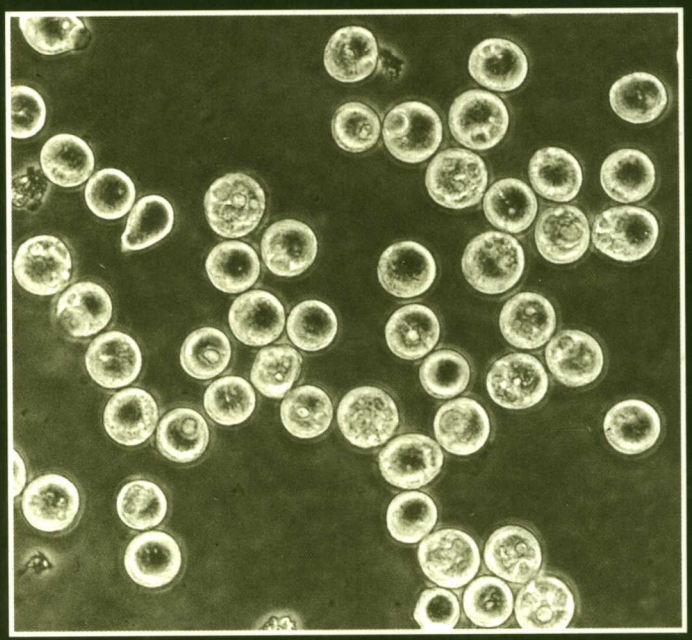
INTERNATIONAL REVIEW OF CYTOLOGY

A SURVEY OF CELL BIOLOGY

Edited by

Kwang W. Jeon



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A Survey of Cell Biology

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Front cover photograph: Cytokinesis of miniprotoplasts. (For more details, see Chapter 1, Figure 4b.)

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Cell Model Systems in Plant Cytoskeleton Studies

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Cytoskeletons play an essential role in cellular functions in both animal and plant cells. In studies of the molecular mechanisms of their functions, a variety of cell model systems, mainly of animal cells, have yielded much information. With plant cells, cell model systems have mostly been restricted to studies on the mechanism of cytoplasmic streaming. Recently, however, there have been several reports of studies employing plant cell model systems to investigate plant cytoskeletons that have revealed new concepts about their structure and functions. To promote and support a general understanding of cell model systems, this review attempts to categorize them, present currently known information on the structure and function of plant cytoskeletons, and offer a possible role of cell model systems in future studies of plant cytoskeletons.

KEY WORDS: Cytoskeleton, Cell model, Plant cell, Tobacco BY-2 cell, microtubule, actin filament.

I. Introduction

Since Szent-Györgi (1949) introduced the first cell model system, a glycerinated model of skeletal muscle, many types of systems have been used to analyze the organization and function of cytoskeletons. In the case of animal cells, many types of cell models have been introduced to study cytoskeletons, whereas model systems for plant cells have been almost restricted to studies on the mechanism of cytoplasmic streaming. Recently, however, several types of plant cell models have been developed and have contributed new concepts to studies of plant cytoskeletons, which form the structures characteristic to plant cells.

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To date, only one review (on model systems of tobacco BY-2 cells by Shibaoka *et al.*, 1996) has focused on the cell model systems of plant cells. In this review, I will survey the model systems of plant cells that have been reported and present a view of cell models in future studies of plant cytoskeletons.

II. Categories of Cell Model Systems

There are many model systems for studying functions of cytoskeletons, and it is not possible to refer to all of them here. Therefore, I will attempt to categorize the model systems with some examples based on their structural aspects. This categorization should help readers understand general concepts of cell model systems and enable them to prepare such systems in their own work.

A. Cell Models

In order to analyze the function of cytoskeletons inside cells, the plasma membrane, which constitutes a barrier against external conditions, should be permeabilized. However, for understanding the function of cytoskeletons of whole cells, cells should retain their intrinsic morphologies even after permeabilization.

