Monoclonal Antibodies Directed against Protoplasts of Soybean Cells: Analysis of the Lateral Mobility of Plasma Membrane–bound Antibody MVS-1

Thomas N. Metcalf III, Marco A. Villanueva, Melvin Schindler, and John L. Wang

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824. Dr. Metcalf's present address is Department of Anatomy and Cell Biology, University of Miami School of Medicine, Miami, Florida 33101.

Abstract. A monoclonal antibody (MVS-1) was used to monitor the lateral mobility of a defined component (M_r ~400,000) of the plasma membrane of soybean protoplasts prepared from suspension cultures of *Glycine max* (SB-1 cell line). The diffusion coefficient (D) of antibody MVS-1 bound to its target was determined ($D = 3.2 \times 10^{-10}$ cm^2/s) by fluorescence redistribution after photobleaching. Pretreatment of the protoplasts with soybean agglutinin (SBA) resulted in a 10-fold reduction of the lateral mobility of antibody MVS-1 ($D = 4.1 \times 10^{-11}$ cm^2/s). This lectin-induced modulation could be partially reversed by prior treatment of the protoplasts with either colchicine or cytochalasin B. When used together, these drugs completely reversed the modulation effect induced by SBA. These results have refined our previous analysis of the effect of SBA on receptor mobility to the level of a defined receptor and suggest that the binding of SBA to the plasma membrane results in alterations in the plasma membrane such that the lateral diffusion of other receptors is restricted. These effects are most likely mediated by the cytoskeletal components of the plant cell.

We have previously reported that binding of soybean agglutinin (SBA)[1] resulted in a decrease of the lateral mobility of wheat germ agglutinin (WGA) bound to the plasma membrane of protoplasts derived from SB-1 soybean cells (19, 32). This decrease in the mobility of the WGA–receptor complexes was reflected by a reduction in the average value of the diffusion coefficient (D), as determined by the method of fluorescence redistribution after photobleaching (FRAP). Using immunofluorescence and peptide mapping techniques, we have recently demonstrated that the SB-1 soybean cells produce SBA (or a closely related homolog), some of which can be localized to the plasma membrane of the protoplast.[2] To the best of our knowledge, this is the first report of an endogenously produced protein, SBA, which can modulate the dynamic properties of its own membrane. For this reason, it was of interest to analyze, in detail, the chemical components and mechanism(s) involved in the modulation process.

Since lectins bind to a heterogeneous population of glycoconjugates on the cell surface (28), the D values determined for the WGA–receptor complexes most probably reflect ensemble averages rather than the behavior of any single diffusing species in the membrane. The availability of a monoclonal antibody, directed against a given component of the plasma membrane, would provide a unique opportunity to refine our previous analysis of the modulation of receptor mobility by SBA. We have generated several hybridoma clones, each secreting a monoclonal antibody directed against a component of the soybean protoplast.[3] One such monoclonal antibody, designated MVS-1, has been characterized in terms of its cell surface binding properties and its antigenic target. The use of antibody MVS-1 and its monovalent Fab fragment in the analysis of the mobility of plasma membrane proteins to soybean cells is reported in the present communication.

Materials and Methods

Cell Culture and Protoplast Isolation

The SB-1 line of soybean (*Glycine max*) cells was kindly provided by Dr. G. Lark (Department of Biology, University of Utah, Salt Lake City, UT) and was grown in suspension cultures as previously described (19).[3] Cellulase (Calbiochem-Behring Corp., La Jolla, CA) and pectinase (Sigma Chemical Co., St. Louis, MO) were used to remove the cell wall in the preparation of protoplasts (19).[3] After this enzymatic digestion, the protoplasts were washed by centrifugation (460 *g* for 4 min) and resuspended in 5 ml of buffer A (10 mM $CaCl_2$, 0.55 M sorbitol, 50 mM Tris-HCl, pH 7.5). Fluorescence microscopy after Calcofluor staining (19) and scanning electron microscopy (19) of the protoplasts showed neither the characteristic fluorescence indicative of cell wall material (23) nor cellulose microfibrils (3), respectively.

