side of a ring-shaped mature phragmoplast are illustrated. thin line, preexisting microtubules; broken line, Polymerized unlabeled tubulin; thich line, polymerized DTAF-tubulin.

CHAPTER II.
ISOLATION OF POLYPEPTIDES WITH MICROTUBULE-TRANSLOCATING ACTIVITY FROM PHRAGMOPLASTS OF TOBACCO BY-2 CELLS

INTRODUCTION

Although little is known about the molecular mechanisms responsible for the organization of phragmoplast microtubules and the accumulation of the vesicles at the plane of formation of the cell plate, several lines of evidence support the hypothesis that the mechanisms involve the microtubule-associated motility systems. During cytokinesis in endosperm cells of Haemanthus, organelles in the phragmoplasts move towards and away from the equatorial plane, suggesting that motility systems for the transport of organelles are associated with the phragmoplast (Bajer et al. 1987). Electron microscopic observations indicating the close association of vesicles with microtubules of the phragmoplast support the hypothesis that vesicles that include cell-plate materials are transported to the equatorial plane along tracks laid down by phragmoplast microtubules (Kakimoto and Shibaoka 1988). The involvement of microtubule-associated motility systems in the organization of the phragmoplast was suggested by the study described in chapter I in which I demonstrated that phragmoplast microtubules were translocated away from the equatorial plane at the expense of energy from GTP or ATP. Although these movements of organelles or microtubules in the phragmoplast can be accounted for by the activities of motor proteins that generate forces that act to cause microtubule-related movement, such motor proteins have not been identified.

Microtubule-associated motor proteins, such as dynein, kinesin and kinesin-like proteins, have been reported to be required for a variety of cellular processes, such as flagellar movement, the transport of organelles and mitosis (Gibbons, 1981; Sawin and Scholey, 1991; Paschal and Vallee, 1987; Vale et al., 1985). Recent studies on microtubule motor proteins in higher plant cells have focused on kinesin and have relied on the use of antibodies

against bovine brain kinesin, or strategies involving the polymerase chain reaction (PCR). Cai et al. (1993) identified a 100-kDa polypeptide that reacted with a monoclonal antibody against kinesin in pollen tubes of Nicotiana tabacum and they purified this protein. The purified 100-kDa polypeptide had microtubule-stimulated ATPase activity and bound to microtubules in an ATP-sensitive manner, as dose kinesin itself. Another study, employing PCR, resulted in the identification of a gene, katA, encoding a kinesin-related polypeptide of 90 kDa in Arabidopsis thaliana (Mitsui et al. 1993). In spite of these studies, no direct evidence has been presented to demonstrate that these kinesin-related or kinesin-like polypeptides have microtubule-translocating activity. Without the isolation and characterization of proteins with the microtubule-translocating activity, we cannot expect to have a full understanding of the molecular mechanisms of microtubule-associated motility, which may be important in both cell division and cell morphogenesis in higher plant cells (Bajer et al., 1987; Williamson, 1991).

In this chapter, I attempted to identify proteins that are associated with the phragmoplast and have the ability to translocate microtubules. A well established method for synchronization of the cell cycle (Kakimoto and Shibaoka, 1988) enabled us to isolate motor proteins directly from phragmoplasts that had been isolated from tobacco BY-2 cells in telophase.

MATERIALS AND METHODS

Culture and synchronization of the cell cycle of tobacco BY-2 cells

Tobacco BY-2 cells (Nicotiana tabacum 'Bright Yellow 2') were cultured in modified Linsmaier and Skoog's medium (LS medium) supplemented with 3% (w/v) sucrose, 370 mg/ml KH_2PO_4, 1.0 mg/ml thiamin hydrochloride and 0.2 mg/ml 2,4-dichlorophenoxyacetic acid (Nagata et al., 1981). The cell cycle of BY-2 cells was synchronized by treatments with aphidicolin and propyzamide (Kakimoto and Shibaoka, 1988).

Extraction and fractionation of phragmoplast-associated proteins

BY-2 cells that had been released from treatment with propyzamide were shaken gently in the abovementioned LS medium supplemented with 0.3 M mannitol, 1% (w/v) cellulase Onozuka RS (Yakult Pharmaceutical Co. Ltd., Takarazuka, Hyogo), and 0.1% (w/v) pectolyase Y-23 (Seishin pharmacentical Co. Ltd., Tokyo, Japan) for 100 min under oxygen-enriched conditions that allow the progression of mitosis (Kakimoto and Shibaoka, 1992). Protoplasts, the majority (> 80%) of which were in telophase, were pelleted, washed twice with a 0.5 M solution of mannitol, and suspended in a glycerination buffer [50 mM K-PIPES, pH 7.0, 1 mM $MgSO_4$, 10 mM EGTA, 60% (v/v) glycerol, 2 mM DTT, 1 mM PMSF, 5 μg/ml leupeptin, 3 μg/ml pepstatin A, 50 μg/ml TAME]. Cells in the glycerination buffer were kept on ice for 5 min and then washed three times with PME buffer (75 mM K-PIPES, pH 7.0, 1 mM $MgSO_4$, 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 50 μg/ml TAME). During the repeated washing, plasma membranes of protoplasts were ruptured and complexes composed of a phragmoplast and two daughter nuclei were released (Fig. 1). Such released complexes will be called isolated phragmoplast-complexes for convenience. The isolated phragmoplasts were extracted in four different ways to yield four different kinds of extract. The complexes were pelleted by centrifugation, mixed with an equal volume of (1) PME buffer, (2) PME buffer that contained 2.5 mM

MgATP and 2.5 mM MgGTP, (3) PME buffer that contained 0.3 M NaCl, or (4) PME buffer that contained 2.5 mM MgATP, 2.5 mM MgGTP and 0.3 M NaCl. Each suspension was then homogenized with glass homogenizer on ice. Homogenates were centrifuged for 1 hr at 4 °C in Beckman TLA-100.2 rotor at 60000 rpm. The four kinds of extract obtained by these procedures were named (1) control extract, (2) ATP/GTP extract, (3) NaCl extract, and (4) ATP/GTP/NaCl extract, respectively. Each extract was dialyzed against PME buffer for 6 hr at 4 °C and the resultant precipitates removed by centrifugation. The concentrations of protein in the dialyzed extracts were 0.52, 0.73, 1.18, and 1.35 mg/ml, respectively. The dialysates were kept on ice until they were assayed for their ATPase- or GTPase- activities. The ATP/GTP/NaCl extract (number 4 above) was further fractionated. The extract was desalted by gel filtration with a column of Sephadex G-25 (Pharmacia LKB, Uppsala, Sweden) and the proteins were applied to a column of DEAE-Sephacel (Pharmacia) that had been pre-equilibrated with PME buffer that contained 0.1 mM ATP and 0.1 mM GTP. The column was washed with the same buffer and adsorbed proteins were eluted stepwise with PME buffers that contained 80, 160, and 400 mM NaCl. The flow-through fraction and eluted fractions were dialyzed against PME buffer and were assayed for ATPase- and GTPase-activities and for microtubule-translocating activity. The concentrations of protein in the flow-through fraction, and in the fractions eluted by 80, 160, 400 mM NaCl were 0.19, 0.38, 0.23 and 0.59 mg/ml, respectively. The concentration of protein was determined by the method of Bradford (1976) in each case.

Isolation of microtubule-binding polypeptides

Tubulin was prepared from bovine brains by a two-cycle assembly and disassembly procedure that was followed by purification on a phosphocellulose column (Shelanski, 1973). The brain tubulin obtained was stored at -70 °C until use. A 35-ml aliquot of the ATP/GTP/NaCl extract was applied to a column of DEAE-Sephacel. After being washed, the column was eluted with PME buffer that contained 200 mM NaCl, and the eluate was retained. The retained eluate was applied to a column of Sephadex G-25 that had been pre-

equilibrated with 100 mM PME buffer (100 mM K-PIPES, pH 7.0, 1 mM MgSO4, 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 50 μg/ml TAME) and a flow-through fraction was centrifuged for 20 min at 100,000 g, at 4 °C to remove any precipitate. The resultant supernatant was supplemented with 10 μM taxol. Each 250 μl of supernatant with taxol was mixed with 30 μl of a suspension of microtubules that had been assembled from a 1 mg/ml solution of purified brain tubulin that contained taxol at 10 μM. The mixture was centrifuged for 15 min at 50,000 g, at 15 °C. The supernatant and pellet were designated S1 and P1, respectively. The P1 was suspended in 100 mM PME buffer that contained 10 μM taxol and centrifuged for 15 min at 50,000 g to yield S2 and P2. The P2 was suspended in 100 mM PME buffer that contained 20 μM taxol and 0.3 M NaCl (100 mM PME-NaCl buffer) and centrifuged for 15 min at 50,000 g to yield S3 and P3. This procedure was repeated with P3 to yield S4 and P4. The P4 was suspended in 100 mM PME-NaCl buffer that contained (1) 10 mM MgATP, (2) 10 mM MgGTP, (3) 10 mM MgAMPPNP, (4) 10 mM MgGMPPNP, (5) 8.3 mM MgATP plus 1.6 mM MgAMPPNP, or (6) 8.3 mM MgGTP plus 1.6 mM MgGMPPNP and then centrifuged for 15 min at 100,000 g at 15 °C. The resulted supernatants were designated E1, E2, E3, E4, E5, and E6, respectively. Each fraction E1 through E6 was analyzed by SDS-PAGE on a 7.5% gel by the standard method (Laemmli, 1970).

Assays of ATPase and GTPase

An aliquot (40 μl) of each extract from isolated phragmoplasts (in the experiment for which results are shown in Fig. 2) or the fractionated ATP/GTP/NaCl extract (Fig. 6) was mixed with 5 μl of PME buffer that contained 20 μM taxol or with 5 μl of PME buffer that contained brain microtubules (2 mg/ml) stabilized with 20 μM taxol, and mixture were incubated at 30°C for 5 min. To start the reaction, 5 μl of 10 mM [g-32P] MgATP or [g-32P] MgGTP (0.5 μCi/μmol) were added to each mixture. After incubation at 30 °C for 15 min, the reactions were terminated by addition of silicotungstic acid, and unhydrolyzed nucleotides were removed with charcoal as described by Bryce et al. (1991). Cerenkov radiation from released 32P was quantitated by scintillation counting for determination of the enzymatic activity.