Permeabilized cells that retain their intrinsic morphologies sometimes show remarkable contraction upon addition of ATP (Hoffmann-Berling, 1954a). In some cases, movements or contractions are restricted to a portion of a cell, such as chromosome movement (Cande and Wolniak, 1978), cytokinesis (Hoffmann-Berling, 1954b), cytoplasmic streaming (Shimmen and Tazawa, 1983a), and axonemal movements of ciliate or flagellate cells (Gibbons and Gibbons, 1972). In order to study the organization of cytoskeletons throughout a cell, cells are permeabilized and purified cytoskeletal proteins are introduced into them to study the protein distribution and/or the regulatory mechanisms of the cytoskeletal organization (Asada *et al.*, 1991; Brinkley *et al.*, 1981; Snyder and McIntosh, 1975; Vantard *et al.*, 1990).

B. Isolation of Cytoskeletal Structures

Since Mazia and Dan (1952) succeeded in isolating spindles from sea urchin eggs, the isolation of functional and structural units consisting of cytoskeletons has become a powerful method. Spindles and centrosomes (Mitchison

and Kirschner, 1984) have been isolated from animal cells and spindles (Cande and McDonald, 1985; Yasuhara *et al.*, 1992), phragmoplasts (Kakimoto and Shibaoka, 1988), and cortical microtubules (Sonobe *et al.*, 1994) from plant cells. In some cases, the functions of the isolated organelles could be reactivated *in vitro*, for example, nucleation of microtubules (MTs) in isolated centrosomes (Mitchison and Kirschner, 1984) and spindle elongation (Cande and McDonald, 1985). These studies helped clarify some of the molecular mechanisms of these functions. Isolation of such organelles often facilitated identification of the protein(s) involved in their functions (Asada and Shibaoka, 1994; Toriyama *et al.*, 1988; Yasuhara *et al.*, 1992).

Interesting phenomena not observed in intact cells have often been observed in artificially modified cells. For example, in a cytoplasmic droplet isolated from *Characean* cells that was still "alive," motile fibrils and rotating chloroplasts were observed (Jarosh, 1956; Kamiya and Kuroda, 1957). Although these movements apparently differed from cytoplasmic streaming, the basic mechanism responsible for them was thought to be a mutually common one, namely, the motive force was generated by interactions between actin and myosin. Analyses of these "artificial" movements have yielded much information on the mechanism of cytoplasmic streaming (Kuroda, 1990). Analyses using isolated cytoplasm were also carried out for *Physarum* (Kuroda, 1979) and ameba (Taylor *et al.*, 1973). The motility of isolated (or naked) cytoplasm showed clearly that contractile elements existed in the endoplasm (sol) and that they could generate a motive force. Caffeine drops isolated from *Physarum* plasmodium were also used to study the regulatory mechanisms of cytoplasmic streaming (Sato *et al.*, 1981). Miniprotoplasts that are prepared from protoplasts of plant cells by eliminating vacuoles show cytokinetic cleavage and are classified in this category (see Section III.F).

C. *In Vitro* Reconstruction

A series of experiments using isolated chromosomes clarified the presence of motor proteins at the kinetochore (Hyman and Mitchison, 1991) and the significant roles of MT dynamics during mitosis (Coue *et al.*, 1991; Koshland *et al.*, 1988). Thus, *in vitro* reconstruction of cytoskeletal functions may allow us to explain the mechanism in "molecular" terms. Moreover, if a protein(s) responsible for the function of cytoskeletons can be purified, we may be able to reconstruct the function *in vitro* using only purified proteins . A typical example is the so-called "*in vitro* motility assay," in which sliding between actin and myosin or a MT and its motor proteins can be visualized (Kron and Spudich, 1986; Sheetz and Spudich, 1983a; Vale and Yano-Toyoshima, 1988). The reconstruction of centrosome-like

structures from which MTs radiated has been done using purified proteins (Toriyama *et al.*, 1988).

