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ACTIN AND MICROFILAMENTS IN CULTURED SOYBEAN CELLS

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by

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ABSTRACT

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The lateral mobility of a mouse monoclonal antibody, termed MVS-1, bound to a protein ($M_r \cong 480,000$) on the plasma membrane of protoplasts derived from the soybean (Glycine max) cell line SB-1, was determined by fluorescence redistribution after photobleaching. The diffusion coefficient of the antibody-antigen complex was $D \cong 3.2 \times 10^{-10}$ cm^2/s. Addition of the drugs cytochalasin B and colchicine, along with the soybean agglutinin pretreatment, caused a total restoration of mobility to the original value. These results suggested that the binding of soybean agglutinin to the plasma membrane of soybean protoplasts induces alterations in the cytoskeleton which, in turn, restrict the mobility of other plasma membrane molecules.

One of the structures hypothesized to be involved in the modulation phenomenon, the actin cytoskeleton, was found to be organized into well-defined microfilament networks in SB-1 cells and protoplasts. The microfilament networks, in all cases, consisted of: a) a network surrounding the nucleus; b) thick cables irradiating from the nuclear arrays; and c) a finer network extending toward the periphery of the cell. These networks contained filaments of 7 nm average diameter

which bound to the S-1 subfragment of myosin in an ATP-dependent manner.

The main component of the microfilament network, actin, was characterized and had the following properties:

(a) a polypeptide molecular weight of 45,000;

(b) an isoelectric point of 5.9 corresponding to the cytoplasmic γ isoform;

(c) peptide maps similar to those of rabbit muscle actin;

(d) immunological cross-reactivity with antibodies directed against calf thymus actin; and

(e) binding to deoxyribonuclease I.

These studies indicate that actin from SB-1 cells is very similar to the actins isolated from other sources, and that it is assembled into well-defined cytoskeletal networks capable of interacting with myosin molecules. This cytoskeleton is hypothesized to play a role as a submembranous structure that restricts the lateral mobility of plasma membrane receptors in soybean protoplasts after the endogenous lectin, soybean agglutinin, binds to the cell surface.

DEDICATION

TO THE LORD

For giving me inspiration, courage, and patience to obtain my goals.

TO MY WIFE AND DAUGHTER

For making my life wonderful all these years despite all the stressful times.

TO MY PARENTS

For their constant sacrifice in hopes of giving me an education, which provided me with an example to follow, and inspired me to pursue graduate study.

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AFC-12	5-N-(dodecanoyl)-aminofluorescein
ATP	adenosine-5'-triphosphate
ATPase	adenosine-5'-triphosphatase
BSA	bovine serum albumin
CB	cytochalasin B
COL	colchicine
Con A	concanavalin A
cpm	counts per minute
CT	calf thymus
D	diffusion coefficient
DEAE	diethylaminoethyl
DNAse	deoxyribonuclease
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid
F-actin	filamentous actin
FRAP	fluorescent redistribution after photobleaching
G-actin	globular actin
HLA	human leukocyte antigen
HMM	heavy meromyosin
Ig	immunoglobulin
kb	kilobase
mRNA	messenger ribonucleic acid
NBD-PC	1-acyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole) aminocaproyl phosphatidyl choline

NBD-PE	N-4-nitrobenzo-2-oxa-1,3-diazolephosphatidyl ethanolamine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pI	isoelectric point
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
S-1	subfragment 1 from myosin
SBA	soybean agglutinin
SDS	sodium dodecyl sulfate
T-PBS	tween-phosphate buffered saline
TX-100	triton X-100
WGA	wheat germ agglutinin

CHAPTER 1

LITERATURE REVIEW

LITERATURE REVIEW

Lateral Mobility of Plasma Membrane Components in Animal Cells

The "fluid mosaic" model (1) of the plasma membrane postulates that both lipids and proteins can undergo translational movement throughout the lipid matrix. The lateral motion of lipids within their own matrix is rapid when the lipids are in a fluid-like phase; however, they can also exhibit a restricted mobility whenever a transition into a gel-like phase occurs (2). Integral membrane proteins, defined operationally as those protein components that require detergent for extraction, can also exhibit lateral diffusion; the rate of diffusion depends on a number of factors such as the size of the protein, protein-protein interactions, and reversible anchorage to cytoskeletal structures.

Recent studies have shown that plasma membrane components can exhibit two distinct classes of lateral mobility: (a) lateral diffusion as a consequence of Brownian motion (3-5), and (b) directional flow on the cell surface (6,7). The initial demonstration of lateral mobility was provided by the classical experiments of Frye and Edidin (3) in which the fusion of mouse and human cells led to intermixing of the major histocompatibility antigens from the two species in the heterokaryon. More recently, the technique of fluorescence redistribution after photobleaching (FRAP) (8), has been used to measure the lateral mobility based on diffusional fluxes for a number of proteins (9), lipids (9), and glycolipids (10,11). In this technique, the cell plasma membrane is fluorescently labeled. A fluorescent microscope is

used to localize the cell and collect the emitted fluorescence. A small area on the cell membrane is then briefly bleached via a laser beam, resulting in a decrease in the monitored fluorescence at that particular spot. The gradual redistribution of fluorescent molecules into that area of the cell can be measured as a function of time and the resulting rate of recovery of fluorescence can be used to calculate a diffusion coefficient (D) (7,9,12). Typical values of diffusion coefficients have been found to range from 10^{-8} to 10^{-12} cm^2/s for proteins on membranes, and from 10^{-8} to 10^{-9} cm^2/s for membrane phospholipids and glycolipids. When a particular diffusing species of protein was observed to have an immobile component of diffusion (9), it was usually ascribed to interactions of the particular protein component with submembranous cytoskeletal structures (11-14).

Receptor-Cytoplasmic Interactions in Animal Cells

It is now believed that the directional movement of receptors occurs via an assembly of cytoplasmic proteins that altogether form a tripartite, metastable structure that consists of: a) certain surface receptors to which external ligands can bind; b) microfilaments and accessory proteins that can lead to the systematic movement of such receptors; and c) microtubules that are responsible for reversible anchorage of the receptors. The postulation of this type of mechanism for the movement of receptors arose from a number of studies that can be divided into four categories: a) the observation of receptor redistribution as exemplified by the phenomenon of patch and cap formation in lymphocytes (6), and its inhibition by the microfilament-disrupting drug cytochalasin B (15); b) studies in which the binding of the lectin Concanavalin A (Con A) modulated the mobility of cell surface receptors (16) and the reversal of this lectin-induced modulation by colchicine which disrupts microtubules (17); c) fluorescence and electron microscopic observation of microfilaments and microtubules that were co-localized with cell surface receptors after their redistribution (18-20); and d) the demonstration of the interaction between cytoskeletal components and plasma membrane proteins (21-27).

Directional flow was first studied in the phenomenon of patch and cap formation in mouse lymphocytes (6); after cross-linking surface receptors with ligands such as antibodies to surface immunoglobulin (Ig), the redistribution of the receptors into patches and caps could be inhibited by the addition of cytochalasin B (15). In addition, the

binding of the lectin Concanavalin A (Con A) to the lymphocyte surface was capable of inhibiting the patch and cap formation induced by the binding of anti-surface Ig (16). This modulation was later shown to be inhibited by the addition of the drugs, colchicine and cytochalasin B (17).

FRAP has been used to study the modulation by lectins of the mobility of cell surface receptors (28,29). Henis and Elson (28) measured the lateral mobility of surface Ig on lymphocytes in the presence of Con A-bound platelets. They found a 6-fold decrease in the lateral mobility (from 5.3×10^{-10} to 8×10^{-11} cm^2/s). Pretreatment of the cells with colchicine induced a restoration to 40% of the original D value; similarly, when cytochalasin B was used, a restoration to 30% of the original D was observed. The combination of both drugs reversed the Con A-induced modulation of mobility completely. The effect of Con A was propagated since it was independent of the distance of the receptors from the sites of the bound Con A-platelets. These results suggested the involvement of both microfilaments and microtubules in the Con A-induced modulation of mobility.

Although the mechanism by which the modulation of lateral mobility is still not completely understood, three possibilities were proposed:

a) The structure of the cytoskeleton remains unchanged while its specific interactions with the proteins are somehow enhanced.

b) The cytoskeleton changes to a state of higher affinity for the receptor, directly or through a linker molecule.

c) The stabilization of the cytoskeletal matrix, which results in a more rigid structure that restricts the mobility of other receptors.

Some evidence (30) suggests that the localized binding of ligands on cell surface receptors alters the viscoelastic properties of the membrane. Although these studies support model c, evidence for accumulation of actin, myosin, and tubulin beneath patches (18-20), and the demonstration of the direct interaction between cytoskeletal and external surface molecules (21-27) appears to favor models a and b. For example, electron microscopic studies have documented abundant microtubule arrays lying immediately beneath Con A-induced caps in ovarian granulosa cells (19). A similar observation has been reported by Yahara and Kakimoto-Sameshima (20) in which immunofluorescently-stained microtubules were found to co-migrate with caps induced by anti-surface Ig antibodies.

Finally, it has been shown that transmembrane proteins with cytoplasmic and external portions exist in the plasma membrane of cells (24,25,31-34). This type of topology immediately predicts a direct way in which signal-transduction mechanisms may occur through the interaction between surface molecules and cytoskeletal components. Experimental evidence suggests that this interaction may exist directly through actin (21,22,24-26) or via a mediating protein (35,36). In fact, it has been demonstrated that a number of transmembrane proteins such as the murine major histocompatibility complex (21), lymphocyte surface Ig (22), and a glycoprotein from mammary ascites microvilli (24,25), interact with actin. On the other hand, other transmembrane proteins may interact with the cytoskeleton in an

indirect way. For example, the cytoplasmic portion of Band 3 of the erythrocyte interacts with ankyrin (23), and a T-lymphoma transmembrane glycoprotein interacts with fodrin (37). Different cell systems seem to have a particular mode of membrane-cytoskeletal interaction (37); thus, different mechanisms of this interaction may be necessary to satisfy the specific structural and dynamic requirements for different cell functions.

Lateral Mobility of Proteins in the Plant Cell Plasma Membrane

Much of the evidence that led to our present understanding of receptor mobility has come from studies in which plant lectins such as Con A were used to induce and modulate the movement of mammalian cell surface receptors. Only recently has attention been paid to the interactions of plant-derived lectins with receptors of the plant cell.

Lateral diffusion coefficients (D) have been obtained by FRAP analysis for the lectins wheat germ agglutinin (WGA), soybean agglutinin (SBA), and Con A bound on the plasma membrane of protoplasts derived from the soybean cell line SB-1 (38). In addition, it has been demonstrated recently that SB-1 cells produce an endogenous lectin whose biochemical and immunological properties closely paralleled those of seed SBA (39). Immunofluorescence studies performed with an antibody raised against seed SBA showed that the SB-1 lectin can be found on the plasma membrane of soybean protoplasts. The diffusion coefficient of this endogenous membrane component has also been determined (40). The results from all these experiments, summarized in Table 1, yielded several important conclusions.

First, there is a class of "fast" moving receptors on the plasma membrane of the soybean protoplast with diffusion coefficients of 3 X 10^{-10} cm^2/s. The lectin WGA bound to its receptors, and anti-SBA bound to endogenous SB-1 lectin on the cell surface, represent this class of receptor mobility (Table 1). These results are similar to and comparable with mammalian cell systems, where a class of fast moving receptors (D values 1.2-6.9 X 10^{-10} cm^2/s) has been observed.

TABLE 1

Lateral Diffusion Coefficients of Lectin Probes on the Plasma Membrane of Soybean Protoplasts.

Probe	D x 10^{+10} (cm^2/s)	% Recovery[1]	Reference
"Fast"			
WGA	3.0	84	14,24
Anti-SBA IgG	2.5	55	16
"Slow"			
SBA	0.46	91	14,24
Con A	0.72	78	14,24

[1] Ratio of the mobile receptor population to the total receptor population (x 100) after photobleaching recovery.

Some examples include Fab fragments of anti-HLA antibodies bound on human lymphocytes (41), anti-surface Ig bound on mouse lymphocytes (28), and rabbit anti-IgE bound to IgE on mast cells (42).

Second, a class of "slow" moving receptors was also observed. SBA and Con A bound to their receptors on the plasma membrane of the protoplasts represent these "slow" moving species, yielding D values of 4×10^{-11} cm^2/s. These values are analogous to those obtained for Con A and succinyl-Con A bound to their receptors in a variety of mammalian cells such as mouse 3T3 fibroblasts (29), mouse embryo fibroblasts (43), chicken embryo fibroblasts (44), and rat myoblasts (5). The diffusion coefficients of these lectin-receptor complexes ranged from 0.6 to 4×10^{-11} cm^2/s. In conclusion, there is a clear similarity in the classification of lateral mobility of receptors in both plant and animal cell systems.

Modulation of the Lateral Mobility of Plasma Membrane Components in Plant Cells

In addition to the comparison of D values, there appears to be another striking similarity between animal cells and soybean cells in terms of the modulation of receptor mobility. Pretreatment of soybean protoplasts with SBA decreased the lateral mobility of surface-bound WGA to a "slow" value (from 3×10^{-10} to 5×10^{-11} cm^2/s, Table 2) (38). The addition of the drugs colchicine or cytochalasin B along with the SBA pretreatment partially returned the lateral mobility of surface-bound WGA to a "fast" value ($D = 1.3 \times 10^{-10}$ cm^2/s) (45) (Table 2). In contrast, the photoinactivated derivative of colchicine, lumicolchicine, which does not bind to microtubules (46), did not have any effect in reversing the SBA-induced modulation. These results are strikingly similar to those obtained for the effect of Con A on receptor mobility in animal cells (47). It is important to note that SBA is a protein endogenously produced by the soybean cell (39). Therefore, in this case, an endogenous protein was capable of modulating the dynamic properties of its own membrane.

The diffusion coefficient of endogenous SB-1 lectin, probed by rabbit anti-seed SBA, was 2.5×10^{-10} cm^2/s (Table 1). This D value is similar to that obtained with WGA bound on the soybean protoplast (Table 1), and to the mobility values obtained in mammalian cell systems for a number of antibodies bound on homogeneous surface antigens (28,41,42).

The D value of endogenous SB-1 lectin was considerably faster, however, than the D value of exogenously added SBA. In making such a

TABLE 2

Effect of Drugs on the SBA-induced Modulation of the Lateral Mobility of Lectin and Antibody Probes on the Plasma Membrane of Soybean Protoplasts (38,40).

Probe	Treatment	D x 10^{+10} (cm^2/s)	% Recovery[1]
WGA	SBA	0.46 (slow)	34
WGA	SBA Colchicine	1.3 (fast)	37
WGA	SBA Lumicolchicine	0.35 (slow)	36

[1] Ratio of the mobile receptor population to the total receptor population (x 100) after photobleaching recovery.

comparison, it should be noted that rabbit anti-seed SBA probes a single, defined component integral to the plasma membrane (SB-1 lectin) (39), whereas exogenously added SBA can bind to a large number of receptors, some of which could induce the modulation of receptor mobility.

In summary, the above results strongly suggest that exogenously-added seed SBA on the plasma membrane of soybean protoplasts may result in alterations of cytoskeletal structures which, in turn, restrict the mobility of other receptors such as those for WGA.