Motility assays

Brain tubulin labeled with 5- (and-6) carboxytetramethylrhodamine succinimidyl ester and minus-end-marked microtubules were prepared essentially as described by Hyman et al. (1990). Fluorescent microtubules were prepared from a 1:2 mixture of rhodamine-labeled tubulin and non-labeled tubulin by the addition of an equal volume of PME buffer that contained 60% glycerol and 10 mM MgSO4. To prepare the minus-end-marked microtubules, the above-mentioned 1:2 mixture was briefly incubated and diluted 1:3 with a 1:1 mixture of non-labeled tubulin and non-labeled NEM-treated tubulin. In experiments for which results are shown in Figures 3 and 4, fluorescent microtubules were mixed with the fraction that had been eluted from the DEAE-Sephacel column with 80 mM NaCl, which we named the 80 mM NaCl fraction, and the mixture was placed on a coverslip. The coverslip was inverted and placed on two strips of Parafilm M (American National Can, Greenwich, U.S.A.) that had been sticked on a glass slide to make a small flow-chamber. The chamber was perfused with PME buffer that contained 5 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) and then with the PME buffer supplemented with 20 μM taxol, oxygen-scavenging enzymes (0.2 mg/ml catalase and 0.06 mg/ml glucose oxidase), 3.6 mg/ml glucose and 3 mM MgATP or 3 mM MgGTP. The coating of coverslips with bovine serum albumin facilitated the smooth movement of fluorescent microtubules, as described by Hyman (1990). In the experiment for which results are shown in Figure 6, a fraction extracted from the P4 fraction with 100 mM PME-NaCl buffer that contained 5 mM MgATP and 5 mM MgGTP was used. A coverslip was coated with the fraction in a moisture chamber on ice for 10 min. The flow-chamber with the coated coverslip was perfused with PME buffer that contained 5 mg/ml bovine brain albumin and then with PME buffer that contained fluorescent microtubules. After the removal of unattached microtubules by perfusion with PME buffer that contained oxygen-scavenging enzymes and glucose, the chamber was perfused with PME buffer that contained 3 mM MgATP or 3 mM MgGTP together with oxygen-scavenging enzymes and glucose.

The fluorescent microtubules were observed with an Olympus fluorescence microscope equipped with an SIT camera (model C2400-08, Hamamatsu Photonics K. K., Hamamatsu, Japan). Images of fluorescent microtubules were processed by the ARGUS-100 system (Hamamatsu Photonics K. K.) and recorded at intervals of 2 to 4 min. The rate of microtubule gliding was estimated from the change in the positions of microtubules that had glided linearly. A graph of time versus distance was plotted and the mean rate of gliding was determined from the slope of the line which was fitted to the data by the least-squares method.

Immunofluorescence microscopy

Isolated phragmoplast-complexes were fixed for 40 min in PME buffer that contained 3.7% formaldehyde and then washed three times with PBS. Fixed samples were suspended in PBS-BSA (PBS that contained 1% bovine serum albumin) and to the suspension was added mouse monoclonal antibodies against chick brain a-tubulin (Amersham, Buckinghamshire, U.K.) and the suspension with the antibodies was incubated for 30 min at room temprature. The samples were washed three times with PBS-BSA and then incubated for 30 min with FITC-labeled rabbit antibodies against mouse IgG (ICN Immunobiologicals, Lisle, U.S.A.) in PBS-BSA. After being washed with PBS three times, they were suspended in a 1:1 mixture of PBS and glycerol and observed with Olympus fluorescence microscope.

Chemical reagents

ATP, GTP, ITP, UTP, CTP, AMPPNP and GMPPNP were obtained from Sigma Chemical Co.. They were disolved in 50 mM K-PIPES (pH.7.0) at 100 mM and stored at -20°C. Solutions of Mg-nucleotides or Mg-nucleotide analogues were prepared immediately before use by mixing a nucleotide or a nucleotide analogue with an equimolar amount of MgSO4 in solution. 5- (and-6) carboxytetramethylrhodamine, succinimidyl ester was obtained from Molecular Probes Inc. (Eugene, OR, USA). Taxol was obtained from Calbiochem Co. (La Jolla, CA, USA).

RESULTS

GTPase and ATPase activity in extracts derived from the isolated phragmoplasts

Phragmoplast-complexes isolated from tobacco BY-2 cells (Fig. 1) were homogenized in four kinds of extraction buffer. The homogenization of isolated phragmoplasts in PME buffer did not result in complete disruption of the complex. The supernatant of the homogenate, i.e., the control extract, had both GTPase activity and ATPase activity, but these activities were not stimulated by the addition of microtubules (Fig. 2A). The homogenization of isolated phragmoplast-complexes in PME buffer that contained ATP and GTP also did not result in the complete disruption of the complexes, but homogenization in PME buffer that contained 0.3 M NaCl caused complete disruption, irrespective of the presence in the buffer of ATP or GTP. The GTPase and ATPase activities of supernatants of these homogenates, i.e., the ATP/GTP extract, the NaCl extract and the ATP/GTP/NaCl extract, were distinctly enhanced by microtubules (Fig. 2A).

The ATP/GTP/NaCl extract, in which activities of both GTPase and ATPase were doubled by the addition of brain microtubules, was fractionated by column chromatography on DEAE-Sephacel, and the fractions were assayed for GTPase and ATPase activities. As shown in Figure 2B, 80 mM NaCl eluted considerable amounts of GTPase and ATPase, the activities of which were enhanced by microtubules.

The movement of microtubules caused by phragmoplast-associated proteins

The fraction eluted from the DEAE-Sephacel column by 80 mM NaCl, i.e., the 80 mM NaCl fraction, was tested for its ability to translocate microtubules. The fraction was mixed with fluorescent microtubules and then MgGTP or MgATP was added. The addition of the fraction to microtubules caused bundling or aggregation of microtubules, an indication of the presence in the fraction of microtule-bundling factors. On the addition of MgGTP to the microtubule bundles (or aggregates) that had adhered to a

coverslip, the microtubules in bundles began to disperse (Fig. 3A) and the dispersed microtubules glided (Fig. 3A and Fig. 4A) at an average rate of 1.06 μm/min (n= 22,s.d.=0.40). MgATP caused similar but less extensive changes in the shape of the bundles of microtubules (Fig. 3b). The difference between the effects of MgGTP and MgATP was more clearly apparent after prolonged incubation (Fig. 3C and D). MgATP supported the gliding of single microtubules (Fig. 4B) and the average rate of ATP-induced gliding was 0.63 μm/min (n=19, s.d.=0.29). MgITP, MgCTP and MgUTP each caused neither the dispersion of microtubules in bundles or aggregates nor the translocation of single microtubules.

The ATP- or GTP-dependent gliding of microtubules was also observed when fractions that had been eluted from the DEAE-Sephacel column in 160 mM or 400 mM NaCl were used. However, the number of gliding microtubules was smaller than that when the 80 mM NaCl fraction was used, suggesting that the concentration of motor proteins in the later fractions was lower than that in the earlier fraction (data not shown).

The polarity of gliding was determined with minus-end-marked microtubules (see Materials and Methods). Microtubules glided with their marked minus-ends in the lead (data not shown; cf. Fig. 7). No gliding headed by plus ends was ever observed.

Purification of polypeptides that bind to and dissociated from microtubules in nucleotide-sensitive manner

A fraction of the ATP/GTP/NaCl extract that had been eluted from the DEAE-Sephacel column by 200 mM NaCl was used for the isolation of proteins with the ability to bind to microtubules. The fraction was mixed with microtubules, which had been assembled from brain tubulin and stabilized with taxol. The mixture was incubated in the absence of nucleotides, and centrifuged. The microtubules in the pellet were found to be associated with a number of polypeptides (Fig. 5; P1). Most of them, including major polypeptides of 150 kDa and 100 kDa, were released from the microtubules by 300 mM NaCl (Fig. 5; S3), but polypeptides of 125 kDa and 120 kDa (collectively referred to as 125-kDa polypeptides for convenience) and other minor polypeptides remained bound (Fig.

5; P4). The 125-kDa polypeptides were recovered in the supernatant of a suspension of the P4 fraction in PME buffer that contained 0.3 M NaCl and 10 mM MgATP or 10 mM MgGTP (Fig. 5; E1 or E2) but not in the supernatant of the suspension in buffer that contained 0.3 M NaCl and 10 mM AMPPNP or 10 mM GMPPNP (Fig. 5; E3 or E4). Depolymerized tubulin and a minor polypeptide of 94 kDa were recovered in the supernatants (Fig. 5). The amount of tubulin in the E1 or the E2 fraction was larger than that in the E3 or the E4 fraction, indicating that MgATP or MgGTP enhanced the depolymerization of taxol-stabilized microtubules. It is probable that dissociation from microtubules of the 125-kDa polypeptides makes microtubules more labile. In the presence of AMPPNP, excess ATP caused the dissociation of 125-kDa polypeptides from microtubules while excess GTP did not (Fig. 5;E5 and E6). Despite the resemblance between the 125-kDa polypeptides and kinesin in the nucleotide-sensitivity in association with and dissociation from microtubules, neither SUK4, a monoclonal antibody that recognizes the kinesin heavy chain from a wide variety of eukaryotic cells (Ingold et al., 1988), nor a polyclonal antibody against kinesin from bovine adrenal medulla cells (Murofushi et al., 1988) recognized the 125-kDa polypeptides during Western blot analysis (data not shown).

Microtubule-translocating activity of the 125-kDa polypeptides

A fraction that contained 125-kDa polypeptides as the major polypeptides was prepared by extracting the P4 fraction with a solution that contained 5 mM MgATP, 5 mM MgGTP and 0.3 M NaCl, and it was tested for its ability to translocate microtubules. On addition of MgATP or MgGTP, microtubules on a coverslip coated with the fraction that contained 125-kDa polypeptides started to glide. Microtubules glided with their minus-ends in the lead (Fig. 6). Microtubules glided at an average rate of 1.28 μm/min (n=10 ,s.d.=0.22) in the presence of MgATP (Fig. 7A) and at a rate of 0.50 μm/min (n=10 ,s.d.=0.15) in the presence of MgGTP (Fig. 7B). Although this fraction contained several polypeptides in addition to the 125-kDa polypeptides, such as a 94-kDa polypeptide and 55-

kDa brain tubulin (E1 or E2 in Fig. 5), the fractions extracted with AMPPNP or GMPPNP that also contained 94-kDa polypeptide and 55-kDa brain tubulin (E3 and E4 in Fig. 5) did not cause microtubules to glide. The fractions with 125-kDa polypeptides and microtubule-translocating activity (E1 and E2 in Fig. 5) did not exhibit the microtubule-bundling activity that was associated with the 80 mM NaCl fraction (see Fig. 3). The fraction of microtubule-associated proteins that had been released from microtubules by 0.3 M NaCl (S3 in Fig. 5) caused bundling of microtubules, but it did not support the movement of microtubules.

DISCUSSION

Mechanochemical enzymes that generate the force that translocates microtubules are known to have ATPase or GTPase activity and such activity is enhanced by microtubules (Kuznetsov and Gelfand, 1986; Cohn et al., 1987; Omoto and Johnson, 1986). Therefore, we tried to obtain an extract with ATPase and GTPase activity that was enhanced by microtubules. We found that an extract prepared by homogenizing phragmoplasts isolated from tobacco BY-2 cells in a buffer that contained MgATP, MgGTP and a high concentration of NaCl had both ATPase and GTPase activities and that these activities were doubled by the addition of brain microtubules. The ATPase and GTPase that were activated by microtubules were adsorbed to a DEAE-Sephacel column whereas those that were not activated did not adsorb to the column. Thus, the microtubule-activated ATPase and GTPase were predominantly present in a fraction that was eluted from the DEAE-Sephacel column, onto which an extract from isolated phragmoplasts had been loaded, by a solution of NaCl. This fraction, the DEAE-fractionated extract, caused brain microtubules to glide in the presence of ATP or GTP. This result strongly suggested that microtubule-activated ATPase and GTPase, present in this fraction, were mechanochemical enzymes. Microtubules glided with their minus ends in the lead, an indication that the gliding was driven by plus-end-directed motor protein(s).