III. Cell Models of Plant Cells

A. Cytoplasmic Streaming

A subject that has been well documented among the roles of cytoskeletons of plant cells is cytoplasmic streaming. Because the details of the mechanism have been presented in many original papers and reviews (Kamiya, 1981; Kuroda, 1990; Shimmen and Yokota, 1994), I will only refer to the studies that employed cell model systems for examining cytoplasmic streaming.

1. Perfused Cells

The internodal cell of *Characeae* has played a central role in studies on the mechanism of cytoplasmic streaming. The cell has a large cylindrical shape and a large central vacuole. Kamiya and Kuroda (1955) first applied a perfusion technique to internodal cells and this technique was later improved (Tazawa, 1964). The perfused cells are still alive because only the vacuole is perfused. Although such vacuole-perfused cells have contributed to the study of vacuole functions (see Shimmen *et al.*, 1994), it was not thought to be an ideal cell model for studying the mechanism of cytoplasmic streaming because exogenously applied substances exerted their effects through the tonoplast. Subsequently, a perfused cell from which the tonoplast was removed was made by perfusing the cell with a solution that contained ethyleneglycol-bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) (Tazawa *et al.*, 1976; Williamson, 1975). In ATP-depleted tonoplast-free cells, movements of organelles that are tightly associated with actin bundles were reactivated upon addition of Mg-ATP (Williamson, 1975) and the movements were regulated by Ca^{2+} (Shimmen *et al.*, 1984). Tonoplast-free cells have also been used for detecting myosin activities by introducing organelles derived from heterogenous cells (Adams and Pollard, 1986; Kohno and Shimmen, 1988; Shimmen and Tazawa, 1982a; Sheetz and Spudich, 1983b), skeletal muscle myosin (Shimmen and Yano, 1984), and myosin-coated beads (Sheetz and Spudich, 1983a). Cytoplasmic streaming was also reactivated on an internodal cell that had been cut open longitudinally (Kuroda, 1983).

2. Permeabilized Cells

Another type of cell model of *Characeae* cells was a plasma membrane-permeabilized cell model. Shimmen and Tazawa (1983a) succeeded in per-

meabilizing *Characeae* cells by inducing rapid plasmolysis. When the cell is transferred from a solution with low osmolarity to one with high osmolarity in the presence of EGTA, the plasma membrane rapidly becomes detached from the cell wall and this causes breakage of the plasma membrane. To make the plasma membrane labile, EGTA must be added to both solutions and all treatments must be performed under cold conditions. As a result, ATP in the cytoplasm flows out and the cytoplasmic streaming stops. It can be reactivated by adding ATP. This method is considered to be useful for permeabilizing the plasma membrane of other plant species because it does not seem to require cells as large as *Characean* cells. In fact, successful permeabilization of the plasma membrane has been reported for potato tubular slices (Ponstein *et al.*, 1990), isolated and *in situ* mesophyl cells of Barley leaves, suspension-cultured cells of *Catharantus roseus* (Mimura and Shimmen, 1992), and protoplasts of suspension-cultured soybean cells (Saleem and Cutler, 1986), although these studies did not focus on the cytoskeleton. If the cell wall is partially digested and then permeabilized by osmotic change, it is also expected that large molecules such as proteins will be introduced. Permeabilization of *Characeae* cells using detergent (Shimmen and Tazawa, 1982a) and electroporation (Shimmen and Tazawa, 1983b) has also been reported.

3. Cytoplasmic Droplets

When one end of an internodal cell is cut, endoplasm flows out to form droplets that are surrounded by membrane. The cytoplasmic droplets display various patterns of movements; that is, moving motile fibrils and rotation of chloroplasts and nuclei (Jarosh, 1956; Kuroda and Kamiya, 1975), which are not seen *in vivo*. Also, the chloroplasts continue to rotate even after removal of the surface membrane of the droplets in the presence of Mg-ATP. Moreover, the chloroplasts are reactivated by heavy meromyosin prepared from skeletal muscle after suppression of the function of the putative *Nitella* myosin (Kuroda and Kamiya, 1975), indicating that their rotation was induced by interaction between actin and myosin. Movements of fibrils that were identified as actin filaments were also observed in squeezed cytoplasm (Higashi-Fujime, 1980).