[1] *Abbreviations used in this paper:* CB, cytochalasin B; COL, colchicine; Con A, concanavalin A; *D*, diffusion coefficient; FRAP, fluorescence redistribution after photobleaching; SBA, soybean agglutinin; WGA, wheat germ agglutinin.

[2] Malek-Hedayat, S., S. A. Meiners, T. N. Metcalf III, M. Schindler, J. L. Wang, and S.-C. Ho. Manuscript in preparation.

[3] Villanueva, M. A., T. N. Metcalf III, and J. L. Wang. Manuscript submitted for publication.

Preparation and Labeling of Immunochemical Reagents

The generation of hybridoma clone MVS-1 and the isolation and characterization of its immunoglobulin product (antibody MVS-1) have been described.[3] Monovalent Fab fragments were prepared from purified antibody MVS-1 as described (20) using papain (ICN Pharmaceuticals, Inc., Irvine, CA) except that digestion was limited to 2 h at 37°C. The Fab fragment was purified by DEAE cellulose chromatography (20). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed according to Laemmli (13) with running gel of 10% (wt/vol) and stacking gel of 4% (wt/vol) acrylamide. Samples were dissolved in 2.3% (wt/vol) sodium dodecyl sulfate, 5% (vol/vol) β-mercaptoethanol and 60 mM Tris, pH 6.8 and boiled for 5 min. For nonreducing conditions, the β-mercaptoethanol was omitted. After electrophoresis, the gels were fixed for 30 min in 10% (vol/vol) trichloroacetic acid and stained with Coomassie Brilliant Blue.

Seed SBA was purified following the method of Allen and Neuberger (1). Rabbit antiserum was produced against the purified seed SBA, and monospecific antibodies directed against SBA were isolated by affinity chromatography. The details of these procedures are described elsewhere.[2]

Fluorescein-derivatized WGA and SBA were obtained from Vector Laboratories (Burlingame, CA). The immunochemical probes—antibody MVS-1, its Fab fragment, and monospecific rabbit anti-SBA immunoglobulin—were labeled with morpholinorhodamine isothiocyanate (Research Organics, Cleveland, OH) as described (20) with the following modifications. After dialysis against bicarbonate-buffered saline (8.0 g NaCl, 1.96 g Na_2CO_3, 2.66 g $NaHCO_3$ per liter), pH 9.5, a 30-fold molar excess of dye (5 mg/ml in dimethylsulfoxide) with respect to protein was added to the sample and incubated for 16 h in the dark at 4°C. The reaction was terminated by adding glycine to a final concentration of 0.1 M, followed by dialysis against bicarbonate-buffered saline, pH 9.2, with one change of buffer. Unincorporated free dye was removed by gel filtration on a Sephadex G-25 column (35 × 1.2 cm) equilibrated in bicarbonate-buffered saline, pH 8.5. The fluorescently derivatized material, which retained antigen-binding activity, was concentrated by ultrafiltration and stored at −20°C.

Binding of Lectin and Antibody Probes to Protoplasts

Protoplasts were prepared for photobleaching by the following procedure: (*a*) protoplasts (5×10^5/0.5 ml) were incubated with the fluorescently derivatized protein probe for 1 h at room temperature; (*b*) the protoplasts were washed three times by centrifugation (460 *g* for 4 min) and resuspension in 1 ml of buffer A; (*c*) after washing, the protoplasts were suspended in 100 μl of buffer A.

The sequential binding of protoplasts with lectin and fluorescent antibody was done by pretreatment of the protoplasts (5×10^5/0.5 ml) with SBA (5, 50, or 250 μg/ml) (1) or WGA (250 μg/ml) (Miles Laboratories, Inc., Naperville, IL) for 1 h at room temperature. The protoplasts were washed by centrifugation (460 *g* for 4 min), resuspended in buffer A, and labeled with the fluorescent probe as described above.