The extract from the column of DEAE-Sephacel contained 125-kDa and 120-kDa polypeptides that co-sedimented with brain microtubules in the absence of ATP and GTP and that dissociated from the sedimented microtubules in the presence of ATP or GTP. The 120-kDa polypeptide was always accompanied by the 125-kDa polypeptide and its level was always lower than that of the 125-kDa polypeptide, as judged by analysis by SDS-PAGE. These polypeptides were indistinguishable in microtubule-association and -dissociation assays, a result that suggests that the 120-kDa polypeptide was a proteolytic product of the 125-kDa polypeptide that had retained the domains required for interaction with microtubules, or that these two polypeptides were constituents of a single motor

molecule. Whichever possibility is correct, these two polypeptides appeared to be closely related to each other. Thus, they were collectively designated 125-kDa polypeptides. We made use of the finding that the 125-kDa polypeptides cosedimented with microtubules and were dissociated from sedimented microtubules in an ATP- or GTP-dependent manner for further purification of the 125-kDa polypeptides. The partially purified 125-kDa polypeptides caused microtubules to glide with their minus ends in the lead just as the fraction after chromatography on DEAE-Sephacel did. Although the preparation of partially purified 125-kDa polypeptides contained some minor microtubule-associated polypeptides in addition to the 125-kDa polypeptides, a fraction that contained only these additional microtubule-associated polypeptides, namely, the fraction extracted from sedimented microtubules with a solution that contained NaCl with or without AMPPNP or GMPPNP, did not cause microtubules to glide. These observations strongly suggest that the 125-kDa polypeptides were responsible for the translocation of microtubules.

Bundles and aggregates of microtubules formed in the crude extract, namely, the extract after fractionation on DEAE-Sephacel, extensively dispersed in the presence of GTP while they dispersed to only a limited extent in the presence of ATP and the rate of gliding of dispersed microtubules was greater in the presence of GTP than in the presence of ATP (Fig. 3). The observation that distinct unbundling of microtubules was caused only by GTP can be explained by hypothesizing that the crude extract contained microtubule-bundling factors which is sensitive to GTP but not to ATP. The rate of GTP-induced gliding of microtubules was greater in the crude extract than in the purified 125-kDa polypeptide preparation. The crude extract appeared to contain factors which stimulated the GTP-dependent translocation of microtubules caused by the 125-kDa polypeptides. The rate of ATP-induced gliding of microtubules was lower in the crude extract than in the purified 125-kDa polypeptide preparation. One of the possible explanation for this difference is that the crude extract contains ATP-dependent motor proteins that support plus-end-headed translocation of microtubules and such proteins reduce the rate of ATP-dependent

minus-end-headed translocation by the 125-kDa polypeptides. Studies to characterize microtubule-associated proteins other than the 125-kDa polypeptides are needed to explain the difference between the crude extract and the purified 125-kDa polypeptide preparation in the ability to cause unbundling and translocation of microtubules.

We demonstrated previously that the translocation of microtubules occurrs in the phragmoplast of membrane-permeabilized tobacco BY-2 cells (Asada et al. 1991). In this system, microtubules in the phragmoplast are translocated in a direction away from the equatorial plane and the translocation is effectively induced by GTP and is less effectively induced by ATP. Although the 125-kDa polypeptides caused the translocation of single microtubules more effectively in the presence of ATP than in the presence of GTP, it is still possible that the 125-kDa polypeptides are involved in the translocation of phragmoplast microtubules since the situation in which phragmoplast microtubules find themselves is not the same as that in which single microtubules do. The observation that the sliding of microtubules in bundles was caused more effectively by GTP than ATP seems to lend support to this possibility. To examine this possibility further, the effects should be examined of antibodies against the 125-kDa polypeptides on the translocation of phragmoplast microtubules in membrane-permeabilized cells, as well as on that of microtubules in vitro.

It is also possible that the 125-kDa polypeptides are responsible for the transport of vesicles or tubular ER in the phragmoplast scince these organelles are transported toward the site of formation of the cell plate, namely, toward the plus ends of phragmoplast microtubules (Heplar 1982), and the 125-kDa polypeptides have a plus-end-directed motor activity. Since conventional kinesin is a well known plus-end-directed motor protein that mediates the transport of membrane-bounded organelles (Vale et al. 1985), it seems appropriate to compare the 125-kDa polypeptides with conventional kinesin. The 125-kDa polypeptides are very similar to conventional kinesin. These proteins resemble one another in terms of molecular mass, sensitivity to nucleotides of binding to microtubules, and the polarity and nucleotide

specificity of motor activity. In spite of such resemblance, the 125-kDa polypeptides failed to cross-react with antibodies against conventional kinesin (Ingold et al. 1988; Murofushi et al., 1988). Moreover, the rate of translocation of microtubule by the 125-kDa polypeptides was far lower than that caused by conventional kinesin (Vale et al., 1985; Porter et al., 1987). Therefore, at this stage, it is difficult to draw any conclusions about the relationship between the 125-kDa polypeptides and conventional kinesin. Characterization of the molecular structure of the newly identified 125-kDa polypeptides should tell us whether the polypeptides belong to the kinesin family or not. Studies to clarify the relationsip between these proteins in terms of molecular structure are in progress. These studies should help to clarify the similarity between the 125-kDa polypeptides and, for example, the 100-kDa kinesin-immunoreactive polypeptide that was recently purified from pollen tubes of Nicotiana tabacum, from which BY-2 cells were originally obtained (Cai et al., 1993).

FIGURES

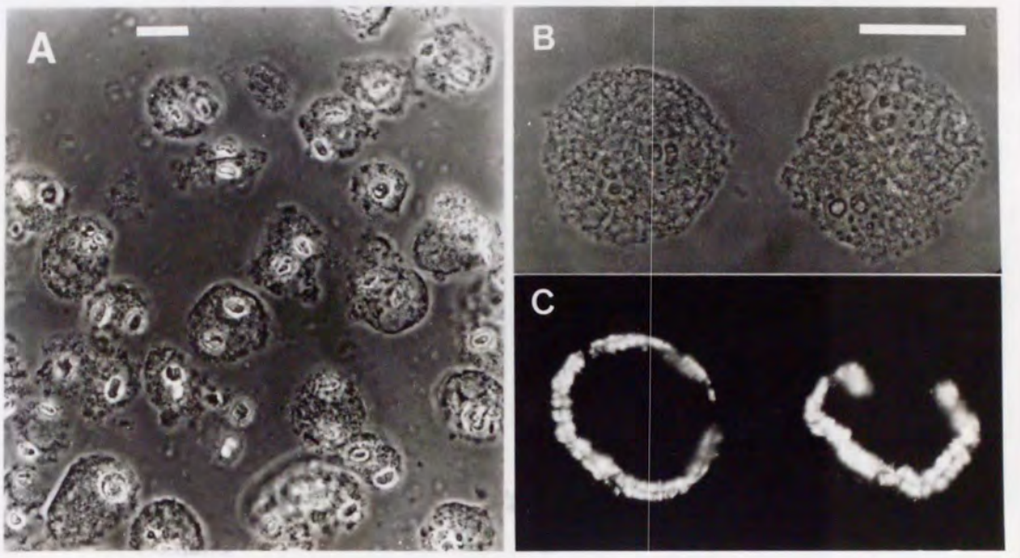


Fig. 1 Phragmoplasts with daughter nuclei isolated from tobacco BY-2 cells. (A, B) Phase-contrast images of isolated phragmoplasts. (c) image of tubulin-specific immunofluorescence of B. Bar, 30 μm

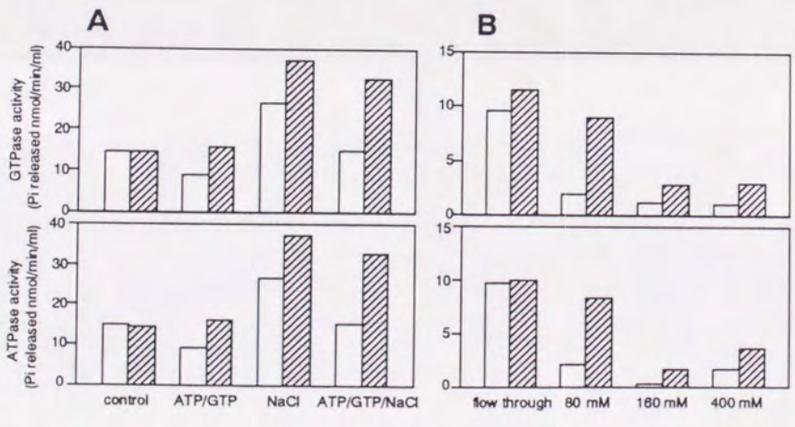


Fig. 2 Enhancement by microtubules of GTPase and ATPase activities in extracts from isolated phragmoplasts. The shaded bars are microtubule-stimulated ATPase or GTPase activity and the open bars are ATPase or GTPase activity in the absence of microtubules. (A) Extracts were prepared by homogenizing isolated phragmoplasts with PME buffer (control), PME buffer with 2.5 mM MgATP and 2.5 mM MgGTP (ATP/GTP), PME buffer with 0.3 M NaCl (NaCl), or PME buffer with NaCl together with MgATP and MgGTP (ATP/GTP/NaCl). (B) The ATP/GTP/NaCl extract was fractionated by column chromatography on DEAE-Sephacel. The flow-through fraction and fractions that were eluted stepwise from the column with 80, 160, and 400 mM NaCl, respectively, were assayed.