Lateral Mobility of Lipids in the Plant Cell Plasma Membrane

The lateral diffusion coefficients on the plasma membrane of soybean (SB-1) protoplasts of the fluorescent lipid analogs, N-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE), and 5-N-(dodecanoyl)-aminofluorescein (AFC-12), have been determined by the technique of FRAP (8) at 18°C (48). At low concentrations of NBD-PE (40 µg/ml), a single diffusion coefficient was obtained ($D = 2.6 \times 10^{-9}$ cm^2/s) (Table 3). When the protoplasts were labeled with a higher concentration of NBD-PE (100 µg/ml), two diffusing species of lipid were observed. The "fast" component had a D value of 2.8×10^{-9} cm^2/s (Table 3), similar to that obtained at 40 µg/ml. This component represented approximately 35% of the initial fluorescence (see % Recovery in Table 3). The new "slow" component had a D value of 0.46×10^{-9} cm^2/s, and corresponded to approximately 20% of the initial fluorescence. The remaining 40% of the probe was found to be immobile. Control experiments suggested that the "slow" component was not an artifact of the experimental procedures (48).

The "fast" component of lipid mobility is similar to D values obtained for lipid probes in a variety of mammalian cell systems where mobility values have ranged 10^{-8}-10^{-9} cm^2/s (9). On the other hand, the second component is below this range of values. Two possibilities were considered to explain the presence of the second "slow" component: (a) it represents a consequence of membrane flow (49) between the plasma membrane and the intracellular membranes; (b) it represents a true diffusion coefficient and suggests the presence of distinct, immiscible lipid domains in the plasma membrane of the protoplast, one

TABLE 3

Lateral Diffusion Coefficients of Lipid Probes in the Plasma Membrane of Soybean Protoplasts (48).

Probe	conc. µg/ml	First Component D x 10^{+9} cm^2/s	First Component % Recovery	Second Component D x 10^{+9} cm^2/s	Second Component % Recovery[1]	Total % Recovery[1]
NBD-PE	40	2.6	45	--------None--------		45
NBD-PE	100	2.8	35	0.46	21	56
AFC-12	40	22	71	--------None--------		71
AFC-12	100	25	69	--------None--------		69

[1] Ratio of the mobile receptor population to the total receptor population (x 100) after photobleaching recovery.

gel-like and one fluid-like. To distinguish between these possibilities, the mobility of a small lipid probe (12 carbon atoms), was analyzed. Previous reports (50-52) indicated that a small probe should partition preferentially into the fluid phase of lipid bilayers. When protoplasts, labeled with various concentrations of AFC-12, were subjected to FRAP, a single "fast" diffusion coefficient was obtained ($D = 25 \times 10^{-9}$ cm^2/s), even at high concentrations (Table 3). The fraction of probes which were mobile in the plasma membrane (~70%) was similar to that seen for the total mobile fraction of NBD-PE, which indicates that both probes sampled similar compartments. Thus, the data suggest that the second "slow" component of NBD-PE diffusion represents the diffusion of the probe in a gel-like domain of the plasma membrane.

A possible factor in the detection of two D values may be the higher steroid-to-phospholipid ratio, 1:1 in the plant membrane (53), as opposed to 0.36:1 found in 3T3 cells (54,55), and/or the substitution of stigmasterol, sitosterol, and campesterol for cholesterol as the major steroids in plant membranes (53). An increased sterol content will induce a more rigid (gel-like) structure (less ordered below and more ordered above the transition temperature) which would have a direct effect on the lateral mobility of components present in such domain. Because these conditions are not as prevalent in mammalian cells, restricted mobilities in small domains are most likely averaged when measured by FRAP, resulting in a single diffusion coefficient.

On the basis of the above results, it was proposed that the plasma membrane of soybean protoplasts is comprised of two distinct, immiscible gel- and fluid-like lipid domains at 18°C.

In the context of the previous analysis of cell surface components in soybean cells (38,40,45), these results suggest that certain plasma membrane receptors, such as those for WGA, have a higher affinity for more fluid domains, while receptors for soybean agglutinin prefer a more aggregated or ordered lipid environment. This is supported by the observation of a 6- to 7-fold variation in lipid mobility between the two mobile species. The physiological role of the lipid domains and their possible role in the process of ligand-receptor mediated endocytosis remain to be determined.

Cytoskeletal Structures in Plant Cells

Microfilaments are composed of double helical polymers of the monomer actin, whose molecular weight varies slightly from species to species but generally ranges from 42,000 to 45,000 daltons. Actin microfilaments can be readily identified under the electron microscope as filaments of 6-10 nm in average diameter. The interaction of these microfilaments with myosin proteolytic subfragments bearing the heavy chain, yields a characteristic complex with the appearance of arrowheads along the length of the filament (56,57, for reviews on microfilaments see 58-60).

Primary observations of a microfilament network in plants came from analyses of thin sections which revealed fine filamentous structures having an average diameter of 7 nm (61,62). These structures were subsequently confirmed to be actin microfilaments by the reversible binding of heavy meromyosin (HMM) and the subfragment 1 of myosin (S-1), which yielded the characteristic arrowhead decoration of the filament (63-72). In addition, the specific binding of phallotoxins to filamentous actin (F-actin) (73) has been used as a tool in the identification of microfilaments in plant cells (74-79).

Characean cells carry out extensive cytoplasmic motions (cytoplasmic streaming) that are responsible for the movement of particles and organelles (80). It has been shown that this phenomenon is brought about by the movement of myosin molecules over actin microfilaments (81). In Nitella, the presence of fibrillar structures attached to the moving chloroplasts were visualized by light (82) and electron microscopy (83). These structures were located parallel to the direc-

tion in which the streaming occurs. These fibrils were shown to consist of bundles of microfilaments of 5-6 nm in diameter (83), and they were composed of F-actin, as jugded by their ability to form arrowhead complexes with HMM and S-1 (63,84,85). Furthermore, the addition of cytochalasin B to the cells caused the streaming to cease (86).

Similarly, in higher land plants actin microfilaments have been found to be more abundant in tissues where cytoplasmic streaming occurs extensively (74). Parthasararhy and Mulethaler (61) examined elongating parenchyma cells in twelve species of plants and found that microfilaments were present at the periphery of these particular cells. In addition, they were oriented parallel to the longitudinal axis in the direction in which growth occurs. Metcalf et al. (72) found an increased amount of actin microfilaments in radicles and root tips of soybean seedlings and mature plants as compared with the cotyledons and petioles. Pesacreta et al. (74) found microfilament bundles in vascular parenchyma cells of conifer roots using a fluorescently-labeled phallotoxin. In contrast, other tissue types from the same plants such as the cortex, where cytoplasmic streaming was non-existent, did not possess the microfilament bundles. The presence of actin microfilaments has also been demonstrated in Vicia (67), Amaryllis (68), Haemanthus (69,70), Lycopersicon (71), and Trifolium (72).

In algae, cytoplasmic streaming and organelle orientation (87) occurs via an actomyosin system (81). In higher land plants, an analogous system may exist, although direct evidence of such a complex

has not been reported; only one report exists on the isolation of a myosin-like molecule from tomato endocarp tissue (71, 88).

A role of actin microfilaments in the mitotic process has also been proposed. Rhodamine-phalloidin staining has revealed actin microfilaments associated with the preprophase band of onion (75). An intricate array of microfilaments was observed in cycling suspension cultures of Medicago sativa and Vicia hajastana (76). Three types of microfilament populations interconnected with each other during interphase were observed: a) a peripheral network close to the plasma membrane; b) large subcortical cables; and c) a meshwork surrounding the nucleus. All these networks disappeared at the onset of mitosis and reappeared after cytokinesis. It was proposed that the nuclear associated meshwork (thereby termed as "nuclear basket") could be a center for microfilament initiation and/or organization. Traas et al. (77) observed similar arrays of F-actin in interphase cells of Daucus carrota suspension cultures. They demonstrated that the actin network does not disappear during mitosis, but rather organizes differently. The microfilament network was observed associated with the preprophase band, the mitotic spindle, and the phragmoplast. Schmit and Lambert (78) further showed that a cortical actin microfilament network is present throughout mitosis in endosperm cells of Haemanthus. It was proposed that this microfilament network acts as a deformable cage, and may help in the guidance of cytoplasmic vesicle transport to the nucleus during mitosis in higher plant cells.

Microtubules, the other main component of the cytoskeleton, are long hollow structures of 25 nm in diameter (for reviews see references 60,79,89). They are composed of the protein tubulin, which is

an α-β heterodimer of 110,000 molecular weight. Each subunit is found in a one-to-one ratio and are almost identical in molecular weight (M_r = 55,000 daltons).

Tubulin-like proteins have been isolated from a variety of plant tissues such as Vigna seedlings (90), fern sperm flagella (91), cultured tobacco cells, (92), and Paul's Scarlett Rose (93). The protein isolated in the latter case was capable of assembling into microtubular structures in vitro. Although it appears that the biochemical properties of plant tubulin closely resemble those of animal tubulin, it has been reported that tubulins from different plant species are immunologically unrelated among their α- and β-subunits, with the α-subunits having the most divergence (94). In addition, they showed differential binding of colchicine with respect to each other and, in general, less binding than that observed with bovine brain tubulin.

The observation of microtubules in plant cells both by electron and immunofluorescence microscopy has been documented extensively (reviewed in 60). Electron microscopic analyses of plant tissue have revealed arrays of microtubules underlying the plasma membrane and running parallel to cellulose microfibrils (95-97). This and the fact that upon treatment with anti-microtubule reagents this orientation is lost have led to the proposal that microtubules play a role in the guidance and orientation of cellulose microfibril deposition. This role however, has not been conclusively demonstrated. Besides cortical microtubules, cytoplasmic microtubules in higher plant cells have also been observed by indirect immunofluorescence (98-100). Cytoplasmic microtubules at different stages of the mitotic cycle have been

observed associated with the preprophase band, interphase arrays, the mitotic spindle, and the phragmoplast (60,79). Anti-microtubule reagents have been shown to induce abnormalities in cell division and differentiation in higher plants (60,101,102). Thus, they appear to play a role in these cellular processes as well.

Microtubules have also been proposed to be involved in cell elongation and growth. A current hypothesis on their organization at interphase proposes that these assemblies form dynamic helices. These helices could exist in a number of orientations according to the particular cellular stage (79). Support for this hypothesis arises from immunofluorescently-stained microtubules in the form of right- and left-handed helices along the length of cells. These arrays have been observed in onion and Urtica root hairs (104), cortical Raphanus cells (105), and cotton fibers (106). In addition, the disruption of the orientation of the helical pattern has been shown to result in abnormal growth (107).

Microfilaments and microtubules, the main cytoskeletal components, and their respective protein components actin and tubulin, have unquestionably been identified in plant cells. A detailed characterization of their biochemical properties should improve our understanding of cytoskeletal functions in plant cells.

Actin in Plant Cells

The monomeric component of microfilaments is the protein actin. Monomeric actin (G-actin) occurs mainly in three isoforms (α, β, and γ) which are identified by their isoelectric points (108). α-Actin (pI-5.6) is found exclusively in muscle cells, whereas β- (pI-5.8) and γ- (pI-5.9) actins are present in all non-muscle cells. Although these are the isoforms typically found in many species examined (109,110), other isotypes have also been reported. Chicken gizzard actin has been shown to have a slightly more basic pI than that of chicken brain γ-actin, whereas the main actin species from Physarum polycephalum was found to migrate with a slightly more acidic pI than that of rabbit muscle actin (109). When a high resolution two dimensional gel electrophoresis system was used to analyze actin from Physarum, it was found that the previously reported main species consisted of three closely spaced isoforms. In addition, a different new more basic isoform was found that differed by 0.4 pH units from the main actin triplet (111). Actins with more basic isoelectric points than the main α-, β-, and γ-isotypes have also been reported to occur in B5 nerve cultured cells (112). They were observed as unstable species with a lifetime of less than 2 hours, although it was acknowledged that they may be non-obligate precursors of the usual β- and γ-isoforms which lack the N-terminal acetylation. Finally, a single actin species from Tetrahymena pyriformis was reported to have a pI 0.2 pH units more basic than that of rabbit muscle actin, and a slightly higher molecular weight on sodium dodecyl sulfate polyacrylamide gels (M_r = 48,000) (113).

The isoelectric point of actin from plants has not been reported so far. Although it is well documented that actin is present in plants (60-79), the isolation and characterization of the actin polypeptide from plant tissues has been a difficult task. This is due to the low amounts present and/or the presence of potent proteases which are released as soon as the tissue is broken. Only a few reports describing a partial characterization of the molecule from plant tissue exist in the literature. Ilker et al. (114) isolated a molecule from wheat germ by gel filtration chromatography. Their material was identified as actin by the following criteria: a) solubility properties; b) co-migration of the polypeptide with rabbit muscle actin in SDS polyacrylamide gels; c) immunological cross-reactivity with anti-turkey gizzard actin antiserum; and d) ability to form needle-shaped fibrils similar to those observed from sea urchin actin. A molecule with the biochemical characteristics of actin has been isolated from tomato endocarp tissue (71,88) by ammonium sulfate fractionation and gel filtration chromatography. The protein exhibited a molecular weight of 42,000 daltons in SDS polyacrylamide gels. Before the column fractionation step, tomato extracts were shown to assemble into filaments of 6 nm in diameter capable of ATP-reversible binding with the S-1 subfragment of rabbit myosin. These extracts were also capable of activating the Mg^{2+} ATPase activity of the rabbit S-1 subfragments by 10-fold. After the column fractionation however, this ATPase activation decreased to 2.5-fold and the microfilament preparations were reported to be very labile. In a later report, Vahey (115) described the isolation of a protein of 72,000 daltons from tomato which inhibited the ability of F-actin to stimulate the low ionic

strength Mg^{2+} ATPase activity of the rabbit S-1 subfragment of myosin. This protein was hypothesized to be responsible for the low levels of ATPase activation by tomato actin found previously (71).

Metcalf et al. (116) isolated a protein from soybean seedlings after extraction in a low ionic strength buffer and ion exchange chromatography. The fraction containing material reactive with anti-calf thymus actin antibodies was further purified by affinity chromatography on a Sepharose column coupled with the same antibodies. The purified polypeptide comigrated with calf thymus actin in SDS polyacrylamide gels. In addition, ^{125}I-labeled fractions of anti-actin reactive material from the ion exchange column were immunoprecipitated with the same antibodies. The immunoprecipitated material was shown to contain a single major polypeptide with an electrophoretic mobility identical to that of calf thymus actin. Although the purified polypeptide was not used to prepare microfilaments, Metcalf et al. (72) have described in a different report the observation by electron microscopy of 7 nm diameter microfilaments from soybean seedling squashes. These microfilaments were capable of ATP-reversible binding with HMM to yield arrowhead complexes.

Taken together these results suggest that actin from higher plants shares the structural and functional properties of actin from animal cells and other eukaryotic organisms.

Despite the above-mentioned biochemical similarities of plant actin with other organisms, an increased divergence has been found between the deduced amino acid sequences obtained from genomic clones of soybean and maize actin, and the amino acid sequences of other actins (117,118). Nagao et al. (119) first reported the existence of

a small multigene family of actin-related sequences in soybean. Subsequently, Shah et al. (117,118) isolated actin genes from genomic libraries of maize and soybean, and their nucleotide sequences were determined. The deduced amino acid sequences from two soybean actin genes (named Sac1 and Sac3), and from one maize actin gene (Mac1) showed a high degree of homology but increased divergence with those of other eukaryotic actins such as mold, protist, and animal actins. For example, a recent report describing the deduced amino acid sequence of actin from Tetrahymena pyriformis showed a sequence homology of only 73% with soybean actin (113), the highest divergence for an inter-species actin reported so far. Previous to this report the highest divergence found in species other than plants was between yeast and Drosophila actin (86.4% amino acid sequence homology) (117).