Platelets (American Red Cross, Lansing, MI) derivatized with SBA were prepared as described (35) using paraformaldehyde as fixative. For photobleaching experiments, 5×10^5 protoplasts were incubated with 2×10^8 SBA-coated platelets for 1 h at room temperature. The protoplasts were washed by centrifugation and then labeled with fluorescently derivatized antibody MVS-1 as described.

In photobleaching experiments where the effect of drugs was examined, cells (5×10^5) were preincubated with 1 μM colchicine (COL, Sigma Chemical Co.) or lumicolchicine (prepared as described [34]), or 10 μg/ml cytochalasin B (CB, Sigma Chemical Co.), for 30 min at room temperature. After washing, the protoplasts were treated with lectin or antibody reagents as described above, except that the concentration of each drug was maintained throughout.

Incorporation of the fluorescent lipid, 1-acyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole)aminocaproyl phosphatidylcholine (Avanti Polar Lipids, Inc., Birmingham, AL) was done by incubating protoplasts (5×10^5/0.5 ml) with 40 μg/ml fluorescent lipid for 15 min on ice. The cells were washed by centrifugation and resuspended as described above. To test the effect of SBA on lipid mobility, protoplasts labeled with the fluorescent lipid were incubated with 250 μg/ml SBA for 1 h at room temperature. The protoplasts were then washed and resuspended in 100 μl of buffer A.

FRAP

The lateral diffusion coefficients of fluorescent probes on the plasma membrane of soybean protoplasts were determined by the technique of FRAP (12). Glass microscope slides were prepared as previously described (19). A drop of protoplast suspension was placed on a washed slide, mounted with a coverslip, and sealed with warm paraffin wax. The experimental optics and electronics have been described elsewhere (11, 19). Fluorescence emission for nitrobenzodiazole and fluorescein derivatives was monitored with an incident wavelength of 476.5 nm in combination with a Leitz TK510 dichroic mirror and a K530 barrier filter. For rhodamine derivatized probes, the incident wavelength was 514 nm, and a Leitz TK580 dichroic mirror and a K570 barrier filter were used. The redistribution of fluorescence, after a localized photobleaching pulse, was analyzed using a normal-mode analysis, according to the approach of Koppel et al. (12).

Tests for Interaction between Antibody MVS-1 and SBA

The possibility that SBA might bind antibody MVS-1 was examined by the following two experimental protocols. First, gel filtration studies were conducted. The position of elution of ^{125}I-labeled antibody MVS-1[3] (100 μg, 8×10^6 cpm) was analyzed on a column of Sepharose 4B (50 × 1.1 cm) equilibrated in 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5. To test for possible interaction between SBA and antibody MVS-1, a sample of ^{125}I-labeled antibody MVS-1 (100 μg, 8×10^6 cpm) was incubated with 250 μg SBA in 1 ml of 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5 for 30 min before loading onto the column.

The second approach was to pass [^{125}I]antibody MVS-1 over an affinity column of SBA coupled to Sepharose beads. SBA (10 mg) (1) was coupled to cyanogen bromide–activated Sepharose 4B beads (5 ml) (4). ^{125}I-labeled Antibody MVS-1 (50 μg, 4×10^6 cpm in phosphate-buffered saline [PBS]) (8 g NaCl, 1.15 g Na_2HPO_4, 0.2 g NaH_2PO_4 per liter, pH 7.2) was applied to the column. After washing, the bound material was eluted with 0.2 M galactose in PBS.

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

The "antigen enriched" fraction was prepared from 1×10^8 protoplasts.[3] This sample was passed over an affinity column of SBA coupled to Sepharose beads and treated as above. The unbound material and the galactose eluted fractions were concentrated by ultrafiltration and were analyzed for MVS-1 binding by the "solid phase" binding assay using Immulon-2 plates as described.[3] Alternatively, after passing the "antigen enriched" fraction over the SBA–Sepharose column, ^{125}I-labeled antibody MVS-1 (10^7 cpm) was loaded onto the column and eluted. Radioactivity in the following fractions was determined by gamma counting: (*a*) unbound material; (*b*) 0.2 M galactose eluent; (*c*) 0.2 M galactose plus 2 M NaCl effluent; and (*d*) 0.1 M citrate, pH 3.0 eluent. As a control for both experiments, the same sample was applied to an underivatized column of Sepharose 4B and treated as described.