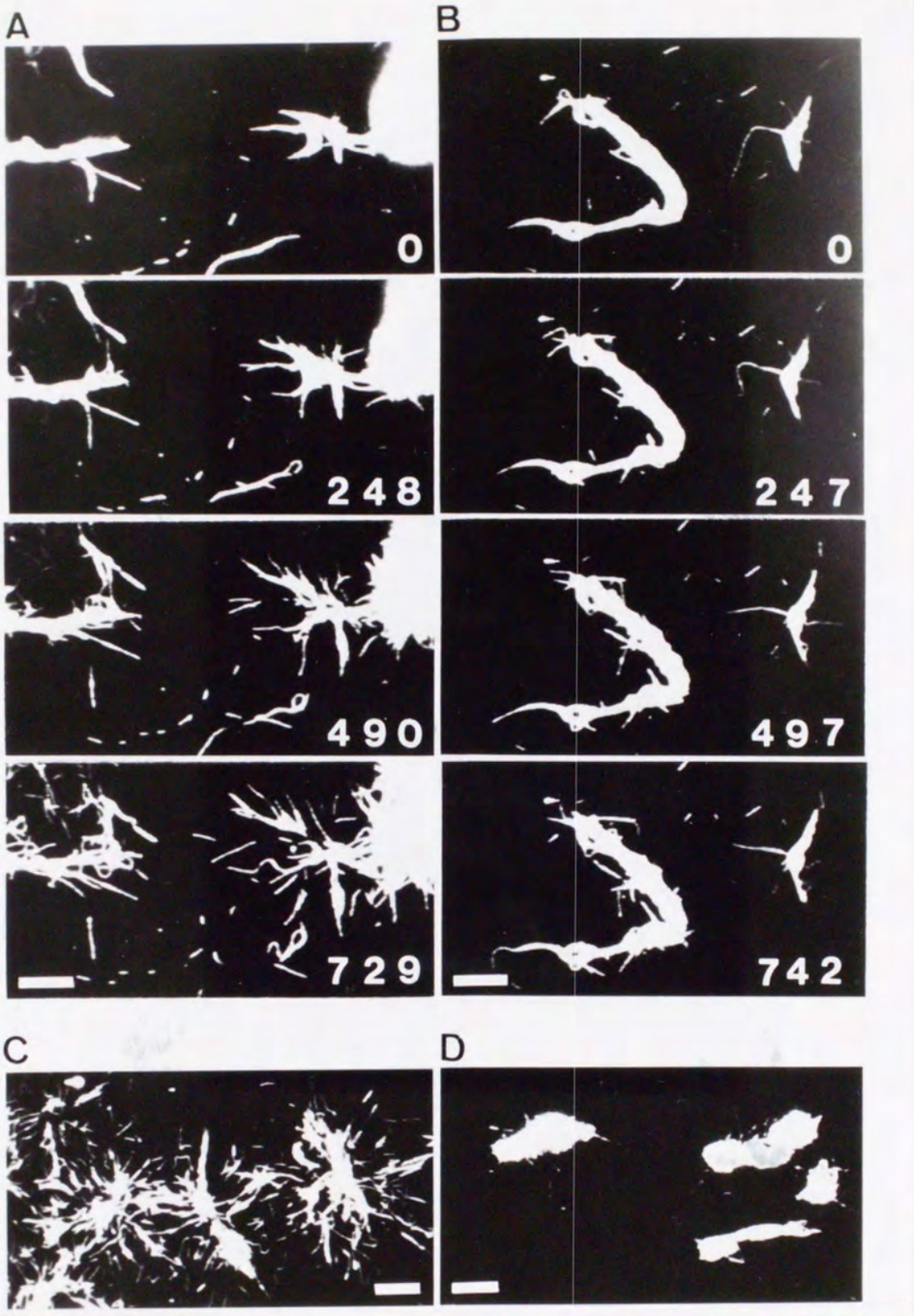


Fig. 3 Aggregates and bundles of microtubules formed upon incubation of microtubules, assembled from bovine brain tubulin, with an extract from isolated phragmoplasts and changes caused by the presence of MgGTP (A and C) or MgATP (B and D). Rhodamine-labeled microtubules were mixed with the 80 mM NaCl fraction (see Fig. 1B) and 3 mM MgGTP or 3 mM MgATP was added to the mixture. The time in seconds after the addition of the nucleotide is indicated in each photographs in A and B. C and D was observed 46 min and 57 min after the addition of the nucleotide, respectively. Bar, 10 μm.

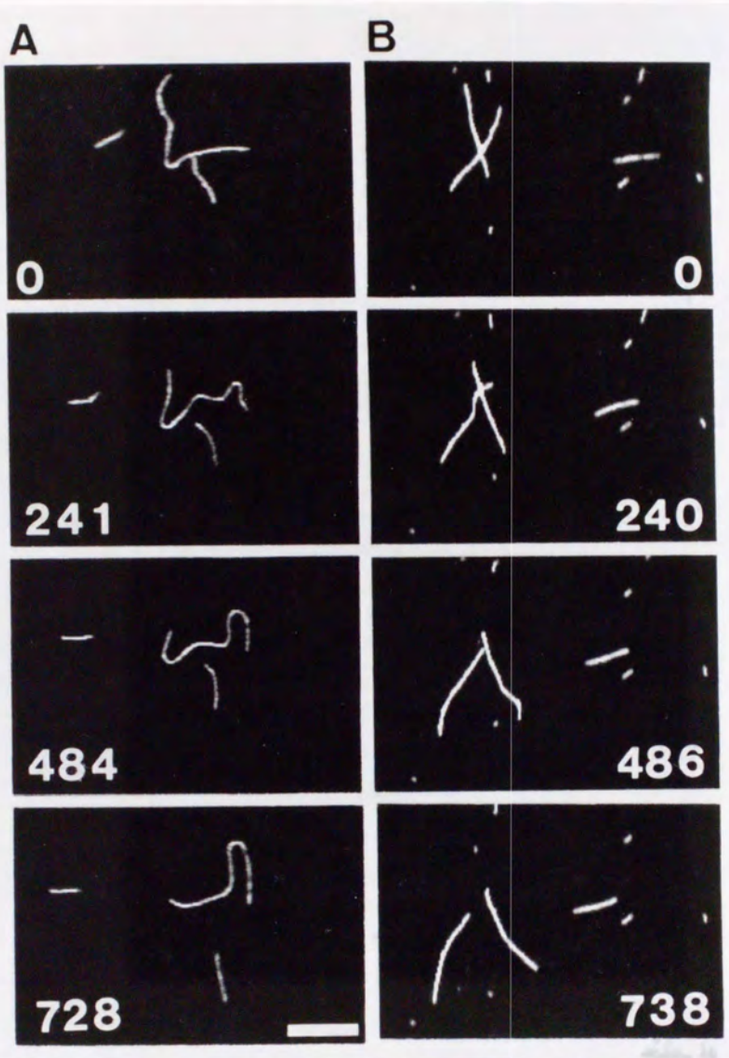


Fig. 4 Gliding of microtubules on a coverslip coated with an extract of isolated phragmoplasts in the presence of MgGTP (A) or MgATP (B). Single microtubules were observed after prolonged incubation of the motility assay for which results are shown in Fig. 3. MgGTP and MgATP were used at 3 mM. Time in seconds after the start of the observation is indicated in each photograph. Bar, 10 μm. Note that most microtubules changed their position in the direction of their long axes. In the presence of MgATP, single gliding microtubules were rarely observed since the bundles or aggregates failed to disperse.

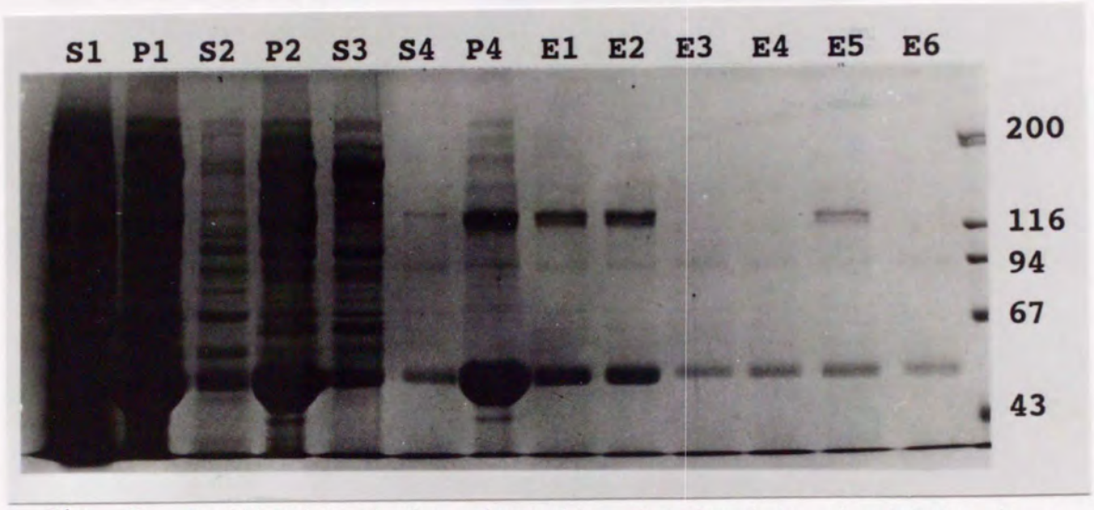


Fig. 5 Purification of the polypeptides that bound to microtubules in a nucleotide-sensitive manner. Microtubules assembled from purified brain tubulin were incubated with a fraction that contained polypeptides that had adsorbed to a DEAE-Sephacel column and had been eluted from the column with 200 mM NaCl. The mixture was centrifuged to obtain a supernatant (S1) and a pellet (P1). The P1 fraction was washed with PME buffer to obtain S2 and P2. The P2 fraction was washed twice with PME buffer that contained 0.3 M NaCl to obtain S3 and P3. The P3 was washed again with the same buffer to obtain S4 and P4. E1, E2, E3, E4, E5, and E6 were obtained by washing the P4 with PME buffer that contained 0.3 M NaCl together with 10 mM MgATP (E1), 10 mM MgGTP (E2), 10 mM MgAMPPNP (E3), 10 mM MgGMPPNP (E4), 8.3 mM MgATP plus 1.6 mM MgAMPPNP (E5), or 8.3 mM MgGTP plus 1.6 mM MgAMPPNP (E6). Each fraction was analyzed by SDS-PAGE on a 7.5% gel. Other details are given in the text. Marker proteins are shown with molecular mass in kDa.

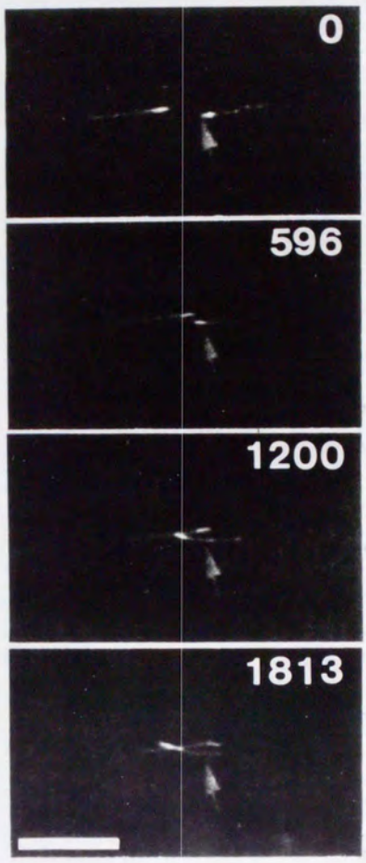


Fig. 6 Gliding of minus-end-marked microtubules caused by partially purified 125-kDa and 120-kDa polypeptides. Minus-end-marked microtubules were attached to a coverslip that had been coated with the fraction prepared by extracting the P4 in Fig. 5 with a solution that contained 0.3 M NaCl and both 5 mM MgATP and 5 mM MgGTP and to the microtubules 3 mM MgATP was applied. Arrows indicate the same position on the video monitor. Time in seconds after the start of the observation is indicated. Bar, 10 μm.

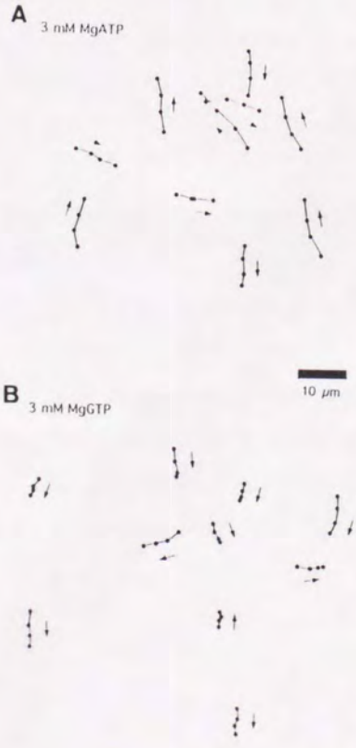


Fig. 7 Gliding of microtubules by partially purified 125-kDa and 120-kDa polypeptides. (A) ATP-induced gliding. (B) GTP-induced gliding. 3 mM MgATP and 3 mM MgGTP were used, respectively. For further details, see legend to Figure 6. The position of ends of gliding microtubules was recorded at 3-min interval. Bar, 10 μm.