Two members of the soybean actin gene family showed a difference of 27% in their nucleotide sequences. This intra-family divergence is also high when compared with the highest divergence reported previously for an actin gene family (15% divergence in nucleotide sequences for Drosophila actin genes (120). A distinct feature that was found in the plant actin genes analyzed was that their first nine amino acids at the amino terminal end were highly conserved even between soybean, a dicot, and maize, a monocot (118). The corresponding sequences in distant animal actin genes are much less conserved. These data suggest that the actin genes from higher plants diverged from a common ancestral gene. The actin(s) arising from these genes may have a certain unique function in the plant.

The increased differences in plant actin gene structure and their unique N-terminal sequences, prompted investigators to look for a

differential expression of these genes (121). Hybridization studies on the six soybean actin genes SAc1, SAc2, SAc3, SAc4, SAc6, and SAc7 indicated the following: a) 2 genes (SAc6 and SAc7) were the most highly expressed accounting for 80% of the actin mRNA; b) SAc3 and SAc1 were found to be expressed at intermediate and low levels, respectively, whereas SAc2 and SAc4 were found to be expressed at extremely low levels; and c) SAc6 was expressed at the same level in root, shoot, and hypocotyl, whereas SAc3 and SAc7 were more highly expressed in shoot than in root and hypocotyl. In all three organs analyzed, SAc3 and SAc1 produced 1.7-kb size transcripts. In Drosophila (122), at least three actin genes produced 1.7-kb size transcripts, two genes produced 1.6- and 1.75-kb size transcripts, and one gene produced 1.7-, 1.95-, and 2.2-kb size transcripts. This indicated that in all cases, the transcripts contained a significant amount of flanking sequences (~1.1-kb are needed for the actin polypeptide alone).

Root, shoot, and hypocotyl contain vascular, cortical, and epidermal tissues, that are continuously undergoing cytoplasmic streaming, and actin has been found in more abundance in these tissues (61,74). It was proposed that the two soybean actin genes that represented the majority of the expressed mRNA (SAc6 and SAc7) might be specific for vascular tissue although no evidence was provided. Therefore, it is still unknown how each of the actin genes relate to the different plant functions in which actin has been implicated such as structure, mitosis, cytoplasmic streaming, and receptor-cytoplasmic interactions.

In conclusion, the actin molecule from plants remains poorly characterized. Although it is similar to its animal counterpart, it is one of the most divergent forms of actin. Based on fragmentary evidence, at least some of its biochemical properties parallel those of animal actin; i.e. similar molecular weight, morphology of assembled microfilaments, specific binding of these microfilaments by phallotoxins and myosin fragments, and immunological cross-reactivity. A more complete characterization of the molecule will shed more light into its similarities and/or differences with animal actin. Furthermore, if indeed the similarities are greater than the differences, the search for its regulatory proteins (reviewed in 123) can be initiated.

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CHAPTER 2

LATERAL MOBILITY OF SURFACE-BOUND MONOCLONAL ANTIBODY MVS-1 ON SOYBEAN PROTOPLASTS

ABSTRACT

Monoclonal antibodies were prepared against protoplasts from soybean (Glycine max) suspension cultures (SB-1 line). One particular antibody, termed MVS-1, bound to a defined protein (M_r = 480,000) on the outer surface of the plasma membrane of the protoplast. The diffusion coefficient of the monoclonal antibody was determined by fluorescence redistribution after photobleaching (D = 3.2 x 10^{-10} cm^2/s). The binding of soybean agglutinin to the protoplasts reduced the lateral mobility of antibody MVS-1 ten-fold (D = 4.1 x 10^{-11} cm^2/s). Soybean agglutinin did not interact with the monoclonal antibody or its antigenic target, ruling out cross-linking between these molecules and the lectin to a set of immobile receptors. When cytochalasin B or colchicine were added along with the soybean agglutinin, the lectin-induced modulation was partially reversed. When both drugs were added together, the lectin-induced modulation of mobility of the antibody-antigen complex was totally reversed. These data are similar to our previous observations on the modulation by soybean agglutinin of the mobility of wheat germ agglutinin receptors. The present study represents a more refined analysis to the level of a single defined receptor on the membrane. These results further suggest that the binding of soybean agglutinin to the plasma membrane of the soybean protoplast induces an altered cytoskeletal structure which, in turn, restricts the mobility of other plasma membrane molecules.

INTRODUCTION

The binding of soybean agglutinin (SBA) to protoplasts derived from a cultured cell line, SB-1, resulted in a decrease in the lateral diffusion coefficient of fluorescently-labeled wheat germ agglutinin (WGA) bound to the plasma membrane of the same cells (1). The lateral mobility of the plasma membrane components was determined using the technique of fluorescence redistribution after photobleaching (FRAP). Because lectins bind to a heterogeneous population of receptors on the cell surface, the lateral diffusion coefficients (D values) obtained for WGA-receptor complexes probably reflect ensemble averages rather than the mobility of a single diffusing species on the membrane. Thus, it was not known whether the decrease in mobility was due to: a) a decrease in the number of fast-moving receptors; b) an increase in the number of slow-moving receptors; or c) an intrinsic change in the mobility of all the receptors.

We have refined these studies on the effect of SBA by the use of a monoclonal antibody designated MVS-1, that binds to a defined component (M_r = 480,000) on the outer surface of soybean protoplasts (2). We now report the analysis of the lateral mobility, and its modulation by SBA, of this antibody-antigen complex in the plasma membrane of soybean protoplasts. This lectin-induced modulation appears to be mediated by the cytoskeletal components of the plant cell.

MATERIALS AND METHODS

Cell Culture and Protoplast Isolation

The SB-1 cell line of soybean (3) was kindly provided by Dr. G. Lark (Department of Biology, University of Utah, Salt Lake City, UT) and was grown in the dark as suspension cultures. Protoplasts were prepared by resuspending a certain volume of 2-day-old cells with fresh 1B5-C culture medium (4), and diluting this suspension with an equal volume of a solution containing 1 mg/ml pectinase (Sigma, St. Louis, MO), 2 mg/ml Cellulysin (Calbiochem, La Jolla, CA), and 100 mg/ml sorbitol (Sigma), pH 5.5. This mixture was incubated for 2 h at 25°C and filtered through a 48 μm nylon mesh. The filtered protoplasts were washed by centrifugation (460 g for 5 min) and resuspension in Modified Gamborg's buffer (4).

Binding of Lectins and Antibody Probes to Protoplasts

The generation and characterization of the monoclonal antibody MVS-1 and its Fab fragment have been described in detail (2,5). The preparation and labeling of the fluorescent reagents have also been described.

Protoplasts (10^6/ml) were incubated for 1 h at 25°C with the fluorescent probe in 50 mM Tris, 10 mM $CaCl_2$, 0.55 M Sorbitol, pH 7.5 (buffer A), followed by centrifugation and resuspension in equal volumes of the same buffer. The sequential binding of lectin and fluorescent antibody was done by pretreatment of the protoplasts with 50-250 μg/ml SBA or 250 μg/ml WGA for 1 h at 25°C. Washing and labeling with the fluorescent antibody were performed as above.

For indirect immunofluorescence, 5 x 10^8 protoplasts resuspended in buffer A were incubated in separate test tubes for 5 h at 25°C with one of the following: a) 50 µg/ml rhodamine-conjugated antibody MVS-1; b) a 1:1 dilution of the culture supernatant from clone 5 (2), followed by a 1:30 dilution of rhodamine-conjugated rabbit anti-mouse IgG (Cappel Laboratories, Cochranville, PA); or c) a 1:30 dilution of rhodamine-conjugated rabbit anti-mouse IgG (Cappel Laboratories). The incubations were followed by washing of the protoplasts 4 times in the same buffer. The fluorescent antibody-treated protoplasts were deposited on microscope slides and visualized under a Leitz (Wetzlar, FRG) fluorescence microscope equipped with a Leitz KP580 dichroic mirror.

Human platelets were coated with SBA (5) using paraformaldehyde as the fixative. For photobleaching experiments, 2 X 10^8 SBA-coated platelets were incubated with 5 X 10^5 protoplasts for 1 h at 25°C, followed by washing and labeling with the fluorescent antibody as described above.

Drug Treatments

Protoplasts were also pretreated with certain drugs that affect cytoskeletal structures. The cells (10^6) were preincubated with 1 µM colchicine (COL, Sigma Chemical Co.), and/or 10 µg/ml cytochalasin B (CB, Sigma Chemical Co.), for 30 min at 25°C. After washing, the protoplasts were treated with lectin or antibody as described above but the concentration of the drugs was maintained throughout. Binding of the fluorescent lipid, 1-acyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole)-

aminocaproyl phosphatidylcholine (NBD-PC, Avanti Polar Lipids) to soybean protoplasts was carried out as described (5).

FRAP Analysis

Analysis of the lateral mobility of fluorescent probes on the plasma membrane of soybean protoplasts was determined by the technique of FRAP (6). The preparation and analysis of samples were carried out as described previously (1,6).

Tests for MVS-1 Antibody and SBA Interaction

The possibility that SBA interacts with antibody MVS-1 was explored by the following experiments. First, ^{125}I-labeled MVS-1 antibody (100 µg, 8 x 10^6 cpm) was applied to a Sepharose 4B (Sigma) column (50 x 1.1 cm), previously equilibrated with 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5, and eluted with the same buffer. A similar experiment was carried out using a mixture of ^{125}I-labeled antibody MVS-1 (100 µg, 8 x 10^6 cpm) preincubated for 30 min with 250 µg SBA in 1 ml 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5, prior to the column fractionation. The position of elution of the main peaks of radioactivity was determined by gamma counting (LKB-Wallac Riagamma, Turku, Finland). Second, 10 mg SBA were coupled to cyanogen bromide-activated Sepharose beads (7) in a final volume of 5 ml. ^{125}I-Labeled MVS-1 antibody (50 µg, 4 x 10^6 cpm) in phosphate-buffered saline (5 mM sodium phosphate, 130 mM NaCl, pH 7.2) (PBS) was applied to the column. After extensive washing with PBS, the column was eluted with 0.2 M galactose in PBS.

Tests for the Interaction Between the Antigenic Target of MVS-1 Antibody and SBA

An "antigen enriched" fraction (2) was prepared from 10^8 soybean protoplasts. Briefly, soybean protoplasts were frozen at -80°C and then thawed to lyse the cells. The lysate was separated into soluble and insoluble fractions by centrifugation at 15,000 g. The insoluble material was extracted with 50 mM potassium phosphate, 0.13 M KCl containing 0.5% (v/v) Triton X-100 (TX-100, Research Products, Int., Elk Grove, IL) in a water bath sonicator for 15 min at 25°C. The TX-100 extracted material was separated into a soluble and insoluble fraction by centrifugation at 15,000 g. The TX-100 soluble material was treated with 5 volumes of isoamyl alcohol to eliminate the detergent (8). The aqueous fraction was shown to contain an enriched amount of the antigenic target of antibody MVS-1 (2) and was termed "antigen enriched" fraction. This fraction was loaded onto an affinity column of SBA coupled to Sepharose beads and treated as above. The unbound, and the galactose-eluted fractions were concentrated in an ultrafiltration device (Amicon Corp., Danvers, MA) using a PM-10 membrane (Amicon Corp., molecular weight cutoff of 10,000). The concentrated fractions were tested for antibody MVS-1 binding activity using a "solid phase" binding assay as described (2), with the exception that Immulon-2 plates (Dynatech, Alexandria, VA) were used. Alternatively, after passing the "antigen enriched" fraction onto the SBA-Sepharose column, ^{125}I-labeled antibody MVS-1 (10^7 cpm) was also passed through and the column washed. The presence of radioactivity was determined in the following fractions: a) unbound material; b) 0.2 M galactose eluent; c) 0.2 M galactose, 0.2 M NaCl eluent; and d)

0.1 M citrate, pH 3.0 eluent. Control experiments were carried out using the same procedure as above, but an underivatized Sepharose 4B column was used instead.

Analytical Procedures

The procedure of Lowry et al. (9) was used to determine protein concentration, with bovine serum albumin (BSA) as a standard.

RESULTS

Lateral Mobility of Antibody MVS-1 on Soybean Protoplasts

Antibody MVS-1 bound to the plasma membrane of SB-1 protoplasts; the fluorescently labeled antibody bound on the soybean protoplasts yielded a ring-like staining, characteristic of a diffuse distribution of the label over the surface of the spherical cell (Fig. 1A and 1B). In contrast, no staining was observed when rhodamine-conjugated rabbit anti-mouse IgG (Fig. 1C) or hybridoma clone 5 supernate followed by rhodamine-conjugated rabbit anti-mouse IgG (Fig. 1D) were used for the incubations. Preliminary results indicate that hybridoma clone 5 is directed against an epitope inside the protoplast (2); therefore, this monoclonal antibody would not be expected to stain intact protoplasts. In some cases, there was also appreciable internal labeling of the protoplasts stained with antibody MVS-1 (Fig. 1B).

The lateral mobility of antibody MVS-1 bound on the plasma membrane of the protoplasts was measured by FRAP, carried out on individual non-agglutinated cells (Table 1). The D value obtained (3×10^{-10} cm^2/s) remained constant over the range of concentrations tested using native immunoglobulin. Furthermore, the same value was observed when monovalent Fab fragments of MVS-1 antibody were used as the fluorescent probe (Table 1). These results indicate that the mobility of antibody MVS-1 belonged to the relatively "fast" class of receptors, exemplified by WGA in a previous analysis (1).

Figure 1. Immunofluorescent staining of SB-1 protoplasts by rhodamine-labeled antibody MVS-1. Soybean protoplasts (5×10^8) were incubated for 5 h at 25°C with: (A and B) 50 µg/ml of rhodamine-conjugated antibody MVS-1 (4); (C) a 1:30 dilution of rhodamine-conjugated rabbit anti-mouse IgG; and (D) a 1:1 dilution of the culture supernatant from hybridoma clone 5 (2), followed by a 1:30 dilution of rhodamine-conjugated rabbit anti-mouse IgG for 2 h. Each incubation was followed by 4 washes in buffer A to remove non-specific binding. The bar represents 25 µm. Phase contrast micrographs are denoted as pH and fluorescence micrographs as fl.

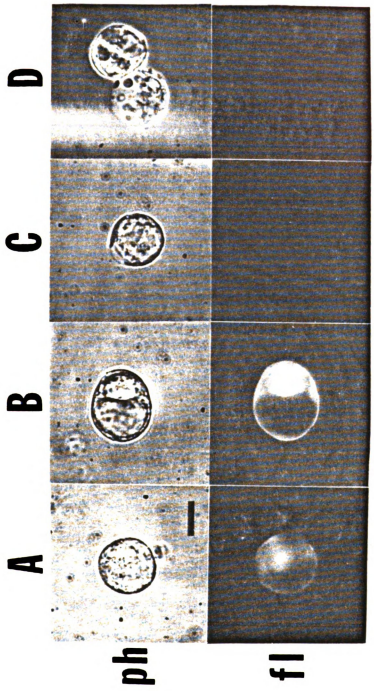

Figure 1

TABLE 1

Lateral Diffusion Coefficients of Fluorescent Antibody MVS-1 and its Fab Fragment on the Plasma Membrane of Soybean Protoplasts.

Probe	Concentration (μg/ml)	$D^1 \times 10^{+10}$ (cm^2/S)	% Recovery[1,2]
Ig	50	3.5 ± 1.6	55 ± 33
	100	2.7 ± 1.3	61 ± 35
	250	3.5 ± 2.1	52 ± 24
Fab	440	3.0 ± 1.1	70 ± 15

[1] Values are expressed as mean ± standard deviation.

[2] Ratio of the mobile receptor population to the total receptor population (x 100) after photobleaching recovery.