Analytical Procedures

Protein concentration, in various samples, was determined by the method of Lowry et al. (16) using bovine serum albumin (BSA) (Sigma Chemical Co.) as a standard.

Results

Lateral Mobility of Antibody MVS-1 and Its Fab Fragment Bound to SB-1 Protoplasts

We have generated several mouse hybridoma clones that secrete antibodies directed at components of protoplasts derived from the cultured soybean cell line, SB-1.[3] One hybridoma clone, designated MVS-1, secreted an immunoglobulin that bound to the outer cell surface of the protoplasts. Under nonreducing conditions, the purified mouse IgG of clone MVS-1 yielded a single predominant band (M_r 150,000) (Fig. 1*d*); in the presence of β-mercaptoethanol, the same material showed two polypeptides (M_rs of 55,000 and 23,000) (Fig. 1*a*). The immunoglobulin fraction was also digested with papain to generate monovalent Fab fragments. Under nonreducing conditions, the material that corresponded to Fab fragments yielded a polypeptide component with the expected molecular weight (M_r 48,000) (Fig. 1*e*). After reduction of the Fab material, both the light chain (M_r 23,000) and the frag-

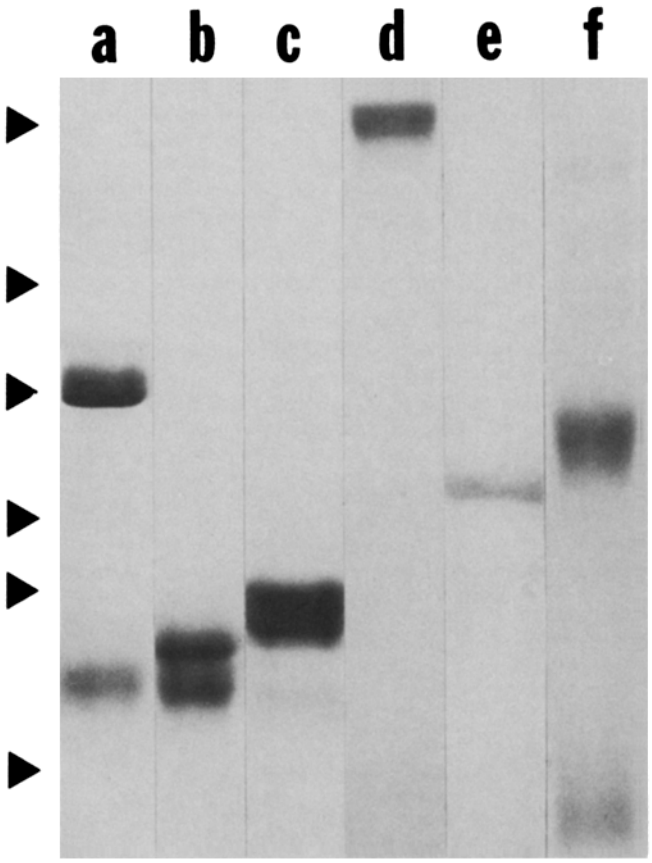

Figure 1. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of immunoglobulin and Fab fragments derived from hybridoma clone MVS-1. The gel consisted of 10% (wt/vol) acrylamide and was stained with Coomassie Blue. Lanes *a, b,* and *c,* electrophoresis in the presence of β-mercaptoethanol; lanes *d, e,* and *f,* electrophoresis in the absence of β-mercaptoethanol. Lanes *a* and *d,* whole immunoglobulin; lanes *b* and *e,* Fab fragment; lanes *c* and *f,* Fc fragment. The arrows indicate the positions of migration of molecular weight markers; from top to bottom: 150,000, immunoglobulin G; 68,000, BSA; 55,000, glutamate dehydrogenase; 40,000, aldolase; 29,000, carbonic anhydrase; and 17,800, myoglobin.

ment (M_r 25,000) of the heavy chain of the immunoglobulin molecule were observed at positions corresponding to their respective molecular weights (Fig. 1 *b*).