CHAPTER III.
PARTIAL AMINO-ACID SEQUENCE AND SUBCELLULAR LOCALIZATION OF 125-kDa MICROTUBULE MOTOR POLYPEPTIDE ISOLATED FROM TOBACCO BY-2 CELLS

INTRODUCTION

As I described in chapter II, a fraction that contained 125-kDa and 120-kDa polypeptide supported a minus-end-headed translocation of microtubules. These polypeptides were similar to kinesin in terms of estimated molecular mass, sensitibity to nucleotides of binding to microtubules, and the polarity and nucleotide specifisity of motor activity. However, the previous study provided no direct evidence that those polypeptides are related to kinesin.

Kinesin was first isolated from the squid optic lobe as a plus-end-directed microtubule motor responsible for the fast axonal transport of vesicles (Vale et al. 1985) and then from a wide variety of sources, including mammalian neuronal tissues (Vale et al, 1985, Brady, 1985), sea urchin eggs (Scholey et al. 1985). One kinesin molecule consists of two heavy chain of 110-134 kDa and two light chain of 60-80 kDa (Bloom et al. 1988; Kuznetsov et al., 1988). The heavy chain have a motor domain that contain a region that include ATP-binding sequence and a microtubule-binding region (Yang et al. 1989).

To date, many polypeptides similar to kinesin heavy chain have been identified by molecular genetic analysis or by other analysis. The kinesin-like or -related polypeptides participate in a wide variety of microtubule-mediated events, including mitosis, meiosis, and nuclear fusion (Endow and Titus, 1992, Skoufias and Scholey, 1993). Recently, from a higher plant, a gene family encoding kinesin-like polypeptides have been identified (Mitsui, 1993). However, functions of kinesin-related polypeptide in higher plant cells are not known.

In this chapter, I show that previously isolated 125-kDa polypeptide is a kinesin-related polypeptide whicn is associated with microtubules in the spindle and the phragmoplast.

MATERIALS AND METHODS

Cell culture and synchronization of cell cycle of tobacco BY-2 cells

Tobacco BY-2 cells were maintained in modified Linsmaier and Skoog's medium as in chapter I and II. For the isolation of phragmoplasts, the cell cycle of BY-2 cells was synchronized by a two step arrest procedure, which employs aphidicolin and propyzamide, as described by Kakimoto and Shibaoka (1992). In the experiment in which the change in the amount of 125-kDa polypeptide during the cell cycle was examined, the cell cycle was synchronized by a single step arrest method which employs only aphidicolin (Nagata et al. 1981).

Polypeptide sequencing

Internal amino acid sequence of 125-kDa polypeptide was determined essentially as described by Aebersold (1989). Approximately 20 μg of the 125-kDa polypeptide and a smaller amount of 120-kDa polypeptide were partially purified from isolated phragmoplasts as described in chapter II, run on SDS-PAGE using 6.5% gel, and blotted on nitrocellulose membrane. The membrane were stained with 0.3% Ponceau S in 3% TCA and the band of 125-kDa polypaptide was excised, destained with 200 μM NaOH, and washed with water. The membrane strip was dissected, blocked with 0.5 % PVP-40 in 100 mM acetic acid and treated with 20 μg/ml of trypsin in 1% hydrogenated Triton X-100 (Calbiochem, San Diego, U.S.A.), 10% acetonitrile, 100 mM Tris-HCl (pH. 8.0) for 13 hr at 37 °C. The peptide fragments were fractionated by reversephase HPLC using C_{18} column. Two hydrophobic peptide fragments were sequenced using gas-liquid-phase protein sequenator.

Polymerase chain reaction (PCR) and DNA sequencing

Following sense primers (1,3) and antisense primers were (2,4) were used:

(1) GT(CTG)CA(GA)TT(CT)(CT)T(TGA)GA(GA)(CT)T(TGA)TA(CT)CAA,

derived from the sequence VQFLELYN in the 125-kDa polypeptide;

(2) TT(CTA)A(GA)(CTA)A(GA)(TA)GT(GA)AA(TGA)AT(CT)TC(GA)TT(TGA)GC, derived from the sequence ANEIFTLLE in the 125-kDa polypeptide; (3) AT(CTA)TT(CT)GC(CA)TA(CT)GG(TA)CAAAC, derived from the sequence IFAYGQT of the consensus ATP-binding site of kinesin relatives; and (4) TC(GA)(CG)(TA)(TA)CC(TG)GC(CTA)A(GA)(GA)TC, derived from the sequence DLAGSE of the consensus MT-binding site of kinesin relatives.

Templates used for PCRs were first strand cDNA that was prepared from poly(A)$^+$ mRNA isolated from tobacco BY-2 cells in mitotic phase. Two rounds of thirty PCR cycles were performed as follws: 94°C for 1 min, 55°C for 2 min and 72°C for 3 min. PCR Products were fractionated in agarose gels, eluted from the gels and subcloned into EcoRV site of pBluescript (Stratagene, La Jolla, USA). They were sequenced by the cycle sequencing method using fluorescent labeled primers and Taq polymerase (Perkin-Elmer cetus, Norwalk, USA) in an apparatus from Applied Biosystems (Urayasu, Japan). Sequences of both DNA strands was obtained by using automatic DNA sequencer.

Preparation of peptide antibody

A peptide of sequence APEDLKVALEDRQK was synthesized by Accord Co., Ltd. (Tokyo, Japan) with an additional N-terminal cysteine. The coupling of the synthetic peptide to carrier protein, the immunization, and the affinity-purification of the peptide antibody were performed essentially as described by Sawin et al. (1992). In brief, the 3 mg synthetic peptide was coupled to 20 mg Keyhole limpet hemocyanin (KLH; Calbiochem, La Jolla, USA) that had been activated by iodoacetic acid *N*-hydroxysuccinimide ester (Sigma, St. Louis, MO). The peptide-conjugated KLH corresponding to 300 μg peptide was injected to a couple of rabbits at intervals of 2 or 3 weeks. The sera was screened on dot blots of the synthetic peptide. The antibody against the synthetic peptide was purified with a column of EAH-Sepharose 4B (Pharmacia LKB, Uppsala, Sweden) to which the peptide was conjugated.. The purified antibody was concentrated using Centricon 10 (Amicon, Beverly, USA) to 1.2

mg/ml, supplemented with 0.1% NaN_3, and stored at 4°C. The peptide antibody prepared was designated as pAb125 for convenience.

Western blot analysis

Phragmoplasts were isolated from protoplasts of BY-2 cells of which cell cycle was synchronized as described by Kakimoto and Shibaoka (1992), except that a buffer containing a detergent (TX-PME buffer; 0.1% Triton X-100, 50 mM K-Pipes, pH 6.9, 1 mM $MgSO_4$, 5 mM EGTA, 1 mM DTT, 1 mM PMSF, 5 μg/ml leupeptin) was used in the isolation procedure. The isolated phragmoplasts were washed once with Triton X-PME buffer, suspended in extraction buffer (20 mM Tris-HCl, pH 7.6, 0.2% SDS, 1 mM PMSF), and vigorously stirred. For the preparation of whole cell extracts, BY-2 cells were frozen and homogenized in liquid nitrogen, and stirred in extraction buffer. The suspensions were centrifuged for 15 min at 368000 g, 4°C. The extracts at the supernatant were mixed with 1/3 volume of a sample buffer (500mM Tris-HCl, pH 6.8, 8% SDS, 20% 2-mercaptethanol, 40% (v/v) glycerol) and stored at -20°C until use.

A protein fraction that contained kinesin was prepared from bovine brain white matter essentially as described by Paschal et al. (1987). In brief, the white matter was homogenized in PME buffer (100 mM K-Pipes, pH 6.9, 1 mM $MgSO_4$, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 100 μg/ml TAME) and the homogenate was centrifuged to yield a clear supernatant. The crude extract was supplemented with 20 μM taxol, incubated at 37°C, and centrifuged to pellet the taxol-assembled microtubules. The pelleted microtubules were washed twice with PME buffer that contain 0.3 M NaCl and 20 μM taxol, and then suspended in PME buffer that contain 0.3 M NaCl, 20 μM taxol, and 10 mM MgATP. The suspension was centrifuged to yield a kinesin-enriched fraction at the supernatant.

SDS-PAGE and immunoblotting was carried out using standard tequniques. Nitrocellulose membranes blotted were blocked with 2% skim milk in PBS. The pAb125 was used at a concentration of 0.6 μg/ml. The monoclonal antibody, SUK4, was used at 1 μg/ml (Ingold et al., 1988). The horseradish peroxidase conjugated secondary antibody was used in the analysis shown in Figure 3A. In

experiment of Figure 3B, biotinylated antibody and horseradish peroxidase conjugated streptavidin were used as second and third probe to amplify faint signal. These probes were obtained from Amersham.

Immunofluorescence microscopy

BY-2 cells at appropriate stage of the cell cycle were packed, suspended in a 1:10 mixture of 37% formaldehyde and 50 mM PME buffer (50 mM K-Pipes, pH 6.9, 2 mM $MgCl_2$, 5 mM EGTA), and rotated for 1 hr at room temperature. Fixed cells were washed twice with 50 mM PME buffer and treated with cell wall digesting solution [0.5% (w/v) cellulase Onozuka RS (Yakult Pharmaceutical Co. Ltd., Takarazuka, Hyogo), 0.05% (w/v) pectolyase Y-23 (Seishin pharmacentical Co. Ltd., Tokyo, Japan), 50 mM K-Pipes, pH 5.5] for 3 min at room temperature. After twice washing with 50 mM PME, cells were permeabilized with 0.1% TX-100 in 50 mM PME buffer for 15 min at room temperature. Cells to be fixed with methanol were placed on a nylon mesh, treated with the cell wall digesting solution for 1 min. After removing the cell wall digesting slution, the cells on the nylon mesh were immersed in 95% methanol at -20°C for 20 min. Cells fixed with formaldehyde or methanol were washed twice with 50 mM PME buffer and suspended in PBS that contained 1% BSA before the incubation with a mixture of pAb125 and monoclonal mouse antibodies raised against chick brain a-tubulin. pAb125 was used at 2.4-6 μg/ml. Secondary antibodies used were FITC-labeled antibodies against rabbit Igs and Texas Red-labeled antibodies against mouse IgG and. Anti-tubulin antibodies and secondary antibodies were obtained from Amersham.

RESULTS

Partial amino-acid sequence of the 125-kDa polypeptide

Two tryptic fragments of the 125-kDa polypeptide were isolated and sequenced (Fig. 1). The sequence of one fragment named p125-50 contained a consensus sequence of the kinesin family, EXYXXXXXDLL (Fig. 1B), that is known to be located between the consensus ATP-binding site and the consensus MT-binding site, within the motor domain, suggesting that the 125-kDa polypeptide is a member of the kinesin superfamily. Thus, the 125-kDa polypeptide was refered to as TKRP125 (125-kDa tobacco kinesin-related polypeptide). The sequence of another fragment named p125-53 was not significantly similar to known polypeptides including members of the kinesin superfamily..

Oligonucleotide primers derived from the sequences of p125-50 and p125-53 were synthesized and used for PCR amplification of tobacco cDNA prepared from BY-2 cells in mitotic phase. The PCR with a sense primer derived from p125-50 and a antisense primer derived from p125-53 resulted in the amplification of a DNA fragment of which size was ~180 bp (data not shown). Sequencing of the DNA fragment revealed that it encodes 63 amino acid of which sequence is more closely related to sequences found in KIP1, BimC, and Eg5 than those in other members of the kinesin superfamily (Fig. 2A).