Modulation by SBA of the Mobility of Antibody MVS-1

When soybean protoplasts were preincubated with SBA, the lateral mobility of antibody MVS-1 decreased about 10-fold (Table 2). Lower concentrations of SBA (5 μg/ml) did not show the modulatory effect. This effect was specific since the same pretreatment using WGA did not cause any variation in the D value of antibody MVS-1 mobility (Table 2). In addition, SBA did not have any effect on the mobility of a fluorescent phospholipid probe bound on the soybean protoplasts.

The modulatory effect on the lateral mobility of antibody MVS-1 produced by localized binding of SBA on a small region of the protoplast surface was investigated using SBA-derivatized platelets. The SBA-platelets bound in a random distribution on the protoplasts but covered only small areas of the cell surface (Fig. 2A, 2B). When the protoplasts were treated under these conditions, the lateral mobility of surface-bound antibody MVS-1 yielded a value of $D = 3.8 \times 10^{-11}$ cm^2/s (Table 2). This value was similar to that given by treatment of the protoplasts with soluble SBA (Table 2). These results demonstrated that similar modulatory effects on the lateral mobility of antibody MVS-1 were produced with both locally bound and soluble SBA.

Immunoglobulins contain carbohydrate in their heavy chains. Therefore, it was important to rule out the interaction between the antibody MVS-1 and SBA. The antibody did not bind to an affinity column derivatized with SBA. In addition, we have also carried out gel filtration analyses of the radiolabeled antibody in the presence and absence of SBA. The position of elution of ^{125}I-labeled antibody MVS-1 was the same under both conditions, showing a main major peak at $M_r = 150,000$ (Fig. 3). This argues against the possibility that

TABLE 2

Effect of SBA on the Lateral Mobility of Antibody MVS-1 Bound on the Plasma Membrane of Soybean Protoplasts.

Probe	Concentration (µg/ml)	Pretreatment	$D^1 \times 10^{+10}$ (cm^2/S)	% Recovery[1,2]
Ig	100	---	3.5 ± 1.6	55 ± 33
Ig	100	5 µg/ml SBA	3.5 ± 1.4	57 ± 16
		50 µg/ml SBA	0.42 ± 0.17	30 ± 19
		250 µg/ml SBA	0.41 ± 0.18	29 ± 7
Ig	100	SBA platelets	0.38 ± 0.18	32 ± 8
Ig	100	250 µg/ml WGA	2.9 ± 1.1	58 ± 23
Fab	450	5 µg/ml SBA	4.1 ± 1.7	59 ± 17
		250 µg/ml SBA	0.40 ± 0.10	28 ± 10
NBD-PC	40	--	65 ± 9	68 ± 24
		250 µg/ml SBA	47 ± 14	62 ± 28

[1] Values are expressed as mean ± standard deviation.

[2] Ratio of the mobile receptor population to the total receptor population (x 100) after photobleaching recovery.

Figure 2. Phase contrast micrographs of SB-1 protoplasts showing SBA-platelets bound on their surface. Human platelets were coated with SBA using paraformaldehyde as the fixative (5). The arrows indicate the position of SBA-platelets. The bar represents 25 μm.

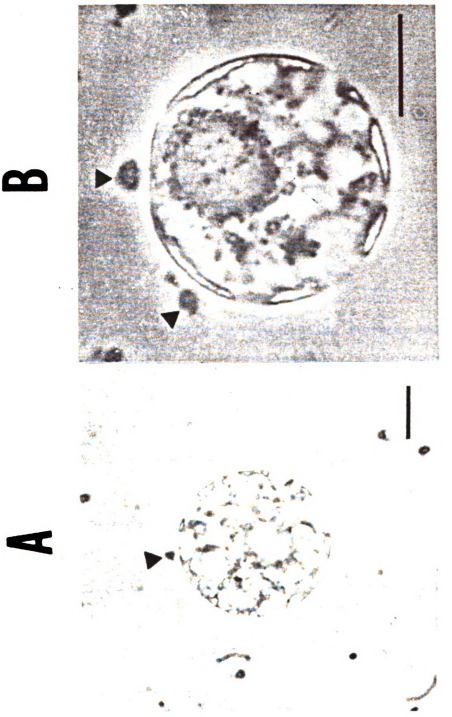

Figure 2

Figure 3. Elution profile of ^{125}I-labeled antibody MVS-1 in the absence (A) and presence (B) of unlabeled SBA on a column of Sepharose 4B. The column (50 x 1.1 cm) was equilibrated and eluted with 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5, at 25°C. In B, ^{125}I-labeled antibody MVS-1 (100 g, 8 x 10^6 cpm) was incubated with 250 µg SBA for 30 min in 1 ml of the equilibration buffer before loading to the column. The arrows indicate the positions of elution of the molecular weight markers: blue dextran (void volume), apoferritin (480,000), catalase (270,000), immunoblogulin G (150,000), and total volume of the column.

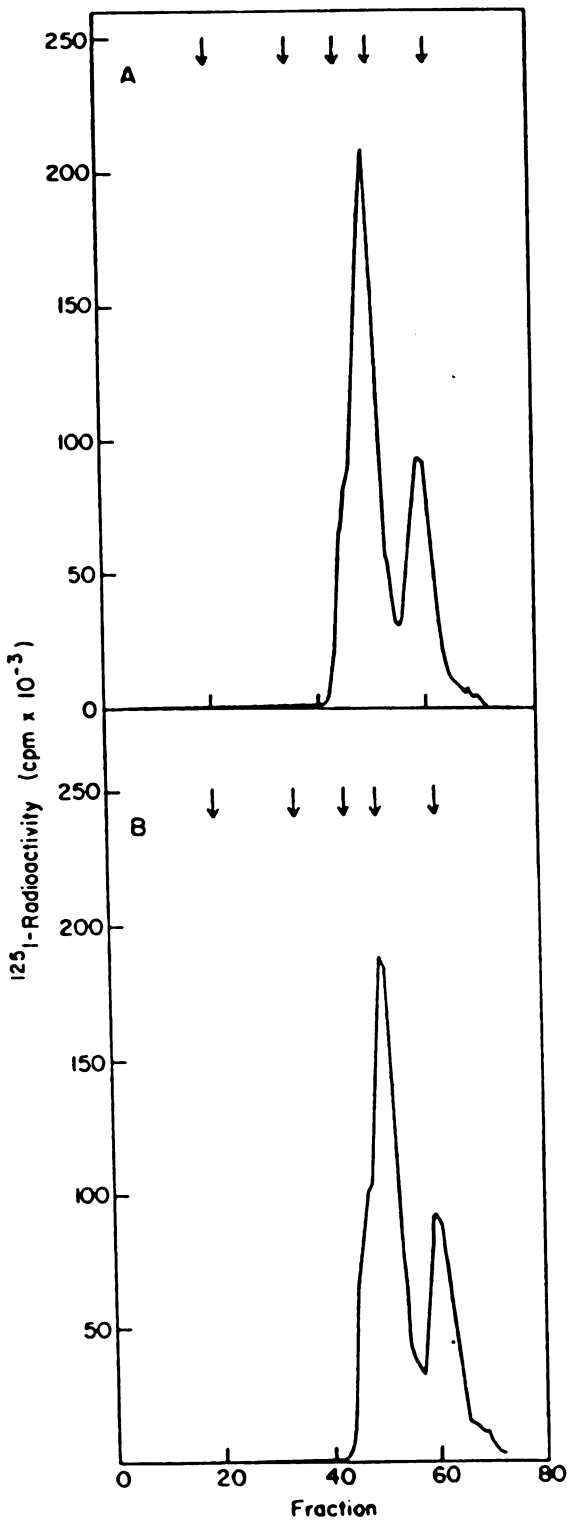
A
B
250
200
150
100
50
0
^{125}I-Radioactivity (cpm x 10^{-3})
0
20
40
60
80
Fraction

Figure 3

cross-linking among the lectin, the antibody, and/or a set of slow-moving receptors causes the decrease in the lateral mobility of the antibody-antigen complex. This conclusion is further supported by the observation that SBA reduced the D value of monovalent Fab fragments of antibody MVS-1, which contain no carbohydrate (Table 2).

In addition, we have carried out experiments to test for the interaction between the antigenic target of the antibody MVS-1 and SBA. A fraction prepared from SB-1 protoplasts previously shown to be enriched in the antigenic target of the antibody (2) was passed over an affinity column derivatized with SBA and the bound material was eluted with galactose. The presence of the antigenic target of antibody MVS-1 was analyzed by a solid phase binding assay (2) on the bound and unbound fractions. The unbound material was shown to retain MVS-1 binding activity (Table 3). Furthermore, when the column was eluted with galactose, no MVS-1 binding activity was detected in the eluate. Finally, the "antigen enriched" fraction was passed through a SBA-Sepharose affinity column, followed by ^{125}I-labeled antibody MVS-1. Essentially all the radioactivity was recovered in the material that did not bind to the column. Fractions eluted with: a) 0.2 M galactose; b) 0.2 M galactose and 0.2 M NaCl; and c) 0.1 M Citrate, pH 3.0, contained little or no radioactivity. The above results indicate that the decrease in the lateral mobility of the antibody MVS-1 by SBA does not result from the cross-linking by SBA of the antigenic target of the antibody MVS-1 to an immobile component.

TABLE 3

Test for the Binding of the Antigenic Target of Antibody MVS-1 to SBA-Sepharose*

Sample	Binding of Antibody MVS-1	Binding of Normal Mouse Immunoglobulin	Specific Binding†
Unbound Material	1,234	103	1,131 ± 54
Galactose Elution	182	187	0
Sepharose Column Unbound Material	1,169	124	1,044 ± 18

* The samples were deposited in wells of microtiter plates, and the binding of antibody MVS-1 was quantitated by the "solid phase" binding assay described (2).

† Specific binding represents binding of normal mouse immunoglobulin subtracted from the binding of antibody MVS-1. The data are given in cpm and represent the averages of triplicate determinations ± standard deviation.

Effect of Cytoskeletal Drugs on SBA Modulation of Mobility

Preincubation of the protoplasts with either COL or CB along with SBA resulted in a partial reversal of the modulatory effect induced by SBA (from 4.1 X 10^{-11} to 1.3 X 10^{-10} cm^2/s) (Table 4). The reversal by each drug alone did not restore the initial value of antibody MVS-1 mobility (Table 1). However, the addition of both drugs in the pretreatment completely abolished the SBA modulatory effect (D = 4.2 X 10^{-10} cm^2/s). The D value of MVS-1 mobility obtained under these conditions was similar to that obtained without SBA or drugs (Tables 1,4). Lumicolchicine, a photoinactivated derivative of COL that does not disrupt microtubules, did not show any reversal of the SBA modulatory effect on the mobility of antibody MVS-1. Finally, the addition of COL and/or CB to untreated protoplasts did not have any effect on the mobility of antibody MVS-1. These results suggest that the binding of SBA to the plasma membrane of the soybean protoplast alters other plasma membrane components, resulting in a restriction of the mobility of certain receptors such as those for antibody MVS-1 and WGA.

TABLE 4

Effect of Drugs on the SBA-induced Modulation of Lateral Mobility of Antibody MVS-1-Bound on the Plasma Membrane of Soybean Protoplasts.

Probe	Concentration (µg/ml)	Treatment	$D^1 \times 10^{+10}$ (cm^2/S)	% Recovery[1,2]
Ig	100	---	3.5 ± 1.6	55 ± 33
		250 µg/ml SBA	0.41 ± 0.18	29 ± 7
		1 µM COL 250 µg/ml SBA	1.3 ± 0.1	39 ± 21
		1 µM lumicolchicine 250 µg/ml SBA	0.50 ± 0.17	24 ± 6
		10 µg/ml CB 250 µg/ml SBA	1.3 ± 0.4	54 ± 11
		1 µM COL 10 µg/ml CB 250 µg/ml SBA	4.2 ± 1.5	46 ± 18
SBA	250	1 µM COL	0.70 ± 0.19	81 ± 20
		10 µg/ml CB	0.49 ± 0.11	78 ± 18
		1 µM COL 10 µg/ml CB	0.64 ± 0.23	73 ± 12
WGA	250	1 µM COL	3.7 ± 0.9	75 ± 19
		10 µg/ml CB	4.0 ± 1.2	80 ± 15
		1 µM COL 10 µg/ml CB	3.1 ± 0.7	72 ± 11

[1] Values are expressed as mean ± standard deviation.

[2] Ratio of the mobile receptor population to the total receptor population (x 100) after photobleaching recovery.

DISCUSSION

The value of the diffusion coefficient of MVS-1 antibody bound on the surface of soybean protoplasts was determined to be $D = 3 \times 10^{-10}$ cm^2/s, corresponding to a relatively "fast" class of mobility as defined by a previous analysis of the lateral mobility of lectins (1). This value did not vary below (50 μg/ml) or above (100-250 μg/ml) saturating concentrations of the antibody (2), suggesting that cross-linking of several antigen molecules by the antibody did not lead to the formation of large immobile patches on the cell surface. These results are further supported by the fact that the same D value was obtained when monovalent Fab fragments of antibody MVS-1 were used for the analysis.

It has been previously reported that pretreatment of SB-1 protoplasts with SBA resulted in a decrease of the mobility of surface-bound WGA (1) and that COL caused a partial reversal of this modulatory effect by SBA (10). Because lectins are known to bind to a heterogeneous population of receptors on the cell surface (11), it was possible that the SBA modulatory effect was caused by: (a) a decrease in the number of fast moving receptors, (b) an increase in the number of slow moving receptors, or (c) a decrease in the intrinsic mobility of all receptors. To obviate these difficulties in interpretation, a similar but more refined analysis was carried out using a monospecific probe (monoclonal antibody MVS-1) which binds to a defined component on the surface of soybean protoplasts (M_r = 480,000) (2). Under these conditions, the modulatory effect of SBA is most easily assigned to a real decrease in the lateral mobility of the monoclonal antibody

receptor. This modulatory effect appears to be specific since WGA did not cause any modulation on the MVS-1 mobility value. The effect was most probably not due to the interaction of SBA with the monoclonal antibody or its target antigen because several specific experiments failed to reveal such interactions (5) (Figure 3, Table 3). Finally, locally bound SBA induced the same modulatory effect, thus showing that the effect was not due to an obstruction caused by surface-bound SBA molecules.

The present results may be similar to the modulation of receptor mobility by lectins on a variety of animal cells (12,13). In these studies the modulatory effect of the lectins was partially reversed by treatment with COL, thus implicating a role of the microtubules. Similarly, cytoskeletal-disrupting drugs (COL and CB) reversed the modulatory effect of SBA on the mobility of surface-bound WGA (10) and antibody MVS-1. The cytoskeletal components microtubules and microfilaments have been identified in a variety of plant cells (14-19), and have been shown to form an intricate network underlying the plasma membrane. Taken together, our present results suggest that the binding of external ligands such as SBA to the plasma membrane of soybean protoplasts may lead to rearrangements in the cytoskeletal network of these cells, altering the dynamic properties of other receptors in a similar fashion as it has been proposed to occur in mammalian cells (20).

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CHAPTER 3

ACTIN IN SOYBEAN CELLS: ISOLATION AND CHARACTERIZATION OF SB-1 CELL ACTIN

ABSTRACT

Cultured soybean cells (SB-1 cell line) were plasmolyzed and lyophilized. Extraction of the dried powder and fractionation yielded a polypeptide with the following key properties: (a) it has a molecular weight of ~ 45,000 and an isoelectric point of ~ 5.9; (b) it is immunologically cross-reactive with rabbit antibodies affinity purified against the M_r 45,000 polypeptide of calf thymus actin; (c) it is eluted from a DEAE-cellulose column at the same ionic strength as Acanthamoeba actin; (d) it yields peptide maps, after limited proteolysis with V8 protease, similar if not identical to those of rabbit muscle actin; and (e) it binds specifically to deoxyribonuclease I. These molecular and binding properties indicate that we have purified a cytoplasmic actin from soybean cells. The actin in cytoplasmic extracts of SB-1 protoplasts formed microfilaments (average diameter ~ 7 nm) which bound S-1 subfragments of myosin in an ATP-dependent manner.