The mobility of fluorescently labeled antibody MVS-1 bound to protoplasts at 20°C was determined by the FRAP method. Photobleaching experiments were done on individual, non-agglutinated cells which showed a diffuse distribution of the fluorescent label over the membrane as observed by fluorescence microscopy. Representative data from an experiment using 100 μg/ml antibody MVS-1 are shown in Fig. 2. This graph shows a semilogarithmic plot of the time course of the first normal mode of fluorophore distribution (12) after a photobleaching pulse. Each point represents a complete fluorescence scan across the protoplast. The inset presents a typical scan across the protoplast before the photobleaching pulse. The peaks indicate that the fluorescent antibody is associated predominantly with the plasma membrane, giving more intense fluorescence at the edges of the cell. The data from this and similar experiments did not show any significant heterogeneity in diffusion rates in terms of deviations from a single exponential decay. Therefore, D values were determined from the initial slope of the semilogarithmic plots. In all instances, mobility was unaffected by multiple bleaches at the same site on the protoplast.

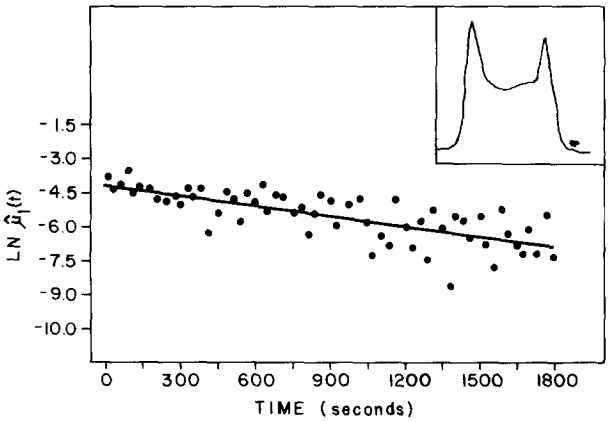

Figure 2. Semilogarithmic plot of $\hat{\mu}_1(t)$ (experimental estimate of the normalized first moment of the fluorophore concentration distribution) as a function of time after the photobleaching pulse. SB-1 protoplasts were labeled with 100 μg/ml of morpholinorhodamine derivatized antibody MVS-1. D, 2.7×10^{-10} cm²/s. The inset shows a scan across the protoplast membrane before the photobleaching pulse.

Table I. Lateral Diffusion Coefficients of Antibody MVS-1 and Its Monovalent Fab Fragment Bound to the Plasma Membrane of Soybean Protoplasts at 20°C

Probe	Concentration	D*	% Recovery*
	μg/ml	$cm^2/s \times 10^{+10}$	
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

* Values are expressed as mean ± standard deviation.

The experiment shown in Fig. 2 yielded a value of 2.7×10^{-10} cm²/s for the diffusion coefficient of antibody MVS-1 (100 μg/ml). As shown in Table I, there was no significant variation in D values when photobleaching experiments were done at lower (50 μg/ml) or higher (250 μg/ml) concentrations of antibody MVS-1. These results indicate that the mobility of antibody MVS-1 on the soybean protoplast belonged to the "relatively fast" group of the two (rather arbitrary) classes of lateral mobility, defined by our previous analysis of lectins (19). The "relatively fast" group was exemplified by WGA with a D value of 3×10^{-10} cm²/s (19).

In previous studies,[3] we had determined the concentration dependence of the binding of ^{125}I-labeled antibody MVS-1. The results showed that the binding reached saturation at a concentration of 100 μg/ml, under conditions corresponding to the FRAP experiments. The lack of variation in D and recovery values with concentration (Table I) also suggests that cross-linking of the antigenic target by antibody MVS-1, at nonsaturating concentrations, does not lead to the formation of large immobile patches. This conclusion is further supported by the results of photobleaching experiments done with monovalent Fab fragments, which yielded D values very similar to those obtained with the intact immunoglobulin (Table I).