Primers derived from the consensus sequences in the presumptive ATP- and MT-binding sites was used in a PCR. Amplified DNA fragments of 450-500 bp encoded at least six kinds of kinesin-related sequences that included consensus sequnces, EXYXXXXXDLL and SSRSH (Fig. 2B). However, none of them encoded the partial amino acid sequence of the TKRP125, suggesting that TKRP125 has unique sequences in ATP- and/or MT-binding site.

Western blot analysis with a peptide antibody against the 125-kDa tobacco kinesin-related polypeptide (TKRP125)

A polyclonal antibody was raised against a synthetic peptide, of which amino-acid sequence was found in the partial sequence of

TKRP125 predicted from sequencing of PCR-amplified cDNA (Fig. 2; APEDLKVALEDRQK). A raised antibody was refered to as pAb125. The partially purified TKRP125 was recognized by pAb125 but not by the monoclonal antibody SUK4 that recognize convensional kinesins of a wide variety of animal cells (Fig. 3A; Ingold et al., 1988). The 120-kDa polypeptide that was always accompanied by TKRP125 was neither recognized by pAb125 nor by SUK4 antibody (Fig. 3A). The pAb125 recognized a single polypeptide of which molecular mass was estimated to be 125 kDa in a extract prepared from phragmoplasts isolated from tobacco BY-2 cells or in a whole cell extracts prepared from the cell in the mitotic phase (Fig. 3B).

The amount of TKRP125 in whole cell extracts was dramatically changed as the cell cycle progressed (Fig. 4). TKRP125 was detected in the extract prepared from cells that had been treated with aphidicolin for 24hr to arrest the cell cycle at the early stage of S phase. After the removal of aphidicolin, the amount of the TKRP125 increased as the cell cycle progressed from S to G2 phase and reached a muximum level at the time when the mitotic index index reached the peak. The amount of TKRP125 polypeptide rapidly decreased when mitotic index began to decrease.

Localization of the 125-kDa polypeptide in tobacco BY-2 cells

Tobacco BY-2 cells at various stages of the cell cycle were fixed with formaldehyde or methanol, and double-stained with an anti-α-tubulin antibody and pAb125. Regardless of the fixative used, pAb125 stained preprophase bands, spindles as well as phragmoplasts from which TKRP125 was originally isolated, but it did not give any significant staining signal in cells with cortical microtubules (Fig. 5 and Fig. 6 A, B). The signal in preprophase bands was always weaker than that in spindles or phragmoplasts. Both of anti-tubulin antibody and pAb125 did not stained the equatorial planes of phragmoplasts, where phragmoplast microtubules are interdigitaing and a number of vesicles are present (Heplar and Jackson, 1968). Interestingly, the width of the equatorial region that was not stained by pAb125 was obviously greater than the width

of the region that was not stained by anti-a-tubulin in cells fixed with methanol (Fig. 6 C, D).

DISCUSSIONS

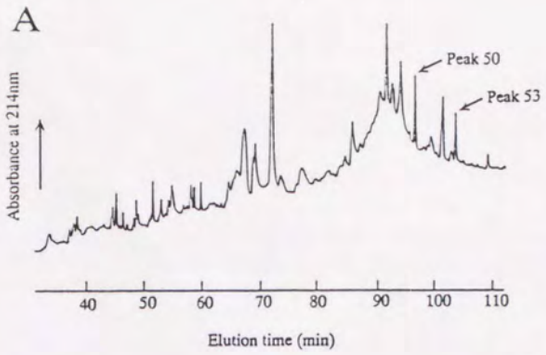
Amino-acid sequencing of the 125-kDa polypeptide revealed that the polypeptide contained a sequence which is conserved in the motor domains of members of the kinesin superfamily. Thus, the polypeptide was named TKRP125 (125-kDa tobacco kinesin-related polypeptide). The PCR using the primers correspond to two peptide sequences of the TKRP125 resulted in the amplification of a DNA fragment that encoded the amino acid sequence that was related to members of the "Bim C subfamily" to which *S. cerevisiae* KIP1, *A. nidulans* bimC, and *Xenopus* Eg5 belong (Endow and Titus, 1992). Thus, it is probable that TKRP125 is a new member of the BimC subfamily. The members of the BimC subfamily have been known to be essential for the organization of bipolar spindles and closely related in the primary sequence of the motor domain (Roof et al. 1992; Hoyt et al. 1992; Le Guellec et al. 1991). TKRP125 is highly expressed during mitosis (Fig. 4) and associated with spindle microtubules (Fig. 5). These features of TKRP125 are comparable to those of members of the BimC subfamily (Hagan and Yanagida, 1990; Sawin et al., 1992).

Because TKRP125 have been isolated from phragmoplasts (chapter II), it have been suggested that this polypeptide is associated with the phragmoplast and contributes to the function of the phragmoplast. Results of immunofluorescence indicated that TKRP125 was distributed along phragmoplast microtubules except the vicinity of the equatorial region. Such an immunolocalization of TKRP125 is just opposite to the predicted localization of the motor that responsible for the microtubule translocation in the phragmoplast (chapter I). However, because antibodies do not penetrate into the equatorial region of phragmoplasts (Gunning, 1982), results of the immunofluorescence studies do not deny the possibility that TKRP125 is buried in the equatorial region. Thus, TKRP125 is still a candidate of the motor for the microtubule translocation in the phragmoplast. It is also possible that TKRP125 is the motor which transport vesicles to the plane of cell plate formation although the staing pattern of TKRP125 is distinct from that of kinesin, a

well characterized vesicle transporter (Hollenbeck and Swanson, 1990; Brady et al.,1991).

The PCR using the primers corresponding to two consensus sequences resulted in the amplification of DNA fragments encoding at least six kinds of kinesin related sequence, implying that tobacco BY-2 cells have multiple kinesin-related transcripts and genes as reported in various types of cells (Endow and Hatsumi, 1991; Mitsui et al., 1993). Characterization of these presumptive motor polypeptides together with TKRP125 should be help us to understand the function of microtubules and the mechanism which regulate the organization of microtbule array. As was pAb125 in the present study, peptide antibodies that recognize short amino-acid sequences specific to motor domain of each presumptive motor polypeptide would be of great help for studies to clarify the function of the peptide.

FIGURES



B

p125-53	GLEEEIVTSANEIFTLLE
p125-50	VQFLELYNEEIQDLL
BIMC	CSFIELYNEELRDLL
CIN8	CSFIELYNEELKDLL
CUT7	CSYYELYNEEIRDLL
KIP1	ISFLELYNENLKDLL
KAR3	CEFIEIYNENIVDLL
EG5	CSLLEIYNEELFDLL
NCD	ATFLEIYNEVLYDLL
KHC-D	VSYYEIYMDKIRDLL
KHC-S	ISYYEIYLDKIRDLL
KHC-U	VSYFEIYMDRIRDLL
⋮	⋮

Fig. 1 Determination of the amino-acid sequence of two tryptic fragments of the 125-kDa polypeptide. (A) HPLC-fractionation profile of tryptic fragments of the 125-kDa polypeptide. Two hydrophobic peptide fragments eluted in Peak50 and Peak53 were isolated for sequencing. (B) Sequences of the peptides eluted in Peak50(p125-50) and Peak53(p125-53). The p125-50 included a consensus sequence of members of the kinesin family, EXYXXXXXDLL. It resembled the partial amino acid sequence of kinesin-like proteins including bimC protein of *A. nidulans*(BIMC), CIN8 protein of *S. cerevisiae* (CIN8), cut7 protein of *S. pombe* (CUT7), KIP1 protein of *S. cerevisiae* (KIP1), KAR3 protein of *S. cerevisiae* (KAR3), Eg5 protein of *Xenopus laevis* (EG5), or ncd protein of *D. melanogaster*(NCD) rather than those of the kinesin heavy chain from *Drosophila*(KHC-D), squid(KHC-S), or sea urchin(KHC-U).

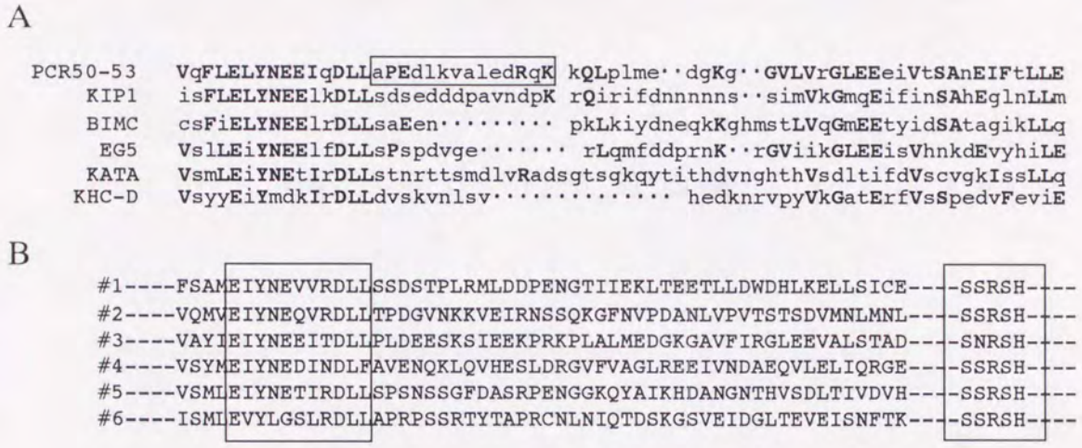


Fig. 2 The partial amino acid sequence of TKRP125 and other kinesin-like-proteins predicted from PCR-amplified tobacco cDNAs. (A) Comparison of the partial amino acid sequence of TKRP125(PCR50-53), KIP1, BIMC, EG5, katA protein of *A. thaliana* and the kinesin heavy chain from *Drosophila*(KHC-D). PCR50-53 was estimated from the sequence of a DNA fragment that was amplified using the primers derived from p125-50 and p125-53 in Figure 1. Amino acids identical to those in PCR50-53 are indicated as bold characters. A synthetic peptide with the enclosed sequence was used to prepare a peptide antibody against TKRP125. KIP1, BIMC, EG5, see legend to Fig. 1. (B) Partial amino acid sequences of kinesin-like-proteins in tobacco BY-2 cells, that were predicted from the sequences of the PCR products amplified using the primers corresponding to the consensus ATP- and MT-binding site of the kinesin family. Limited length of sequences that contain the consensus sequences, EXYXXXXXDLL and SSRSH, are shown. Dashes indicate variable numbers of omitted residues. The sequence of SSRSH is known to be highly conserved in the motor domains of the members of the kinesin family.