INTRODUCTION

In previous studies, we had demonstrated that ligands bound to the plasma membrane of soybean protoplasts exhibited two classes of lateral mobility, arbitrarily classified as "fast" and "slow" (1,2). We had also reported that the binding of exogenously added soybean agglutinin on the protoplast decreased the lateral mobility of a number of "fast"-moving receptors, including those for certain lectins and antigenic targets of monoclonal antibodies. Reagents that are known to disrupt the organization of cytoskeletal components (e.g. cytochalasin B) could abolish this modulation of receptor mobility by soybean agglutinin. It is likely, therefore, that the binding of the lectin to the plasma membrane of protoplasts causes alterations in the cytoskeletal network such that the lateral mobility of other receptors is restricted.

Actin is a ubiquitous protein in eukaryotic cells and actin-like proteins and filaments have been reported in a number of plants (3). In soybeans, the identification and isolation of an actin-like protein which reacted with antibodies directed against calf thymus (CT) actin has been reported (4,5). More recently, Shah *et al.* (6,7) cloned the multigene family from soybean encoding actin-like proteins and reported the complete nucleotide sequence from two of these soybean actin genes. The deduced amino acid sequences showed homology to the sequences of actins from evolutionarily diverged organisms such as *Drosophila melanogaster* and yeast.

To the best of our knowledge, however, soybean actin has not been purified to homogeneity in a form amenable for detailed characterization and comparison with other known actins, particularly in terms of its binding properties. In the present communication, we report the purification of soybean actin, and its molecular and binding characteristics. In the course of these studies, we also developed a procedure for the extraction of proteins from cultured soybean cells and soybean seedlings that minimized proteolysis of the cellular proteins after cell breakage and homogenization.

MATERIALS AND METHODS

Soybean Cell Extracts for Affinity Chromatography

SB-1 cells (Glycine max [L.] Merr. cv. Mandarin) (8) were generously provided by Dr. Gordon Lark (Department of Biology, University of Utah, Salt Lake City, UT) and cultured as previously described (9). The cells from a five-day-old SB-1 culture were filtered on Whatman filter paper and were then plasmolyzed by resuspension and incubation in 0.44 M sorbitol, 50 mM Tris-HCl, 0.5 mM $CaCl_2$, pH 7.5 for 15 min at 25°C. The cells were then vacuum drained to almost dryness and resuspended in ice-cold 10 mM imidazole, 0.1 mM $CaCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 67 μM streptomycin sulfate, 0.57 mM phenyl methylsulfonyl fluoride, 5 mM benzamidine hydrochloride, and 1 Kallikrein inhibitor unit of aprotinin (10) (Boehringer Mannheim, West Germany) per ml of buffer, pH 7.5 (G buffer), containing 0.4 M sorbitol. They were incubated for 10 min at 4°C and drained again. The resulting pellet was frozen at -80°C and lyophilized. The chunks of lyophilized material were reduced to a powder and stored at -10°C. Extracts from the powder were prepared at 4°C by resuspending and stirring 1 g of dry material per 10 ml of G buffer for 20 min with the aprotinin concentration increased to 25 Kallikrein units/ml. The suspension was subjected to centrifugation at 27,000 g for 15 min and the supernate was filtered first through Whatman paper and then through a 45 μm pore diameter Millivex cartridge (Millipore Corporation, Bedford, MA). The filtered solution was applied directly to a deoxyribonuclease (DNAse) I-Sepharose column.

Soybean Cell Extracts for Electron Microscopic Analysis

Protoplasts were prepared by a modified procedure of Constabel (11). Ten ml of a 2 day-old SB-1 culture were added with an equal volume of enzyme solution containing 200 mg cellulase "Onozuka" R-10 (Yakult Honsha Co., Tokyo, Japan), 100 mg pectinase (Sigma Chemical Co., St. Louis, MO), and 1 g mannitol, pH 5.5. After 1 h at 25°C, the protoplast suspension was filtered through a 48 µm nylon filter and pelleted by centrifugation for 4 min at 460 g. The pelleted protoplasts were washed once by gentle resuspension and centrifugation as above in a buffer containing 0.44 M mannitol, 0.1 M piperazine-N, N'-bis[2-ethanesulfonic acid] (PIPES), and 5 mM $MgSO_4$, pH 6.9. The final pellet was resuspended in 0.1 M PIPES, 5 mM $MgSO_4$, 10 mM ethylene glycol-bis-(β-aminoethyl-ether)N,N'-tetraacetic acid (EGTA), 0.01% (w/v) NaN_3, pH 6.9, and the protoplasts were further ruptured by gentle resuspension with a Pasteur pipet. The suspension was allowed to settle for 5 h at 25°C, and the supernatant was centrifuged at 160,000 g for 30 min at 25°C in an air ultracentrifuge (Beckman Instruments, Palo Alto, CA). The resulting pellet was resuspended in a minimum volume of 0.1 M PIPES, 5 mM $MgSO_4$, 10 mM EGTA, 0.01% (w/v) NaN_3, pH 6.9, and deposited on grids for negative staining and electron microscopic analysis.

DNAse I-Sepharose Chromatography

All steps were performed at 4°C. DNAse I (25 mg, Sigma Chemical Co.) was coupled to cyanogen bromide-activated Sepharose 4B beads (4 ml wet volume, Sigma Chemical Co.) overnight with rocking on a platform. The coupling buffer was 0.13 M NaCl, 5 mM sodium phosphate,

pH 7.5 (PBS). The suspension was packed into a column (3.2 x 1.3 cm) and washed extensively with 0.1 M Tris-HCl, pH 8.0, to hydrolyze the unreacted active sites. The column was then washed with 50 volumes of 0.1 M citrate buffer, pH 3.0, and again with 50 volumes of Tris-HCl. Finally, the column was equilibrated in G buffer. Affinity purification of actin was performed following the method of Maekawa et al. (12) with some modifications. After application of the soybean cell extracts, the column was washed with 200 volumes of 0.01% (v/v) Triton X-100 (TX-100, Boehringer Mannheim) in G buffer and 7 volumes 0.75 M urea in G buffer. The bound material was eluted with 2 M urea in G buffer, and the column was further washed with 7 volumes of 6 M urea in G buffer before reequilibration in G buffer. The fractions from the 2 M urea eluate were tested for actin content by immunodot blots with rabbit antibodies against calf thymus (CT) actin followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Biorad Laboratories, Richmond, CA). The fractions containing immunoreactive material were concentrated with a Centricon 30 device (Amicon Corporation, Danvers, MA, 30,000 molecular weight cutoff) and stored at -10°C prior to analysis.

DEAE-Cellulose Chromatography

The fractions containing actin isolated by DNAse I-Sepharose chromatography were labeled with ^{125}I and diluted in DEAE buffer (same as G buffer except that the imidazole was substituted by 5 mM sodium phosphate, pH 7.5). A column (1.4 cm high x 1.1 cm internal diameter) of DEAE-cellulose (DE-52, Whatman Biosystems, Ltd., England) was equilibrated in DEAE buffer and, after application of the sample (3.5

x 10^5 cpm), was washed with DEAE buffer until the radioactivity in fractions collected from the wash yielded a low background level. Elution was achieved by a linear gradient of 0 to 0.3 M NaCl in DEAE buffer. The fractions eluted from the column were analyzed by polyacrylamide gel electrophoresis and autoradiography.

Gel Electrophoresis and Immunoblotting

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out according to Laemmli (13) in minigels (Hoeffer Scientific Instruments, San Francisco, CA) composed of 12.5% acrylamide, and a stacking gel of 4% acrylamide. Samples were diluted at a ratio of 5:1 with concentrated sample buffer (5% (w/v) SDS, 25% (w/v) dithiothreitol, 25% (v/v) glycerol, and 0.25 M Tris-HCl, pH 6.8) and boiled for 5 min; concentrated samples were diluted in regular SDS-PAGE sample buffer (2.3% (w/v) SDS, 5% (w/v) dithiothreitol, 10% (v/v) glycerol, and 60 mM Tris-HCl, pH 6.8). Proteins were visualized by silver staining (14). Western immunoblotting was carried out as described previously (9). After proteins were immobilized on nitrocellulose (Schleicher and Schuell, Inc., Keene, NH) by dot application or electrotransfer, the membranes were blocked with 3% (w/v) bovine serum albumin in PBS for 1 h at 25°C. The membranes were then incubated at 25°C in 15 µg/ml rabbit anti-CT actin antibodies (4,5) in PBS containing 0.01% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20, Sigma Chemical Co.) (T-PBS), followed by horseradish peroxidase- (1:1500 dilution in T-PBS, BioRad Laboratories) or alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1000 dilution in T-PBS, Sigma Chemical Co.). In some cases, the

membrane was probed with rabbit anti-CT actin that had been affinity purified (15) against the M_r 45,000 polypeptide of CT actin. The antibodies bound on the polypeptides were visualized with 4-choloro-1-naphthol and H_2O_2 or nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BioRad Technical Bulletin), depending on the probe used. Isoelectric focusing of the proteins in two-dimensional gel electrophoresis analysis was carried out as described by O'Farrell (16) with a pH gradient of 4 to 7.6. The electrofocused gels were equilibrated for 30 min in regular SDS-PAGE sample buffer and subjected to SDS-PAGE in the second dimension. Proteins were detected by immunoblotting.

For the immunodetection of myosin in SB-1 cells, 0.2 g of extract, prepared in the form of a dried powder as described above, were suspended in 5 ml of 0.6 M KCl, 10 mM PIPES, 5 mM $MgSO_4$, pH 6.9, containing 25 Kallikrein units/ml of aprotinin (Boehringer Mannheim). After stirring for 15 min at 4°C, the suspension was subjected to centrifugation (15,000 g, 15 min, 4°C), and the supernate was mixed with an equal volume of 12.5% trichloroacetic acid to precipitate the proteins. After centrifugation (15,000 g, 5 min, 4°C), the pellet was resuspended in 5 ml of sample buffer. Two µl of 2 M Tris-HCl, pH 9.0, were added to neutralize the final solution. The samples were boiled for 5 min and electrophoresed in 14% acrylamide gels according to the procedure described by Gibson (17). The proteins from the gel were transferred onto nitrocellulose and immunoblotted with a rabbit antiserum raised against chicken gizzard myosin. Chicken gizzard myosin was purified according to the procedure of Harris and Suelter (18). A

white, female New Zealand rabbit was immunized with 800 µg of the purified myosin in complete Freund's adjuvant. A booster injection was given after one month (500 µg myosin in incomplete Freund's adjuvant). After this period, booster injections at one week intervals (300-400 µg myosin in incomplete Freund's adjuvant) were given.

Peptide Map Analysis

Rabbit muscle actin and ^{125}I-labeled SB-1 actin were compared by peptide mapping after limited proteolysis using a slight modification of the procedure reported by Piperno and Luck (19). Rabbit muscle actin and ^{125}I-labeled soybean actin were electrophoresed in adjacent lanes on a 12.5% acrylamide gel and the M_r 45,000 polypeptide from rabbit muscle actin was visualized by brief staining with Coomassie Brilliant Blue and destaining with 50% (v/v) methanol. Slices corresponding to the M_r 45,000 polypeptide were cut out of the gel and equilibrated for 30 min at 25°C in regular SDS-PAGE sample buffer. The slices were then applied to another 12.5% acrylamide gel, followed by the corresponding addition of Staphylococcus aureus V8 protease (Miles Laboratories, Elkhart, IN), and immediately electrophoresed. In some cases, further digestion was allowed for 15 min at the end of the stacking gel. The resulting peptides were visualized after electrophoresis by Coomassie Blue staining and/or autoradiography on XRP-5 film.

Electron Microscopy

Purified rabbit muscle actin was a generous gift from Dr. Kermit L. Carraway (Department of Anatomy and Cell Biology, University of

Miami, Miami, FL). Parlodion (Mallinckrodt Chemical Works, New York, NY) carbon-coated grids were used. Five µl of extract were mixed with an equal volume of a 1 mg/ml solution of S-1 subfragment from myosin (Sigma Chemical Co.) diluted in 0.3 M KCl, 10 mM PIPES, 5 mM $MgSO_4$, pH 6.9 (S-1 buffer). The grid was inverted on the solution and incubated for 30 min at 25°C. After the incubation, the grid was inverted in sequence on the following solutions: a) 5 drops of S-1 buffer; b) 10-drops of 0.2 mg/ml cytochrome c (Nutritional Biochemicals Corp., Cleveland, OH) in 0.1% amyl alcohol; and c) 6 drops of 1% uranyl acetate, allowing the grid to incubate for 10 sec on the last drop of stain (20). The negatively-stained grids were examined on a Phillips 201C electron microscope. The ATP-dissociation of S-1 was carried out following the same procedure except that the S-1 buffer contained 10 mM ATP.

Other Procedures

Radioiodination of the SB-1 actin was carried out by the Chloramine T method as described by Langone (21). The free isotope was separated from the coupled proteins by gel filtration on a Sephadex G-25 (Sigma Chemical Co.) column (17 x 0.5 cm).

RESULTS

Extraction of Soybean Cells

In previous studies, it had been demonstrated that an actin-like protein existed in soybean cells on the basis of immunochemical reactivity with an antiserum directed against CT actin (4). In these studies, homogenates of soybean cells were prepared, filtered, subjected to low and high speed centrifugations, and then partially purified before immunochemical analysis. Alternatively, homogenates of soybean cells could be subjected to ammonium sulfate precipitation and dialysis similar to procedures reported for the purification of actin from tomato (22,23). During the course of the present studies, we found that once the cells are broken, a significant amount of proteolysis occurs as a result of endogenous protease activity (24), even in the presence of protease inhibitors. Therefore, we sought conditions for the preparation of extracts that would minimize: (a) the length of time required between cell breakage and purification; and (b) the extent of proteolysis in the sample.

For cultured SB-1 soybean cells, we found the best procedure to consist of plasmolysis of the cells in the presence of protease inhibitors, followed by lyophilization of the drained material. The resulting chunks of dried material can be pulverized into a powder that appears to be stable until extraction and immediate application onto a column for fractionation. The use of lyophilized dry material minimizes sample volume, time of handling, and proteolysis.

Identification of Actin from SB-1 Cells

Extracts of cultured SB-1 cells were initially fractionated by affinity chromatography on a column of DNAse I-Sepharose. The majority of the protein components did not bind to the column as indicated by a comparison of the SDS-PAGE patterns of the material prior to affinity fractionation and of the material that flowed through the column (Fig. 1, lanes a and b). After extensive washing, the material bound to the column was eluted in two steps: first with 0.75 M urea and then with 2 M urea. The material eluted by 2 M urea contained four major components (Fig. 1, lane c); one of the polypeptides co-migrated with a sample of CT actin (M_r 45,000) (Fig. 1, lane d). The material eluted from the affinity column with 0.75 M urea also contained the M_r 45,000 polypeptide, but was more heterogeneous in terms of other polypeptide contaminants (data not shown).

Rabbit antiserum directed against CT actin (4) was affinity purified on the basis of its binding to the M_r 45,000 polypeptide of CT actin (15). When the material from the 2 M urea elution of the DNAse I affinity column (Fig. 1, lane c) was subjected to SDS-PAGE and immunoblotting with this affinity purified anti-actin antibody, one predominant and two minor bands were observed (Fig. 2, lane a). The predominant polypeptide migrated to a position corresponding to the M_r 45,000 polypeptide of CT actin (Fig. 2, lane b). The polypeptide bands of lower molecular weight revealed by the anti-actin antibodies in both SB-1 and calf thymus preparations represent proteolytic fragments of the M_r 45,000 polypeptide.