Table II. Effect of SBA on the Lateral Diffusion Coefficients of Antibody MVS-1 and its Monovalent Fab Fragment Bound to the Plasma Membrane of Soybean Protoplasts at 20°C

Probe	Concentration	Treatment	D^*	% Recovery*
	$\mu g/ml$		$cm^2/s \times 10^{+10}$	
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	250 μg/ml WGA	2.9 ± 1.1	58 ± 23
Ig	100	SBA–platelets	0.38 ± 0.18	32 ± 8
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

* Values are expressed as mean ± standard deviation.

Modulation of Antibody MVS-1 Mobility by SBA

In previous experiments, we had reported that the binding of unlabeled SBA to SB-1 protoplasts decreased the lateral mobility of a distinct class of mobile molecules as exemplified by a sixfold reduction in the D values of rhodamine-conjugated WGA (19). Since the mobility of antibody MVS-1 bound to the plasma membrane of the soybean protoplast belonged to the "relatively fast" group, similar to WGA, it was of interest to investigate whether SBA could exert its modulatory effect on a single, defined, diffusing component. The results showed that SBA (250 μg/ml) reduced the mobility of MVS-1 ~10-fold ($D = 0.41 \times 10^{-10}$ cm^2/s) (Table II).

This effect of SBA was concentration dependent. High concentrations of SBA (50–250 μg/ml) showed the modulatory effect on the lateral mobility of antibody MVS-1. Low concentrations of SBA (5 μg/ml) were ineffective in reducing the D value of the same fluorescent antibody (Table II). Moreover, this effect on MVS-1 mobility was also specific. Whereas SBA reduced the D value of MVS-1, WGA failed to show the same effect (Table II). Finally, the presence of SBA (250 μg/ml) had no effect on the lateral mobility of a phospholipid probe, 1-acyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole)aminocaproyl phosphatidylcholine, which yielded a D value of 5×10^{-9} cm^2/s (Table II).

Because immunoglobulins are glycoproteins, it was important to establish that antibody MVS-1 did not interact with SBA. Antibody MVS-1 did not bind to an affinity column of Sepharose covalently coupled with SBA. In addition, we have also carried out gel filtration studies of ^{125}I-labeled antibody MVS-1 in the presence and absence of unlabeled SBA. The positions of elution for [^{125}I]antibody MVS-1 under both conditions were essentially identical (corresponding to a molecular species of M_r 150,000, see Fig. 3). It appears, therefore, that the modulation of the mobility of antibody MVS-1 bound on its antigenic target was not due to cross-linking of the mobile protein to the lectin anchored on a set of slow moving receptors. This conclusion is further corroborated by the observation that SBA reduced the D value of monovalent Fab fragments, which have no carbohydrate moiety for SBA binding, of antibody MVS-1 (Table II).