4. Other Plants

There have been few reports employing cell models of other plant cells. Takata (1961) succeeded in reactivating the glycerinated model of *Acetabularia* stalk by Mg-ATP and Ca^{2+}. Cytoplasmic streaming in *Acetabularia* was also studied by employing perfused cell (Nagai and Fukui, 1985) and isolated cytoplasm *in vitro* (Menzel and Elsner-Menzel, 1989). La Claire

(1984) reported that a Triton-permeabilized cell model of the coenocytic green alga *Ernodesmis* showed remarkable cytoplasmic contraction, which was thought to reflect a motile reaction during wound healing of this alga *in vivo*, upon addition of ATP in the presence of 10^{-6} *M* Ca^{2+}.

B. Cortical Microtubules

Since "microtubules" were first found ultrastructurally by Ledbetter and Porter (1963) in the cortical region of pea epicotyl, the orientation of MTs in the cortical region, so-called cortical microtubules (CMTs), has been examined by electron and immunofluorescence microscopy (Gunning and Hardham, 1982; Williamson, 1991; Lloyd, 1987).

CMTs are thought to regulate cellular morphogenesis by controlling the direction of cellulose microfibril deposition on the outer surface of the plasma membrane (Delmer, 1987; Giddings and Staehelin, 1991), and their orientation and organization are regulated by many internal and external factors, such as plant hormones (Shibaoka, 1994), light irradiation (Iwata and Hogetsu, 1989; Laskowski, 1990; Zandomeni and Schopfer, 1993), progression of cell cycle (Ledbetter, 1967), aging (Hogetsu and Ohshima, 1986), and mechanical forces (Williamson, 1990). However, the molecular mechanism of CMT organization remains unsolved.

1. Perfused Cells

Wasteneys and Williamson (1989a) observed MT assembly in perfused *Nitella* internodal cells after introduction of biotinylated brain tubulin. Exogenous tubulin polymerized in the endoplasm to form MT bundles and some of them were associated with nuclei. In the cortical region, a limited number of MTs was observed at the site where a normal array of chloroplasts was retained, whereas a mass of MTs was observed at the edge of a region where chloroplasts had been removed, suggesting the presence of a factor that nucleated MTs at the cortical region. These results suggested the presence of sites responsible for nucleation of MTs in the cortical region. Chloroplasts that associated with the plasma membrane were thought to interfere with exogenous tubulin accessing the sites.

2. Membrane Ghosts

When protoplasts burst after attachment to the surface of polylysine-coated coverslips in a hypotonic solution, they leave fragments of the plasma

membrane on the coverslip (Figs. 1a and 1b). This technique was introduced by Marchant (1978) in studies of CMTs of the green alga *Mougeotia* and was based on a technique used for ameba of slime mold *Dictyostelium* (Clark *et al.*, 1975). Thus, the membrane ghosts have been thought to be a kind of model of CMTs because the CMTs on the ghosts can be manipulated directly. Kakimoto and Shibaoka (1986) have suggested a significant role of the plasma membrane in stabilizing CMTs. CMTs of membrane ghosts of *Mougeotia* were resistant against the m*M* level of Ca^{2+} but were depolymerized by Ca^{2+} after removal of the plasma membrane by treatment with Triton X-100. These results suggested the presence of a factor that conferred stability to CMTs on the plasma membrane. Kakimoto and Shibaoka also observed projections on MTs and cross-bridge structures between neighboring MTs, suggesting a significant role of microtubule-associated proteins (MAPs) in the organization of CMTs. Involvement of a transmembrane protein(s) in stabilization of CMTs was demonstrated by Akashi and Shibaoka (1991). The effect of Ca^{2+} and calmodulin on CMT stability was also reported in the membrane ghosts (Cyr, 1991).