The DNAse I-Sepharose bound material that was eluted with 2 M urea (Fig. 1, lane c) was further analyzed by two-dimensional gels

Figure 1. Purification of SB-1 actin on a DNAse I-Sepharose column. Extracts from SB-1 cells were prepared as described in Materials and Methods and loaded on a DNAse I-Sepharose column; after extensive washing, the column was eluted in sequence with 0.75 M and 2 M urea. Analysis of the polypeptides at different stages in the purification was carried out by SDS-PAGE on a 12.5% acrylamide gel. Lane a, material prior to loading on the column (40 µg); lane b, flow-through from the column (40 µg); lane c, material eluted by 2 M urea (1 µg); lane d, calf thymus actin (1 µg). The arrow indicates the position of migration of a polypeptide (M_r 45,000).

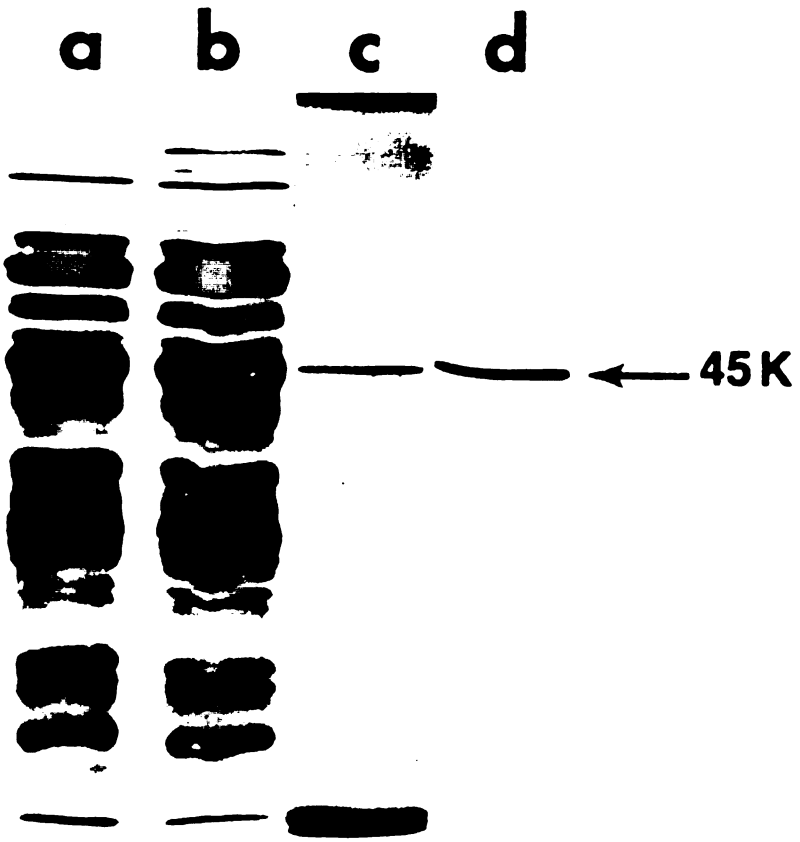

Figure 1

Figure 2. Western immunoblotting analysis of the material eluted from the DNAse I-Sepharose column. Lane a, material eluted by 2 M urea (0.5 μg) and probed with affinity isolated antibodies to the M_r 45,000 polypeptide of calf thymus actin; lane b, calf thymus actin (0.5 μg) probed with the same antibodies. The arrow indicates the position of migration of a polypeptide (M_r 45,000).

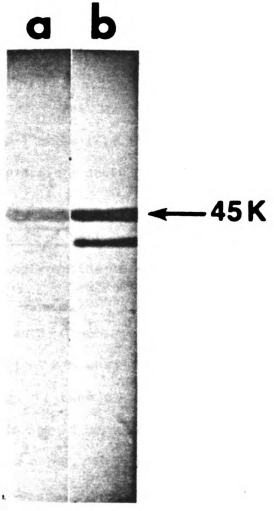

Figure 2

(isoelectric focusing in the first dimension and SDS-PAGE in the second dimension) and immunoblotting with rabbit anti-CT actin (Figs. 3A,B). A single spot with M_r ~ 45,000 and pI ~ 5.9 was revealed. Rabbit muscle actin, a representative of muscle (α) actin, and CT actin, representative of cytoplasmic actins (β and γ), were simultaneously analyzed in a parallel two-dimensional gel. Comparison of the positions of migration of rabbit muscle actin and CT actin (Fig. 3C) with that derived from SB-1 cells (Fig. 3B) indicated that the latter corresponded to the cytoplasmic γ actin group.

DEAE-Cellulose Fractionation of SB-1 Actin

On the basis of the polypeptide molecular weight (M_r ~ 45,000), isoelectric point (pI ~ 5.9), immunological cross-reactivity with affinity purified rabbit anti-CT actin, and DNAse I binding properties, we conclude that the 2 M urea eluted material from the DNAse I-Sepharose column (Fig. 1, lane c) contained soybean actin from cultured SB-1 cells. It should be noted, however, that this fraction (Fig. 1, lane c) contained other components which were not revealed when specific antibody detection methods were used. To further fractionate this material, it was first labeled with ^{125}I; after removal of non-covalently coupled ^{125}I, it was subjected to DEAE- cellulose chromatography.

Analysis of the fractions from the DEAE-cellulose column (Fig. 4) by SDS-PAGE and autoradiography revealed two major peaks of radioactivity that were not associated with polypeptides of M_r ~ 45,000 (Fig. 4, inset lanes 1-7). This is consistent with the previous results obtained from silver staining of the DNAse I-Sepharose bound

Figure 3. Two-dimensional gel analysis of SB-1 actin. A, Western blot of the two-dimensional gel of SB-1 actin probed by rabbit antibodies to calf thymus actin. The arrow points to the single spot detected corresponding to a pI of 5.9 and a molecular weight of 45 kilodaltons (arrowhead on the left); B, magnified view of the data from A; C, two-dimensional gel electrophoresis analysis of rabbit muscle actin (a) and calf thymus actins (b,c) with pI of 5.6, 5.8 and 5.9, respectively. The spots were detected by immunoblotting. The amounts of protein loaded on the gels were 0.5 µg in A, and 4 µg in C.

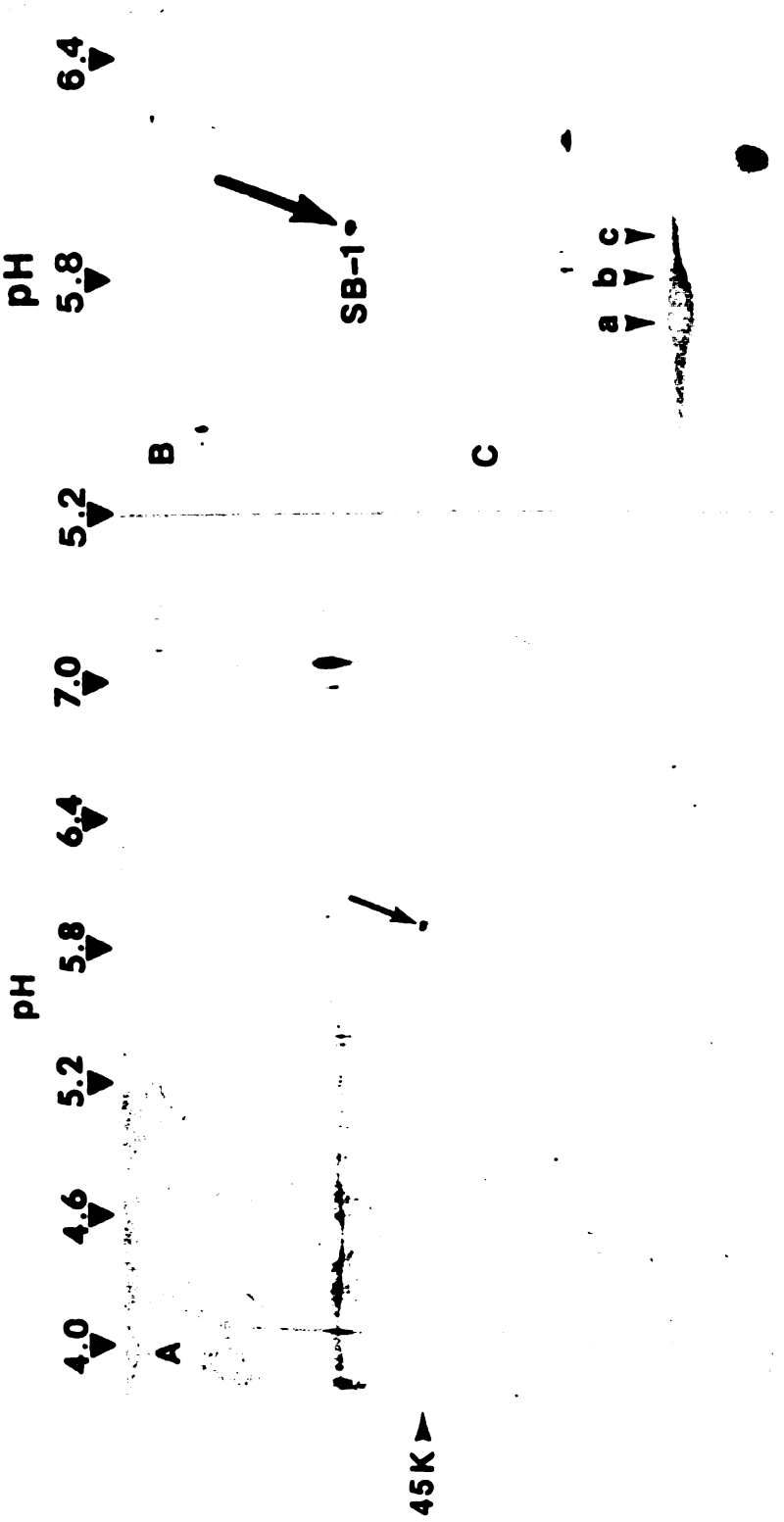

Figure 3

Figure 4. Elution profile of SB-1 actin on DEAE-cellulose columns. The material from DNAse I-Sepharose that was eluted by 2 M urea was radiolabeled with ^{125}I, and 3.5 x 10^5 cpm were applied on a DEAE-cellulose column (1.4 cm high x 1.1 cm internal diameter). After extensive washing, the bound material was eluted with a linear gradient from 0 to 0.3 M NaCl and 300 µl fractions were collected. The inset shows the SDS-PAGE and autoradiographic analysis of fractions 14 (1), 18 (2), 19 (3), 20 (4), 21 (5), 23 (6), 24 (7), 26 (8), 40 (9), 42 (10), 44 (11), and 45 (12); the arrowhead indicates the position of migration of the 45 kilodalton polypeptide corresponding to SB-1 actin. The brackets show the fractions corresponding to the salt concentration at which Acanthamoeba actin elutes (25).

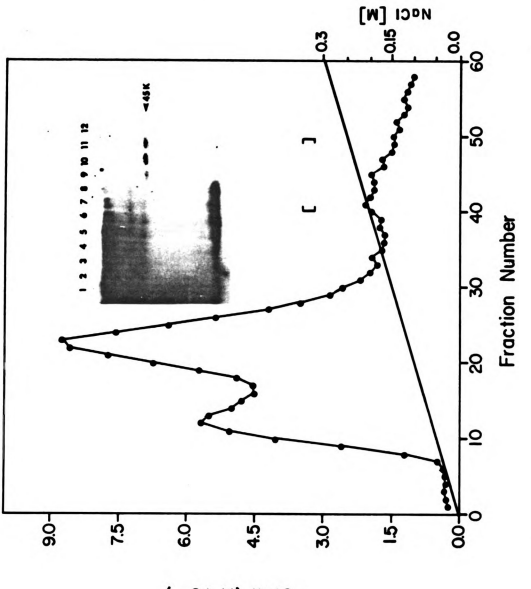
CPM (X 10-3)
9.0
7.5
6.0
4.5
3.0
1.5
0.0
0
10
20
30
40
50
60
Fraction Number
NaCl [M]
0.3
0.15
0.0
1 2 3 4 5 6 7 8 9 10 11 12
~45K
[ ]

Figure 4

material eluted by 2 M urea; the principal bands migrated at the high and low molecular weight ends of the gel (Fig. 1, lane c). The material eluting from the DEAE-cellulose column in fractions 42 through 45 contained a single polypeptide of M_r ~ 45,000 (Fig. 4, inset lanes 9-11). The positions of elution, between 0.2 M to 0.25 M NaCl, correspond closely to that reported for actin from Acanthamoeba (25). Hereafter, this material (Fig. 4, fractions 42-45) is designated SB-1 actin.

Comparative Peptide Map Analysis of SB-1 Actin and Rabbit Muscle Actin

Limited proteolysis with V8 protease (19) was carried out on the SB-1 actin preparation and the peptide fragments were analyzed by SDS-PAGE and autoradiography. In parallel, rabbit muscle actin was similarly treated. When 0.075 µg of V8 protease were used for the digestion, fragments with M_rs of ~ 37,000, ~ 27,000, ~ 20,000 and ~ 17,000 were observed (Fig. 5, lane b). Increasing the amount of enzyme (to 0.25 µg) resulted in the complete degradation of the high molecular weight fragments. In this case, polypeptide bands of M_r ~ 20,000, ~ 17,000 and < ~ 10,000 were observed (Fig. 5, lane d). Digestion of rabbit muscle actin yielded the corresponding fragments (Fig. 5, lane c). These results provide additional evidence that the M_r 45,000 polypeptide represents actin from cultured SB-1 soybean cells.

Actin Microfilaments from Cytoplasmic Extracts of Soybean Protoplasts

Protoplasts from SB-1 cells were prepared and the cells were lysed in a low osmotic medium to prevent rupture of the vacuoles (26). Electron microscopic analysis of material extracted in this manner and

Figure 5. Comparative peptide map analysis of rabbit muscle and SB-1 actin. ^{125}I-Labeled material (2 x 10^5 cpm) from the 2 M urea elution of the DNAse I-Sepharose chromatography, and 5 µg of rabbit muscle actin were run on adjacent lanes in an SDS-polyacrylamide gel. Slices containing the 45 kilodalton polypeptide were cut out from the gel and run on another gel in the presence of Staphylococcus aureus V8 protease. Lanes a and b, rabbit muscle actin and SB-1 actin, respectively, in the presence of 0.075 µg of V8 protease; lanes c and d, rabbit muscle and SB-1 actin, respectively, in the presence of 0.25 µg of V8 protease and digested for 15 min in the stacking gel.

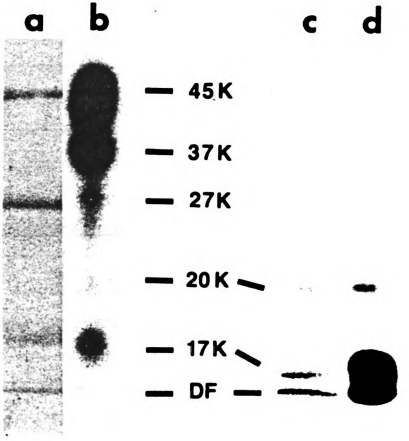

Figure 5

incubated with the S-1 subfragment of myosin revealed microfilaments with the characteristic arrowhead decoration (Fig. 6B). These decorated microfilaments were similar to those obtained when rabbit actin microfilaments and S-1 were incubated together (Fig. 6A). Furthermore, when the incubation of soybean extracts and S-1 was carried out in the presence of 10 mM ATP, the arrowhead pattern was lost. The resulting naked microfilaments had an average diameter of 7 nm, as has been reported for actin microfilaments from other species (27,28). These results indicate that polymerized actin exists in the soybean cell, and that these microfilaments can interact with myosin in an ATP-dependent manner.