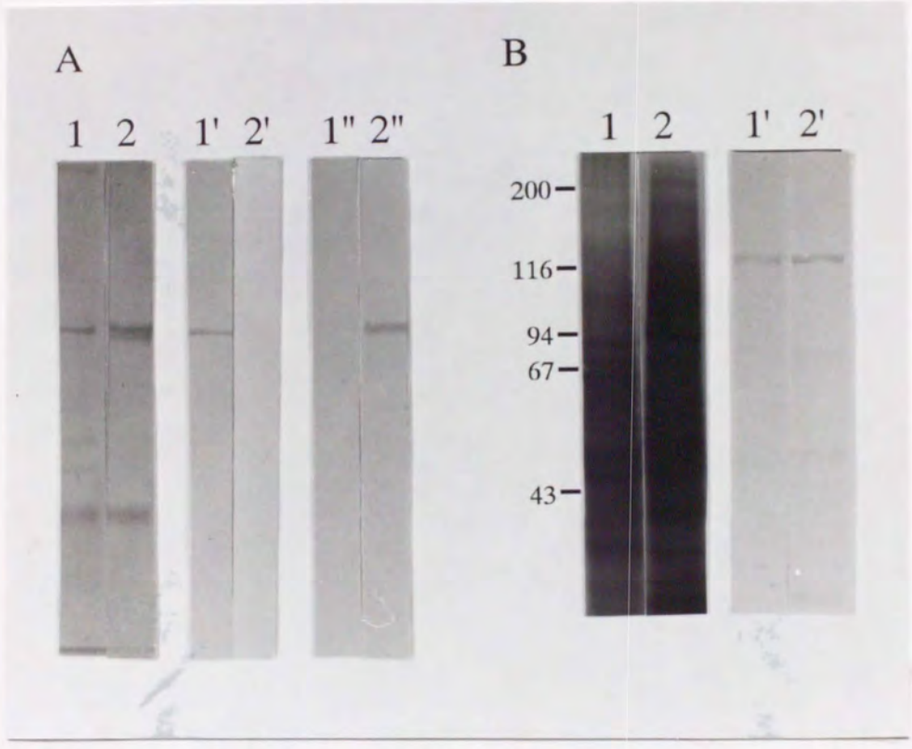


Fig. 3 Reactivity of a peptide antibody against TKRP125 (A) Comparison of the reactivity of SUK4, a monoclonal antibody against kinesin heavy chain, and that of pAb125, an antibody against a synthetic peptide of which sequence was predicted from nucleotide sequence of TKRP125cDNA. Partially purified bovine brain kinesin (1, 1', 1") and TKRP125 (2, 2', 2") were run on 7.5% gel, blotted, and stained with amido black (1, 2), SUK4 (1', 2') or PAb125 (1", 2"). SUK4 that recognize kinesin did not recognize TKRP125 while PAb125 recognized TKRP125 but not kinesin. (B) Specificity of PAb125. A crude extract prepared from isolated phragmoplasts (1, 1') and a whole cell extract prepared from cells in mitotic phase (2, 2') were run on 8.5% gel, and stained with Coomassie Blue (1, 2) or stained with PAb125 (1', 2') after blotting. A band of 125 kDa was strongly stained.

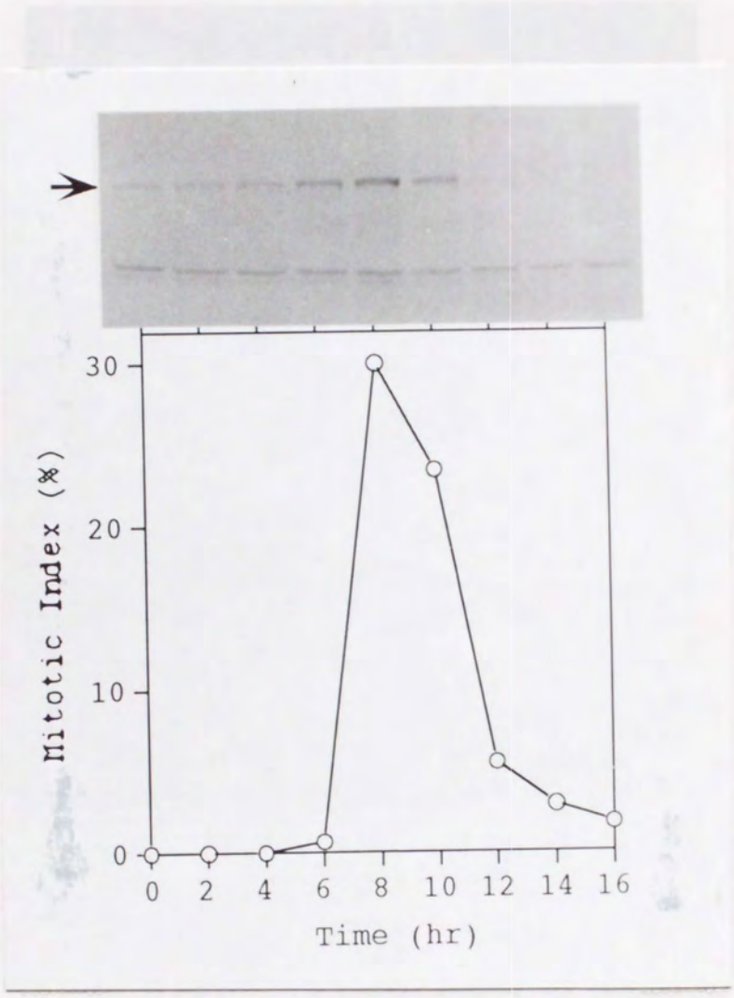


Fig. 4 Change in the amounts of TKRP125 during the cell cycle of tobacco BY-2 cells. An arrow indicate the bands of TKRP125. Lower bands are nonspecific signals.

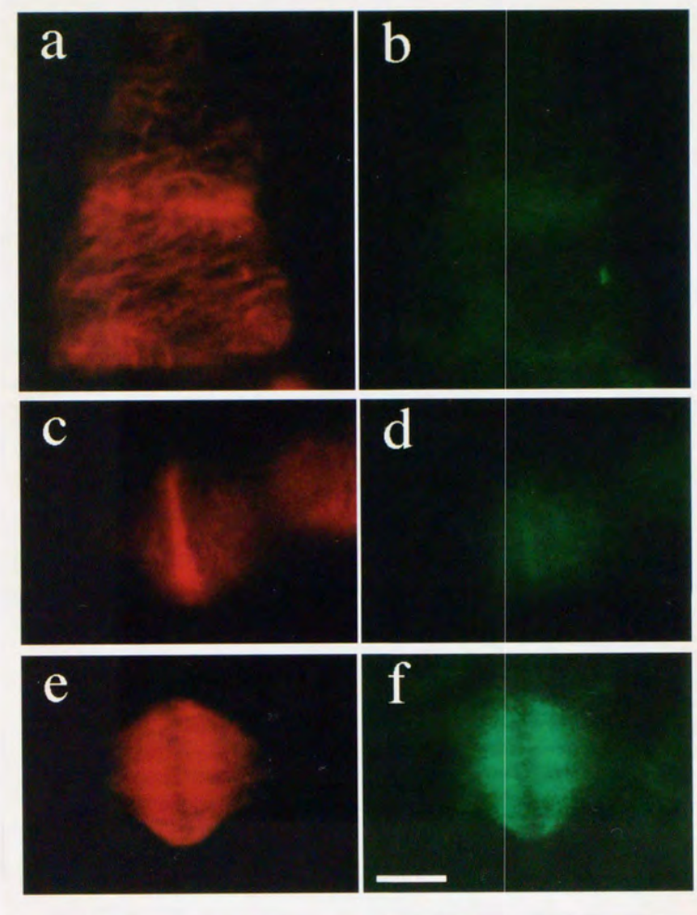


Fig. 5 Localization of TKRP125 in premitotic or mitotic tobacco BY-2 cells. The cells with cortical microtubules (a, b), preprophase band (c, d), and spindle (e, f) were double-stained with anti-a-tubulin antibody (a, c, e) and PAb125 (b, d, f). bar, 10 μm

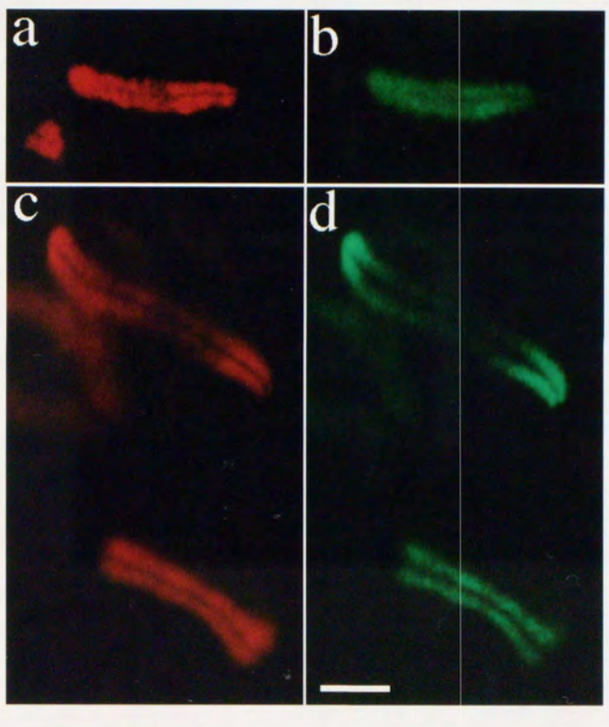


Fig. 6 Localization of TKRP125 in phragmoplasts of tobacco BY-2 cells. The cells with phragmoplasts were fixed with formaldehyde (a, b) or cold methanol (c, d) and double-stained with anti-α-tubulin antibody (a, c) and PAb125 (b, d). The dark band at the equatorial region in TKRP125-stained cells was wider than that in tubulin-stained cells. bar, 10 μm

GENERAL DISCUSSION

The study using the glycerinated tobacco BY-2 cell revealed that the equatorial plane of the phragmoplast strongly incorporate exogenously applied tubulin (chapter I), suggesting the occurrence of the tubulin polymerization at the plus ends of phragmoplast microtubules (chapter I, Fig. 4). Without the mictotubule sliding system that I found and characterized in this study, the oppositely oriented microtubule-array of the phragmoplast could not be maintained. Therefore, the microtubule sliding system seems to play an important role in the organization of the phragmoplast. In chapter I, I suggested that the equatorial plane posseses the mechanism responsible for the microtubule movement. The mechanism probably use a microtubule motor protein that cause minus-end headed movement of microtubules at the expense of the energy of GTP or ATP. I have succeeded to isolate such a microtubule-motor-polypeptide, TKRP125 (chapter II, III). Althogh TKRP125 colocalize with phragmoplast microtubules, it is not clear whether or not TKRP125 is involved in the microtubule sliding in the phragmoplast. To examin this, experiment in which the peptide antibody against TKRP125 is introduced into glycerinated tobacco cells with phragmoplasts should be carry out.

Acknowledgments

I wish to thank professor H. Shibaoka for kind guidance and encouragements throughout the course of this study. I thanks Dr. S. Sonobe for valuable suggestions on the study using glicerinated cells, Dr. T. Kakimoto for helpful discussions and technical advice. I thank Dr. J. Scholey of the University of Colorado for providing the SUK4 monoclonal antibody and Dr. H. Murofushi of the University of Tokyo for providing the antibody against kinesin from bovine adrenal medulla cells. I thank Dr. R. Kuriyama and Dr. M. Kimble of University of Minesota and P.Matudaira of M.I.T. for cooperation in amino-acid sequencicng. I thank Dr. A. Shinohara and Dr. O. Hisatomi of the Osaka University for helpful advice on PCR and DNA sequencing. I thanks Keiko Mayumi, Takahide Yokoi, and Keisuke Matsui of the Osaka University for preparation of figures in this thesis. This study was supported by Japan Society for the Promotion of Science.