CMTs on the membrane ghosts disappeared upon treatment with ATP (Sonobe and Shibaoka, 1990; Katsuta and Shibaoka, 1992). These results and those showing that the arrangement and stability of CMTs were affected by kinase inhibitors (Mayumi and Shibaoka, 1996; Mizuno, 1992) suggested the participation of protein phosphorylation in regulation of CMT organization. Phosphorylation of a 65-kDa plant MAP (Jiang and Sonobe, 1993) that was shown to be colocalized with CMTs by endogenous kinase was found in isolated CMTs (Yamamoto *et al.*, 1994, see below), but its function in CMT organization is not known.

An experimental system in which CMTs were reconstructed has been developed using both membrane ghosts and a cytoplasmic extract of miniprotoplasts of tobacco BY-2 cells (Sonobe and Takahashi, 1994). When CMTs on the ghosts (Figs. 1a and 1b) were removed by incubation with a high concentration (m*M* order) of Ca^{2+} at low temperature (Figs. 1c and 1d) and then CMT-free ghosts were incubated with a cytoplasmic extract of miniprotoplasts, MTs reappeared on the ghosts (Figs. 1e and 1f), but none did when the ghosts were preincubated with trypsin (Figs. 1g and 1h). These results indicated that on the ghosts there was the presence of a factor that facilitates association of MTs with the plasma membrane. The factor was thought to be a membrane-associated protein because a high concentration of KCl prior to incubation with the extract inhibited the reappearance of MTs on the ghosts. In comparison with the system employing the introduction of purified tubulin to a cell model, such as perfused *Characean* internodal cell (Wasteneys and Williamson, 1989a), the cytoplasmic extract is thought to be more complicated, but all components responsible for CMT organization are present. Wasteneys and Williamson