Polypeptides Cross-Reactive with Antibodies Directed Against Chicken Gizzard Myosin

To test for myosin endogenous to SB-1 cells, extracts of the cells were subjected to SDS-PAGE and immunoblotting with antibodies directed against chicken gizzard myosin. This antibody preparation recognized the heavy chain and two of the light chains of the rabbit myosin molecule (Fig. 7, lane b). In SB-1 cell extracts, rabbit anti-chicken gizzard myosin immunoblotted four polypeptides (Fig. 7, lanes d-g), two of which corresponded to the heavy (M_r - 200,000) and light (M_r - 16,000) chains of rabbit muscle myosin. The other two polypeptides detected by the antibody corresponded to M_r - 25,000 and M_r - 12,000 bands. Although the origins of these latter two bands are not known, they were not observed when the same SB-1 extracts were immunoblotted with control preimmune serum, which reacted neither with rabbit muscle myosin (Fig. 7, lane c) nor with SB-1 cell extracts

Figure 6. ATP-dependent binding of the S-1 subfragment of myosin to actin microfilaments from SB-1 protoplasts. Rabbit actin microfilaments or cytoplasmic extracts from SB-1 protoplasts prepared as described in Materials and Methods were incubated with the S-1 subfragment of myosin in the presence and absence of 10 mM ATP. The incubation mixture was deposited on parlodion carbon-coated grids, and negatively stained with uranyl acetate. The characteristic arrowhead decoration is observed along the rabbit (a) and soybean (b) microfilaments. In the presence of 10 mM ATP no binding of S-1 was detected (c). The magnification is: a) 105,442; b) 120,600; and c) 105,000. The bars represent 100 nm.

Figure 6

Figure 7. Identification of polypeptides that immunologically cross-react with antibodies directed against chicken gizzard myosin by Western immunoblotting analysis. Lane a, SDS-PAGE of rabbit muscle myosin (20 µg). Lanes b to h, Western blots. Lane b, rabbit muscle myosin (2 µg) probed with antibodies directed against chicken gizzard myosin; lane c, rabbit muscle myosin (20 µg) incubated with a preimmune serum; lanes d to g, 10, 20, 30, and 40 µl, respectively, of SB-1 cell extract prepared as described in Materials and Methods, probed with antibodies directed against chicken gizzard myosin; lane h, 40 µl of SB-1 cell extract incubated with a preimmune antiserum. The positions of migration of the heavy chain (HC, M_r ~ 200,000) and two of the light chains (LC1, M_r ~ 21,000 and LC2, M_r ~ 16,000) of myosin are indicated by the arrows on the left. The arrows on the right, from top to bottom, show the position of migration of the molecular weight marker proteins phosphorylase b (M_r ~ 92,500), bovine serum albumin (M_r ~ 67,000), ovalbumin (M_r ~ 45,000), carbonic anhydrase (M_r ~ 31,000), soybean trypsin inhibitor (M_r ~ 18,000), and lysozyme (M_r ~ 14,400).

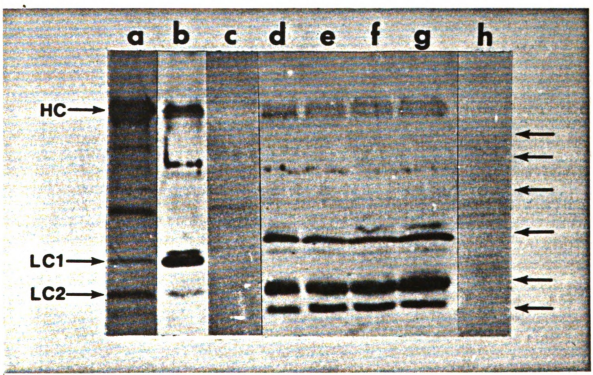

Figure 7

(Fig. 7, lane h). These results suggest the presence of a myosin-like molecule in SB-1 cells.

DISCUSSION

Microfilaments are recognized as ubiquitous cytoskeletal and contractile elements found in virtually all types of cells. Indeed, actin, the major protein component of these fibrillar structures, has been isolated from a variety of animal muscle and non-muscle cells (27), as well as from other organisms such as Mycoplasma pneumonia (29), Chlamydomonas (19), Physarum (30,31), Acanthamoeba (25,32), Saccharomyces cerevisiae (33), and Dictyostelium (34). Numerous ultrastructural observations of microfilaments from plant tissue have been reported, including the observation of ATP-dependent arrowhead decoration of such filaments by heavy meromyosin or the S-1 subfragment of myosin (3,5,22,23). Identification of the actin subunit of these structures have been documented for Nitella (35) and Lycopersicon esculentum (22,23). In practically all cases studied to date, the various actins are remarkably similar to each other and to muscle actin, all consisting of globular subunits of M_r - 45,000.

The present purification of soybean actin with a polypeptide chain of M_r 45,000 extends the list of similar proteins. It confirms the previous identification of an actin-like protein on the basis of reactivity with antibodies directed against CT actin (4), as well as the molecular cloning and sequence studies on the multigene family encoding for actin-like proteins in the soybean (6,7). The purified protein was demonstrated to be the cytoplasmic form of actin (pI - 5.9) by two-dimensional gel electrophoresis. When rabbit muscle actin and CT actin were analyzed simultaneously, one could distinguish muscle α actin (most acidic form of actin) from cytoplasmic forms (β

actin pI ~ 5.8, γ actin pI ~ 5.9) (36-40). On this basis, the actin purified from SB-1 cells was shown to be the non-muscle or cytoplasmic γ form of actin.

In previous attempts to purify soybean actin from seedlings on a DEAE-cellulose column, it was observed that too many proteins co-eluted with actin (4). Similar results were obtained when extracts from SB-1 cells were fractionated the same way. Instead, we took advantage of the known property of actin to bind to DNAse I (41) to fractionate actin from SB-1 cells. The material eluted from a DNAse I-Sepharose column was shown to contain high and low molecular weight proteins in addition to the M_r 45,000 dalton polypeptide (Fig. 1C). However, upon further fractionation of this material on a DEAE-cellulose column, the M_r 45,000 polypeptide could be separated from other impurities. The single polypeptide was eluted at an ionic strength similar to that observed for Acanthamoeba actin fractionated in DEAE-cellulose columns (25). Therefore, the DEAE-cellulose column chromatography could be used as an additional purification step for the SB-1 actin isolation.

Although the deduced amino acid sequence from soybean actin genes have shown an increased divergence compared with actins from other species (6,7,42), the results reported here indicate that soybean actin shares a number of characteristics, biological activities and binding properties that have been characterized for other actins. These include: a) similar proteolytic fragments to those derived from rabbit muscle actin; b) binding to DNAse I; and c) it is assembled into actin microfilaments. This last conclusion is based on two lines of evidence: 1) the average diameter of these filaments was ~ 7 nm;

and 2) these filaments were capable of ATP-dependent binding with the S-1 subfragment of myosin.

In algae, the movement of organelles and cytoplasmic vesicles has been ascribed to an actomyosin system (43). In higher land plants, the existence of such a system has not been demonstrated. However, the ability of the microfilaments to interact with the myosin subfragments in an ATP-dependent manner is suggestive of this possibility. A similar interaction has been observed for tomato (22,23), and soybean and clover roots (5). Furthermore, the isolation of myosin-like molecules from tomato (22,23) and Egeria densa have been reported (44). We have also obtained preliminary evidence for the presence of a myosin-like protein(s) in cultured soybean cells on the basis of immunological cross-reactivity with an antiserum directed against chicken gizzard myosin.

The properties of SB-1 actin are remarkably similar to those of actins from other species. This suggests the possibility of similar interactions between the actin molecule and other actin binding proteins (e.g. capping and bundling proteins (28)), to regulate the form in which it exists in the cell.

The availability of purified actin from soybean cells will now allow us to: (a) study the assembly properties of the plant cell actin in vitro; (b) search for actin binding proteins such as villin, α-actinin, profilin, and vinculin; (c) analyze the regulation of assembly of microfilaments in higher plants by associated proteins; and (d) search for a direct connection between plasma membrane receptors and cytoskeletal microfilaments, as implicated in our previous work on receptor mobility (1,2).

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CHAPTER 4

ACTIN IN SOYBEAN CELLS: A WELL DEFINED ACTIN CYTOSKELETON THAT IS PRESERVED AFTER CELL WALL DIGESTION AND PROTOPLAST LYSIS

ABSTRACT

The microfilament network in cultured SB-1 cells and their protoplasts was observed by staining with fluorescently-labeled phalloidin. In whole cells, three types of networks were identified: a) arrays surrounding the nucleus; b) a network containing large subcortical cables irradiating from the nuclear arrays; and c) a finer network extending towards the periphery of the cell. In addition, some cells that had recently undergone cytokinesis showed microfilaments associated with the phragmoplast. The microfilament network in protoplasts was similar in distribution to that observed in whole cells. When protoplasts were lysed in a low osmotic medium, the vacuoles were released and the staining of the microfilaments co-localized with the remaining "ghost" of the cell. In addition, certain filamentous structures in the debris of lysed cells also stained for F-actin. These results indicate that cultured SB-1 cells contain a well defined actin cytoskeleton that is preserved after cell wall digestion and even protoplast lysis.

INTRODUCTION

Actin is a ubiquitous protein that has been associated with a number of cellular functions such as muscle contraction, amoeboid movement, cell division, and maintenance of cell structure (1).

In plants, the visualization of actin microfilaments by electron microscopy (2-5), and by the specific binding of fluorescent phallotoxins (6-10) has been reported. These microfilaments were more abundant in elongating cells and cells that carry out extensive cytoplasmic streaming (1,11,12). Actin bundles and cables have also been found associated with structures typical of the mitotic process. For example, F-actin has been observed in the preprophase band in onion root cells but subsequently disappeared (6). F-Actin in interphase cells of alfalfa and Vicia hajastana was found around the nucleus, the subcortical region, and near the plasma membrane (7). At the onset of mitosis the microfilaments were observed associated with the preprophase band and the mitotic spindle but subsequently disappeared until after cytokinesis, when they were first observed associated with the phragmoplast. Similar arrays were observed in cultured carrot cells (8) and Haemanthus katherinae endosperm cells (9). In these latter reports, the F-actin network was shown to remain throughout the cell cycle and reorganize during mitosis. The network formed a cage-like structure around the dividing nucleus, and was suggested to play several roles including: a) a coordinated role with the microtubules for the guidance and transport of vesicles throughout the mitoic cycle; b) integrity of the cell cortex; and c) structural dynamics of the cytoplasm-mitotic spindle interface.

These phenomenological observations on actin filaments can best be related to their specific role or function if the chemical properties of the component actin are better characterized. A multigene family of actin genes has been identified in soybean (13), and these genes have been cloned and sequenced (14,15). In addition, we have undertaken studies to characterize the properties of actin at the protein chemistry level (3,16). In the previous communication, we reported the purification of a cytoplasmic actin from the cultured soybean cell line, SB-1 (Chapter 3). We now report the assembly of actin into a well defined network in these SB-1 cells.

MATERIALS AND METHODS

Soybean Cell Culture and Protoplast Isolation

The SB-1 cell line of soybean (Glycine max [L.] Merr. cv. Mandarin) (17) was kindly provided by Dr. Gordon Lark (Department of Biology, University of Utah, Salt Lake City, UT) and was grown in the dark as suspension cultures (18). Protoplasts were prepared as described previously (19,20) with the following modifications: a) the enzyme solution was added with 25 Kallikrein inhibitor units/ml of aprotinin (Boehringer Mannheim, F.R.G.); b) mannitol was used instead of sorbitol; and c) the washing buffer was 0.44 M mannitol, 0.1 M piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES), and 5mM $MgSO_4$, pH 6.9 (buffer A). The resulting protoplasts were filtered through a 48 µm nylon filter and washed by centrifugation (460 g, 5 min) and resuspension in buffer A.

Rhodamine-Phalloidin Staining of Cells and Protoplasts

All incubations were carried out at 25°C and all buffers contained 25 Kallikrein units/ml of aprotinin (Boehringer Mannheim). Soybean cells were permeabilized with 0.01% (v/v) NP-40 (Calbiochem, La Jolla, CA) and 5% (v/v) dimethyl sulfoxide in 0.1 M mannitol, 0.1 M PIPES, 5 mM $MgSO_4$, pH 6.9, according to the procedure of Traas et al. (8). They were stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) at a final concentration of 6×10^{-8} M. Control experiments were carried out by preincubating cells with 10^{-5} M unlabeled phalloidin (Sigma Chemical Co., St. Louis, MO) for 30 min,

followed by the addition of 6 x 10^{-8} M rhodamine-conjugated phalloidin. The cells were deposited on slides, sealed with paraffin wax, and observed under phase contrast and fluorescence using a Leitz (Wetzlar, F.R.G.) fluorescence microscope equipped with a Leitz KP580 dichroic mirror and a 50X water immersion objective. Photographs were taken with Kodak Tri-X film (Eastman-Kodak, Rochester, N.Y.) which was pushed to ASA 3200. The same exposure times used for photographing rhodamine-phalloidin stained cells were used for control experiments.

Protoplasts were stained without permeabilization, immediately after digestion and one wash with buffer A. Lysis of the protoplasts was carried out after the wash in buffer A. The cells were subjected to centrifugation and the protoplast pellet was resuspended in 0.1 M PIPES, 5 mM $MgSO_4$, 10 mM ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA), pH 6.9. The fluorescent phalloidin was added immmediately.

RESULTS

Distribution of F-actin in SB-1 Cells from Suspension Cultures

Cells from SB-1 suspension cultures were permeabilized (8) and stained with rhodamine-conjugated phalloidin. The cells showed a continuous network of microfilaments (Fig. 1). Control incubations, in which excess unlabeled phalloidin was added prior to the rhodamine-phalloidin, showed little or no staining (Fig. 1f).

Careful examination of the cells showed that different types of staining were present in different parts of the cell. For example, the nucleus showed very strong staining suggesting that a dense layer of microfilaments surround it ((N) in Figs. 1b and 1c). Thick filament cables, suggestive of bundles of microfilaments, are derived from the nuclear arrays ((S) in Figs. 1b and 1c). These cables were often observed to be co-localized with transvacuolar strands ((S) in Figs. 1a, 1b, and 1c). Finally, finer fibrillar networks probably composed of thinner bundles of microfilaments, formed the cytoplasmic cables that extended towards the periphery of the plasma membrane (Fig. 1c, arrowhead). The network as a whole appeared to have its center of organization around the nucleus from which the thick cables emerged; these cables reached all the way toward the plasma membrane and appeared to wrap around the whole cell like a cage (Fig. 1c, 2b). In some cells that had recently undergone cytokinesis, the phragmoplast was also observed to stain for F-actin (Fig. 1e (P)). These observations indicate that a well defined microfilament network exists in soybean cells, and that the association of this network with cellular

Figure 1. Staining of SB-1 cells with fluorescent phalloidin showing the distribution of actin microfilaments. Cells from two day-old suspension cultures were stained with rhodamine-conjugated phalloidin as described in Materials and Methods, and visualized under phase contrast and fluorescence microscopy. (a) phase contrast micrograph of an interphase cell showing a transvacuolar strand (S); (b) fluorescence micrograph of the same cell showing heavy staining on the nucleus (N), and thick cables co-localized with the transvacuolar strand (S); (c) fluorescence micrograph of the same cell taken at a different focus, showing thick cables co-localized with the transvacuolar strand (S), and cortical microfilaments (arrowhead); (d) phase contrast micrograph of a cell that just undergone cytokinesis; (e) fluorescence micrograph of the same cell showing strong staining of the phragmoplast (P); and (f) fluorescence micrograph of cells preincubated with 10^{-5} M unlabeled phalloidin followed by rhodamine-phalloidin. All micrographs show the same magnification. The bar represents 25 μm.