Hallenbeck, P. J. and Swanson, J. A. (1990) Radial extension of macrophage tubular lysosomes supported by kinesin. Nature **346**, 864-866

Hardham, A. R. and Gunning, B. E. S. (1980). Some effects of colchicine on microtubules and cell division in roots of Azolla pinnata. Protoplasma **102**, 31-51.

Heplar, P. K. (1982). Endoplasmic reticulam in formation of the cell plate and plasmodesmata. Protoplasma **111**, 121-133.

Hepler, P. K. and Jackson, W. K. (1968). Microtubule and early stages of cell-plate formation in the endosperm of Haemanthus katherinae Baker. J. Cell Biol. **38**, 437-446.

Hoyt, M. A., He, L., Loo, K. K. and Saunders, W. S. (1992) Two Saccharomyces cervisiae kinesin-related gene products required for mitotic spindle assembly J. Cell Biol. 118 109-120

Hyman, A. A. (1990). Preparation of marked microtubules for the assay of the polarity of microtubule-based motors by fluorescence. J. Cell Sci. (Suppl.) **14**, 125-128.

Hyman, A. A., Dreschel, D., Kellogg, D., Salser, S., Sawin, K., Steffen, P., Wordeman, L., and Mitchson, T. (1990). Preparation of modified tubulins. Meth. Enzymol. **196**, 478-485.

Ingold, A. L., Cohn, S. A., and Scholey, J. M. (1988). Inhibition of kinesin-driven microtubule motility by monoclonal antibodies to kinesin heavy chains. J. Cell. Biol. **107**, 2657-2667.

Inoe, S (1968) Organization and finction of the mitotic spindle. In: Allen, R. D. and Kamiya, N. (eds) Primitive Motile Systems in Cell Biology, Academic Press Inc., London, 594-598.

Kakimoto, T. and Shibaoka, H. (1988). Cytoskeletal ultrastracture of phragmoplast-nuclei complexes isolated from cultured tobacco cells. Protoplasma [Suppl.] **2**, 95-103.

Kakimoto, T. and Shibaoka, H. (1992). Synthesis of polysaccharides in phragmoplasts isolated from tobacco BY-2 cells. Plant Cell Physiol. **33**, 353-361.

Keith, C. H., Feramisco, J. R.b and Shelanski, M. J. (1981) Direct visualization of fluorescen labeled microtubule in vitro and in microinjected fibroblast. J. Cell Biol. **88**,234-240

Kimble, M. and Kuriyama, R. (1992) Functional components of microtubule-organizing centers. Int. Rev. Cytol. **136**, 1-50.

Kuznetsov, S. A. and Gelfand, V. I. (1986). Bovine brain kinesin is a microtubule-activated ATPase. Proc. Natl. Acad. Sci. USA **83**, 8530-8534.

Kuznetsov, S. A., Vaisberg, E. A., Shanina, N. A., Magretova, N. N. and Gelfand, V. I. (1988) The quaternary structure of bovine brain kinesin. EMBO J. **7**, 353-356.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685.

Le Guellec, R., Paris., Couturier, A., Roghi, C., Philippe, M. (1991) Cloning by differential screening of a Xenopus cDNA that encodes a kinesin-related protein. Mol. Cell. Biol. **11**, 3395-3398.

Nagata, T., Okada, K., Takebe, I. and Mitsui, C. (1981). Delivery of tobacco mosaic virus RNA into plant protoplasts mediated by reverse-phase evaporation vesicles (liposomes). Mol. Gen. Genet. **184**, 161-165.

Masuda, H., and Cande, W. Z. (1987) The role of tubulin polymerization during spindle elongation in vitro. Cell **49**, 193-202

Mitsui, H., Yamaguchi-Shinozaki, K., Shinozaki, K., Nishikawa, K., and Takahashi, H. (1993). Identification of a gene family (kat) encoding kinesin-like proteins in Arabidopsis thaliana and the characterization of secondary structure of KatA. Mol. Gen. Genet. **238**, 362-368.

Mitison, T. J. and Kirshner, M. W. (1985) Properties of kinetochore in vitro. Microtubule capture and ATP-dependent translocation. J. Cell Biol. 101, 766-777.

Murofushi, H., Ikai, A., Okuhara, K., Kotani, S., Aizawa, H., Kumakura, K., and Sakai, H. (1988). Purification and characterization of kinesin from bovine adrenal medulla. J. Biol. Chem. **265**, 12744-12750.

Omoto, C. K. and Johnson, K. A. (1986). Activation of dynein adenosinetriphosphatase by microtubules. Biochemistry **26**, 419-427.

Palevitz, B. A. and Hepler, P. K. (1974). The control of the plane of division during stomatal differentiation in Allium. Chromosoma **46**, 327-341.

Paschal, B. M. and Vallee, R. B. (1987). Retrograde transport by the microtubule-associated protein MAP1C. Nature (London) **330**, 181-183.

Paschal, B. M., Shpetner, H. S., and Vallee, R. B. (1991) Purification of brain cytoplasmic dynein and characterization of its in vitro properties. Meth. Enzymol. **196**, 181-449.

Porter, M. E., Scholey, J. M., Stemple, D. L., Vigers, G. P. A., Vale, R. D., Sheetz, M. P. and McIntosh, J. R. (1987). Characterization of the microtubule movement produced by sea urchin egg kinesin. J. Biol. Chem. **262**, 2794-2802

Roof, D. M. Meluh, P. B. and Rose, M. D.(1992) Kinesin-related proteins required for assembly of the mitotic spindle. J. Cell Biol. **118**, 95-108

Sawin, K. E. and Scholey, J. M. (1991). Motor proteins in cell division. Trends Cell Biol. **1**, 122-129.

Sawin, K. E., Mitchison, T. J., Wordeman, L. G. (1992) Evidence for kinesin-related proteins in the mitotic apparatus using peptide antibodies. J. Cell Biol. **101**, 303-313

Sawin, K. E., LeGuellec, K., Philippe, M. and Mitchison, T. J. (1992) Mitotic spindle organization by a plus-end-directed microtubule motor Nature **359**, 540-543

Scholey, J. M., Porter, M. E., Grissom, P. M. and McIntosh, J. R. (1985) Identification of kinesin in sea urchin eggs, and evidence for its localization in the mitotic spindle. Nature **318**, 483-486.

Skoufias, D. A. and Scholey, J. M. (1993) Cytoplasmic microtubule-based motor proteins. Curr. Opin. Cell Biol. **3**, 95-104

Shelanski, M. L., Gaskin, F. and Cantor C. R. (1973). Microtubule assembly in the Absence of added nucleotides. Proc. Nat. Acad. Sci. USA **70**, 765-768

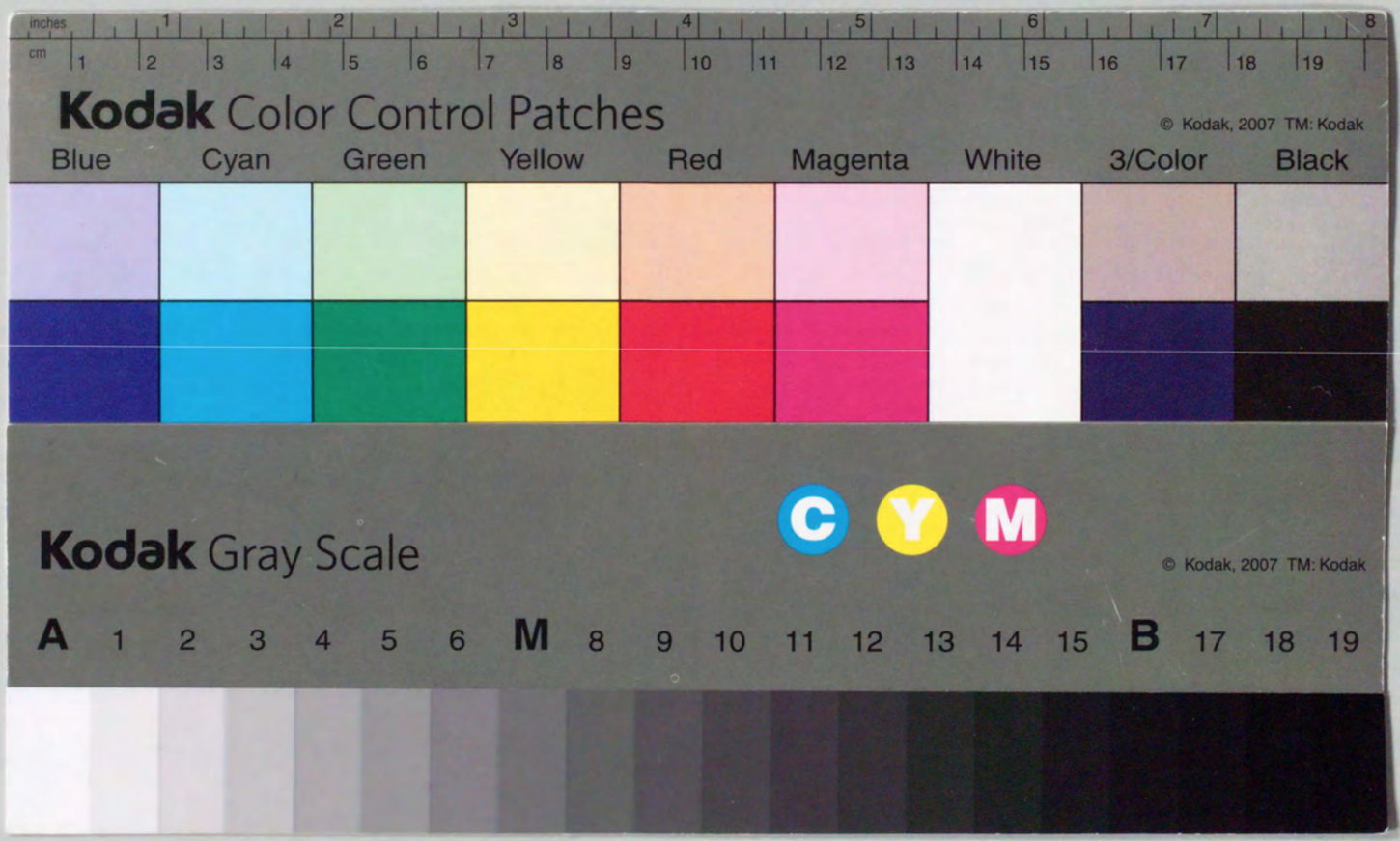
Vale, R. D. and Reese, T. S. and Sheetz, M. P. (1985a). Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. Cell **42**, 39-50

Williamson, R. E. (1991). Orientation of cortical microtubules in interphase plant cells. Int. Rev Cytol. **129**, 135-206.

Yang, J. T., Laymon, R. A., Goldstein, L. S. B. (1989) A three-domain structure of kinesin heavy chain revealed by DNA sequence and microtubule binding analysis. Cell 56, 879-889

Yang, J. T., Saxton, W. M., stewart, R. J., Raff, E. C. and Goldstein (1990) Evidence that the head of kinesin is sufficient for force generation and motility in vitro. Science **249**, 42-47

Zhang, D., Wadsworth, P. and Heplar, P. K. (1990). Microtubule dynamics in living dividing plant cells: confocal imaging of microinjected fluorescent brain tubulin. Proc. Nat. Acad. Sci. USA **87**, 8820-8824.


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19