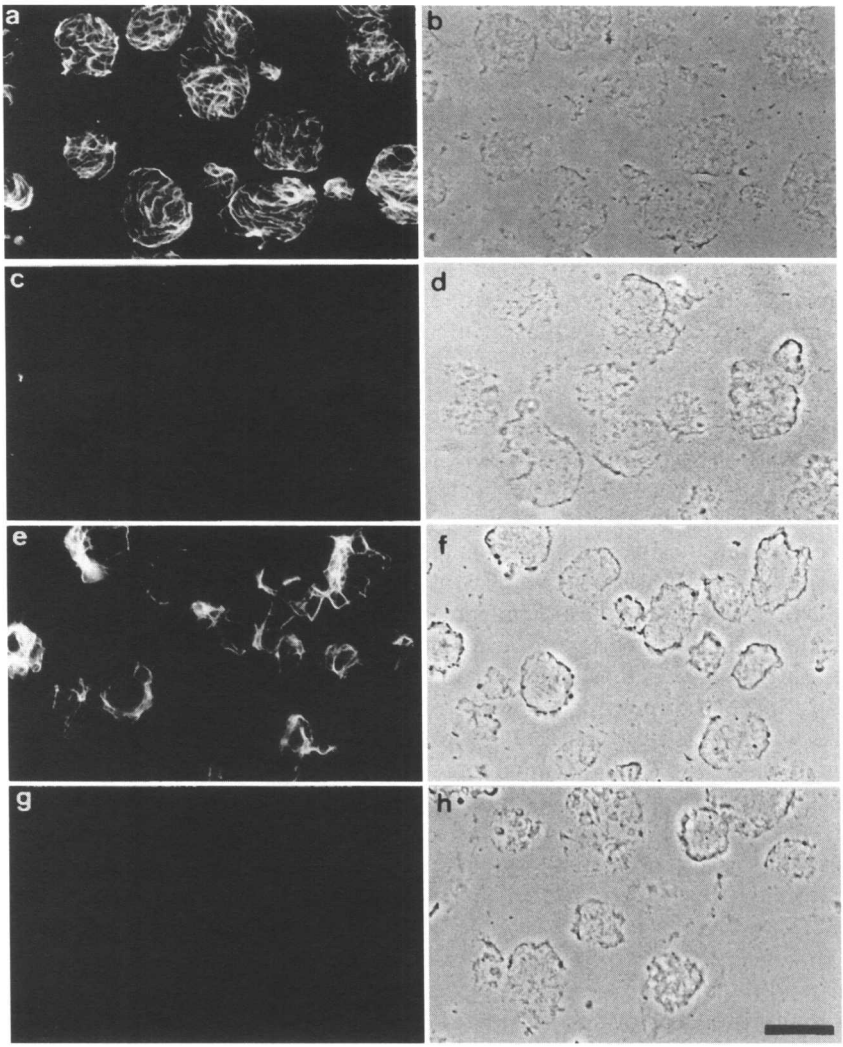

FIG. 1 Fluorescence micrographs of membrane ghosts of tobacco BY-2 cells. Freshly prepared membrane ghosts (a and b) were incubated with cold Ca^{2+}-containing solution to remove preexisting CMTs for 60 min (c and d) and then the ghosts were incubated with the cytoplasmic extract of miniprotoplasts for 10 min (e and f). MTs reappeared on the ghosts but not on the ghosts pretreated with trypsin prior to incubation with the extract (g and h). Fluorescent (a, c, e, and g) and phase-contrast images are shown. Bar = 20 μm.

(1989a) pointed out the possibility that reassembly of exogenous tubulin at the cortical region of the perfused *Nitella* cells without any branching, which had been observed in intact cells during recovery from disassembly of CMT with oryzalin (Wasteneys and Williamson, 1989b), should be due to the absence from purified tubulin of a factor that was essential for CMT rearrangement.

As has been repeatedly demonstrated by electron microscopy (Hardham and Gunning, 1978; Seagull and Heath, 1980; Lancelle *et al.*, 1986; Giddings and Staehelin, 1991), cross-bridge structures exist between CMT and the plasma membrane and between neighboring MTs. Although these structures are thought to play an important role in CMT organization, they have not been biochemically characterized. The system mentioned previously should be a powerful tool for biochemically identifying cross-bridge proteins by detecting their ability to reconstruct MTs on the ghosts.

3. Isolated CMTs

As described previously, in order to understand the mechanism of CMT organization, cross-bridges between CMTs and the plasma membrane and between neighboring MTs must be characterized biochemically. We first attempted to isolate cross-bridge proteins from membrane ghosts because the finding that KCl extraction of membrane ghosts inhibited attachment of MTs seemed to indicate that a high concentration of KCl could release cross-bridge proteins from the plasma membrane. However, there was difficulty in obtaining a sufficient amount of proteins from membrane ghosts. Therefore, we attempted mass isolation of CMTs (Sonobe *et al.*, 1994; Yamamoto *et al.*, 1994).

Protoplasts of tobacco BY-2 cells were gently homogenized in the presence of taxol and separated by centrifugation through a density gradient of Percoll. The uppermost layer in the centrifuge tube contained vesicles to whose membrane CMTs were attached (Fig. 2a). CMTs were isolated from these vesicles by solubilization of the membrane with Triton X-100. Cross-bridge structures remained associated with the isolated CMTs (Fig. 2b). After cycling of depolymerization and polymerization of the isolated CMTs, we found that four kinds of polypeptides (i.e., 200-, 120-, 80-, 65-kDa polypeptides) remained associated with MTs. The 65-kDa polypeptide was previously isolated by Jiang and Sonobe (1993).

C. Phragmoplast

Cytokinesis of higher plant cells occurs by the formation of a cell plate that centrifugally develops after mitosis. The cell plate is thought to be