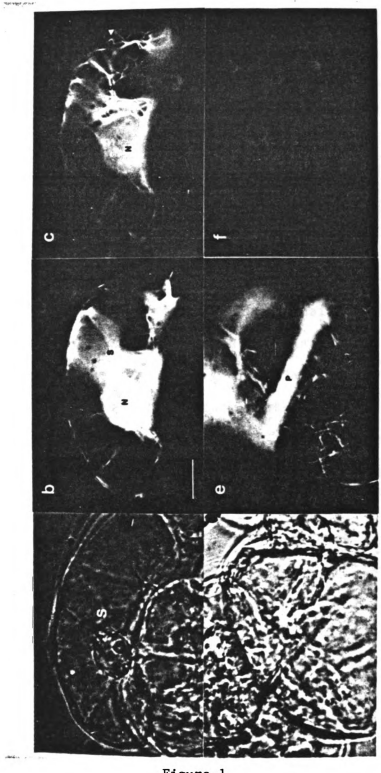

Figure 1

Figure 2. Comparison of the phalloidin staining pattern in SB-1 cells and protoplasts. Protoplasts from 2 day-old suspension cultures were prepared as described in Materials and Methods. Whole cells and protoplasts were stained in parallel with rhodamine-conjugated phalloidin, and observed under phase contrast and fluorescence microscopy. (a) phase contrast micrograph of interphase SB-1 cells; (b) fluorescence micrograph of the same cells showing the cortical microfilaments forming a cage-like structure around the cell; (c) phase contrast micrograph of a protoplast from SB-1 cells; (d) fluorescence micrograph of the same cell showing the staining associated with the nucleus (N), and the periphery of the cell (arrowhead). The bars represent 25 μm.

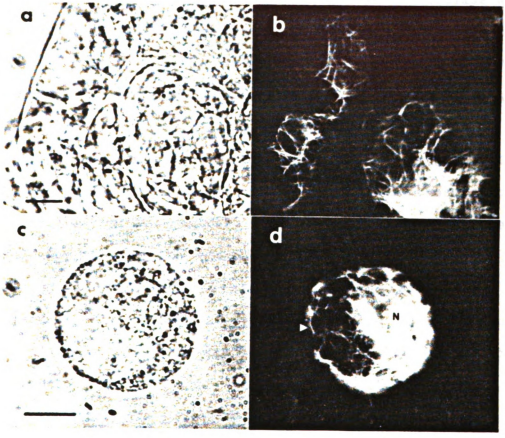

Figure 2

structures is similar to those reported for other cells in culture (7,8).

Comparison of F-actin in Whole Cells and Protoplasts

The cell wall of SB-1 cells was digested with cellulase and pectinase and the resulting protoplasts were incubated with rhodamine-phalloidin. Two distinct cell populations were observed: one type had a continuous well-defined plasma membrane typical of intact protoplasts; the second type had a plasma membrane with a grainy appearance, most probably due to rupture of the membrane. The rhodamine-phalloidin staining could only be detected in those protoplasts with the ruptured membrane, indicating that the fluorescent probe could not penetrate the intact protoplast (data not shown).

In the stained protoplasts, an intricate network of microfilaments, similar to that observed in whole cells, was found (Fig. 2). In both cases there was an extensive network of cortical microfilaments underlying the plasma membrane (Figs. 2b, 2d). In addition, the protoplasts also showed an actin network surrounding the periphery of the nucleus ((N) in Figs. 2d and 3b), giving rise to thick cables (Fig. 3b (S)), and the fine fibrillar networks extending toward the plasma membrane (arrowheads in Figs. 2d and 3b).

Preincubation of the protoplasts with excess unlabeled phalloidin followed by rhodamine-phalloidin yielded no staining, again suggesting that the staining was specific. These data show that the microfilament network is preserved after digestion of the cell wall, and that its distribution within the cell is similar to that found in cells containing the cell wall.

Figure 3. Phalloidin staining pattern in lysed protoplasts from SB-1 cells. Protoplasts from SB-1 cells were resuspended in a hypoosmotic buffer to rupture the plasma membrane, and they were then stained with rhodamine-conjugated phalloidin. (a) phase contrast micrograph of a protoplast with the plasma membrane slightly ruptured and a vacuole (V) just beginning to emerge; (b) fluorescence micrograph showing the same protoplast with the phalloidin stain associated with the nucleus (N), the thick cables (S), and the periphery of the plasma membrane (arrowhead); (c) phase contrast micrograph of a protoplast whose membrane has ruptured significantly and has released the vacuole (V) almost completely; (d) fluorescence micrograph of the same lysed cell showing that the majority of the stain was associated with the "ghost" and no staining in the vacuole (V). The bars represent 25 μm.

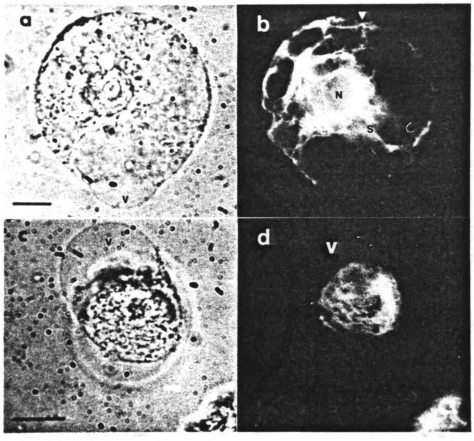

Figure 3

F-actin in Lysed Protoplasts

The actin cytoskeleton appeared to be less intricate around the vacuoles of the cell. When the cells were resuspended in a hypo-osmotic medium, the plasma membrane ruptured and the vacuoles emerged from the protoplast (Fig. 3c (V)), leaving a "ghost" containing the nucleus and the cell envelope. When these osmotically ruptured cells were stained with rhodamine-conjugated phalloidin, the F-actin micro-filaments were observed remaining in the "ghost" of the cell (Fig. 3d). No staining was observed in or around the vacuole (Fig. 3d (V)). This indicated that the actin cytoskeleton is also preserved after rupture of the cell. When lysed cells were pretreated with excess unlabeled phalloidin prior to the rhodamine-phalloidin staining, no fluorescence was observed.

Fluorescent staining was also observed in the debris remaining after the cells were completely lysed (Figs. 4a, 4b). This staining was specific inasmuch as excess unlabeled phalloidin competed with the rhodamine-phalloidin (Fig. 4d). In any case, careful examination of the debris under phase contrast microscopy showed a characteristic beads-on-a-string pattern. The beads are highlighted by the arrowheads in Fig. 4a. The filament (string) was stained with rhodamine-phalloidin (Fig. 4b). Although the identities of these components are not known, one possibility may be that these beads are cytoplasmic vesicles associated with the filaments of actin.

Figure 4. Phalloidin staining of the debris remaining in the medium after lysis of SB-1 protoplasts. Lysed protoplasts were stained with rhodamine-conjugated phalloidin and the debris was analyzed for staining of F-actin. (a) phase contrast micrograph of fibrillar debris that appears to be associated with vesicles (arrowheads); (b) fluorescence micrograph of the same debris showing the stain associated with the filamentous structures. The arrowheads denote the position of the putative vesicles; (c) phase micrograph of fibrillar debris similar to the one shown in (a); (d) fluorescence micrograph of the same debris from (c) preincubated with 10^{-5} M unlabeled phalloidin showing no staining. The bars represent 10 µm.

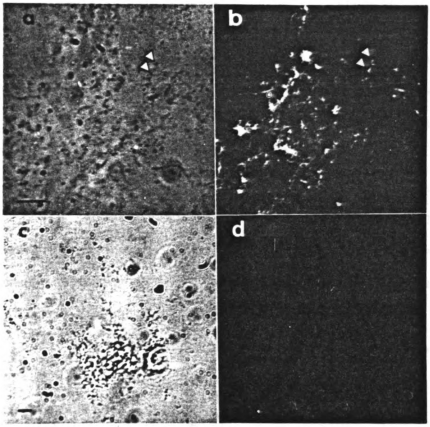

Figure 4

Cell Shape and the Organization of the Actin Network

The actin cytoskeleton appears to conform to the particular shape of the cell, particularly near the cortex. In whole cells with an elongated shape, the actin network showed cortical microfilaments wrapping around the cell throughout its length (Figs. 1c, 2b). In contrast, more or less round cells showed a round network (Figs. 1e, 2b). When the cell wall is digested, round protoplasts are formed. The cortical microfilaments of these protoplasts were observed to extend towards the periphery of the plasma membrane and wrap around the circumference of the protoplast (Figs. 2d, 3b).

When lysed protoplasts were analyzed for the presence of microfilaments, their "ghosts" were observed to contain the bulk of the actin network. These "ghosts" also showed a varied pattern of actin microfilaments depending on their shape. For example, "ghosts" in the aqueous medium had a round appearance (Fig. 3c) with microfilaments extending to fit the corresponding shape (Fig. 3d). However, when these "ghosts" were trapped between the air-water interface of a bubble, they became elongated (Fig. 5a). The microfilaments from these stretched "ghosts" were observed to stretch out according to the shape of the "ghost" (Figs. 5a to 5c). Furthermore, the "ghost"-associated microfilaments showed all three types of associations with the cellular structures. A nuclear network ((N) in Fig. 5b), from which thick cables are derived (arrowhead in Fig. 5b). These cables extended away to form a cortical network near the "ghost" envelope (Fig. 5c). These data suggest that the actin cytoskeleton of SB-1 cells is a structure that conforms to the particular shape of the cell.

Figure 5. Staining pattern of phalloidin, showing the distribution of the actin cytoskeleton in "ghosts" of soybean protoplasts. Lysed protoplasts were stained with rhodamine-conjugated phalloidin. Some of the "ghosts" remaining after release of the vacuoles could be found at the air-water interface of a bubble and these were analyzed for staining of F-actin. (a) phase contrast micrograph of a "ghost" that has been stretched out due to surface tension at the air-water interface; (b) fluorescence micrograph of the same "ghost" showing the microfilaments associated with the nucleus (N), and the thick cables (arrowhead) that derive from it. In addition, cortical microfilaments are observed extending to the periphery of the envelope; (c) fluorescence micrograph of the same "ghost" but focused on the cortical microfilaments which span the periphery of the whole envelope like a "wrap". The pictures in (b) and (c) show the same magnification. The bars represent 25 μm.

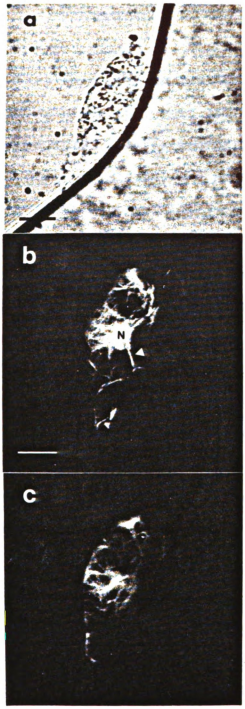

Figure 5

DISCUSSION

The cytoskeleton of the plant cell has been implicated in several functions such as cytoplasmic streaming, cell wall deposition, cell elongation, and mitosis (21,22). Actin microfilaments have been observed as components of the cytoskeleton, and have been identified in a variety of plant cells by electron (2-5,23-27), and fluorescence microscopy (6-10).

The presence of a well-defined actin cytoskeleton observed by staining with fluorescent phallotoxins was recently documented in a variety of dividing cells from plants and suspension cultures (6-9). Three types of microfilament arrays were observed: a) intricate arrays of microfilaments surrounding the periphery of the nucleus; b) thick cables or transvacuolar strands; and e) cortical microfilaments of axial and transverse orientation (7,8). In these studies, F-actin has been observed associated with structures characteristic of the mitotic process; namely, the preprophase band (6), the mitotic spindle (7-9), and the phragmoplast (7,8).

The present study documents the observation of microfilaments visualized by staining with rhodamine-phalloidin in soybean suspension cultures and protoplasts derived from them. The distribution of these microfilaments within the cell consisted of three different types of arrays: a) a network of microfilaments surrounding the nucleus; b) thick cables emerging from the nucleus and extending towards the cell periphery; and c) finer arrays connected with the thick cables and wrapping the cell around the cortex. This distribution was very similar to that observed in suspension cultures of alfalfa, Vicia

hajastana (7), and carrot (8). In addition, some recently divided cells showed actin microfilaments associated with the phragmoplast. These observations provide further support for the existence of an intricate, continuous network of actin microfilaments in plant cells.

Protoplasts derived from SB-1 cell suspension cultures showed a similar distribution of microfilament arrays with a nuclear-associated network emerging into thick cables. These cables, in turn, give rise to finer fibrillar networks around the cortex of the cell. These arrays were preserved even after the protoplasts were lysed and the vacuoles released. The bulk of the microfilaments remained in the "ghost" suggesting that the nuclear array is the main support for the whole structure, with its weakest part in the cortex around the vacuoles where less staining was observed. To the best of our knowledge, these results provide the first documentation for the presence of a well defined actin cytoskeleton in protoplasts and their "ghosts" from plant cells.

The actin cytoskeleton in dividing cells of Haemanthus (9) and carrot suspension cultures (8) was reported to form a cage around the nucleus. This cage was observed to stretch around the mitotic spindle during anaphase chromosome movement, and was compared to a deformable basket (9). This basket was proposed to play an important role in the control of spindle shape and elongation, and the actin bundles were suggested to play a role in the coordinated movement and transport of vesicles.

Our present study provides further evidence that the actin cytoskeleton is a structure that conforms to the shape within which it is contained. This is supported by the following observations: a)

both elongated and round cells had a cytoskeleton with a cage-like structure that conformed to their particular shape; b) the microfilaments in protoplasts conformed to the new round shape of the cell with the cortical microfilaments surrounding the periphery of the plasma membrane; and c) protoplast "ghosts" that were stretched out on the air-water interface of a bubble showed stretched-out microfilament arrays.

The ability to visualize actin filaments in soybean cells provides the opportunity to search for associations in the cytoskeletal structures upon perturbations with exogenous ligands. First, we demonstrated in previous studies that the binding of soybean agglutinin to the plasma membrane of SB-1 protoplasts resulted in the restriction of the mobility of the surface components (20,31). There was evidence implicating the role of cytoskeletal structures in the modulation of receptor mobility (20,32). We can now directly test for alterations in the organization and distribution of the actin network in SB-1 protoplasts upon binding of soybean agglutinin.

Second, we had reported (33) that incubation of SB-1 cells with Rhizobium japonicum resulted in the adhesion of the bacteria to the soybean cells. This binding was specific: a) strains of bacteria that normally infect soybean roots bound to the cells but other strains of Rhizobium did not; and b) the adhesion could be blocked by specific saccharides such as galactose and lactose whereas glucose and mannose had no effect. We would now be interested to determine whether the binding of Rhizobium japonicum to the SB-1 cells results in alterations of the actin cyoskeleton.

Finally, we have purified a cytoplasmic actin from SB-1 cells and have begun to characterize its chemical properties (Chapter 3). The knowledge of the assembly properties of soybean actin and the identification of endogenous proteins that may modulate its polymerization (e.g. villin, severin) (34) will provoke us to ask whether these same parameters can be manipulated in whole soybean cells. The ability to monitor the assembly and distribution states of actin microfilaments by their visualization with fluorescent phallotoxins will clearly facilitate this analysis.

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