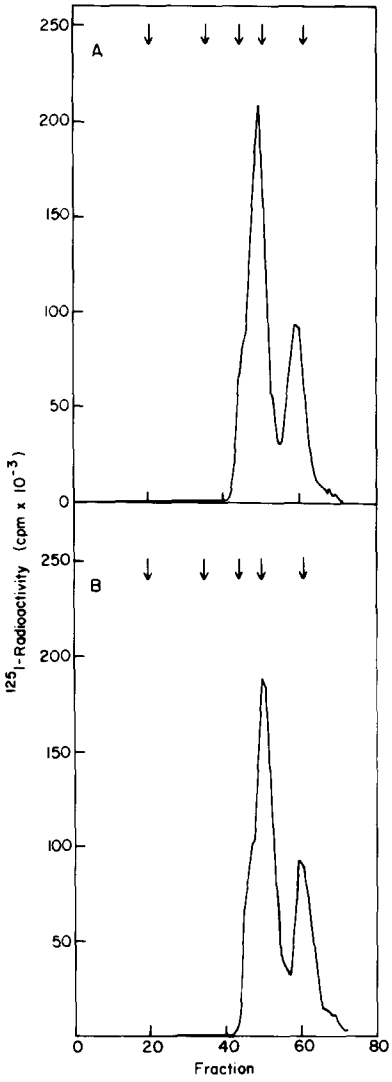

Figure 3. Elution profile of ^{125}I-labeled antibody MVS-1 in the absence (*A*) and presence (*B*) of unlabeled SBA on a column of Sepharose 4B (50 × 1.1 cm). The column was equilibrated in 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5, at room temperature. In *B*, ^{125}I-labeled antibody MSV-1 (100 μg, 8×10^6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

In addition, we have also done experiments to ascertain that the antigenic target of antibody MVS-1 did not interact with SBA. As described previously,[3] the target of antibody MVS-1 can be partially purified by extraction with Triton X-100, followed by removal of the detergent with isoamyl alcohol to yield an "antigen enriched" fraction. This fraction was passed over an affinity column of Sepharose covalently derivatized with SBA. The bound material was eluted with galactose. The presence of the antigenic target of antibody MVS-1 in the bound and unbound fractions was assayed by the "solid phase binding" assay.[3] The material that did not bind to the SBA–Sepharose column retained MVS-1 binding activity (Table III). Moreover, when the column was eluted with galactose, no MVS-1 binding activity was seen in the eluted ma-

terial. This indicates that the antigenic target did not bind to SBA. Further support for this conclusion was obtained by the following experiment. The "antigen enriched" fraction was fractionated on a SBA–Sepharose affinity column. Subsequently, [^{125}I]antibody MVS-1 was passed over the column. Essentially all the radioactivity was recovered in the unbound material. Little or no radioactivity was found in material eluted by: (*a*) 0.2 M galactose; (*b*) 0.2 M galactose plus 2 M NaCl; and (*c*) 0.1 M citrate, pH 3.0. These results indicate that the antigenic target of MVS-1 was not bound to the SBA affinity column. Therefore, the decrease in the lateral mobility of antibody MVS-1 in the presence of SBA does not come about by the cross-linking of the antigenic target of MVS-1 by SBA, to an immobile component.

*Table III. 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‡
	cpm	*cpm*	*cpm*
SBA–Sepharose column			
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.[3]

‡ Specific binding represents binding of normal mouse immunoglobulin subtracted from binding of antibody MVS-1. The data represent the averages of triplicate determinations.

Modulation of Antibody MVS-1 Mobility by Localized Binding of SBA

Platelets, derivatized with SBA, were used to investigate the modulation of mobility of antibody MVS-1 by lectins localized over certain regions of the protoplast. In these experiments, there was a random distribution of SBA–platelets bound to the protoplast, but the SBA-coated platelets covered only a small area of the cell surface (Fig. 4). In a sample of 20 protoplasts, there were ~120 SBA–platelets bound per protoplast, on the average. In contrast, uncoated platelets did not bind to the protoplasts (data not shown). Under these conditions, the lateral mobility of antibody MVS-1 was determined to be 0.38×10^{-10} cm^2/s (Table II). This D value is similar to that obtained previously when protoplasts were pretreated with soluble SBA (see Table II). These data demonstrated that the localized binding of SBA to the plasma membrane of the protoplast resulted in the modulation of mobility of other plasma membrane components in the same fashion as soluble SBA.

Effect of COL and CB on the Modulation of Mobility by SBA

When protoplasts were preincubated with COL (1 μM) before FRAP analysis, the effect of SBA on the lateral mobility of fluorescently labeled antibody MVS-1 was partially reversed (Table IV); the D value increased from 0.41×10^{-10} cm^2/s to 1.3×10^{-10} cm^2/s. This D value is close to that obtained for antibody MVS-1 monitored in the absence of SBA (Table I). Lumicolchicine, a photo-inactivated derivative of COL that does not bind to tubulin (10), failed to yield this reversal of the SBA effect. The D values obtained for surface-bound

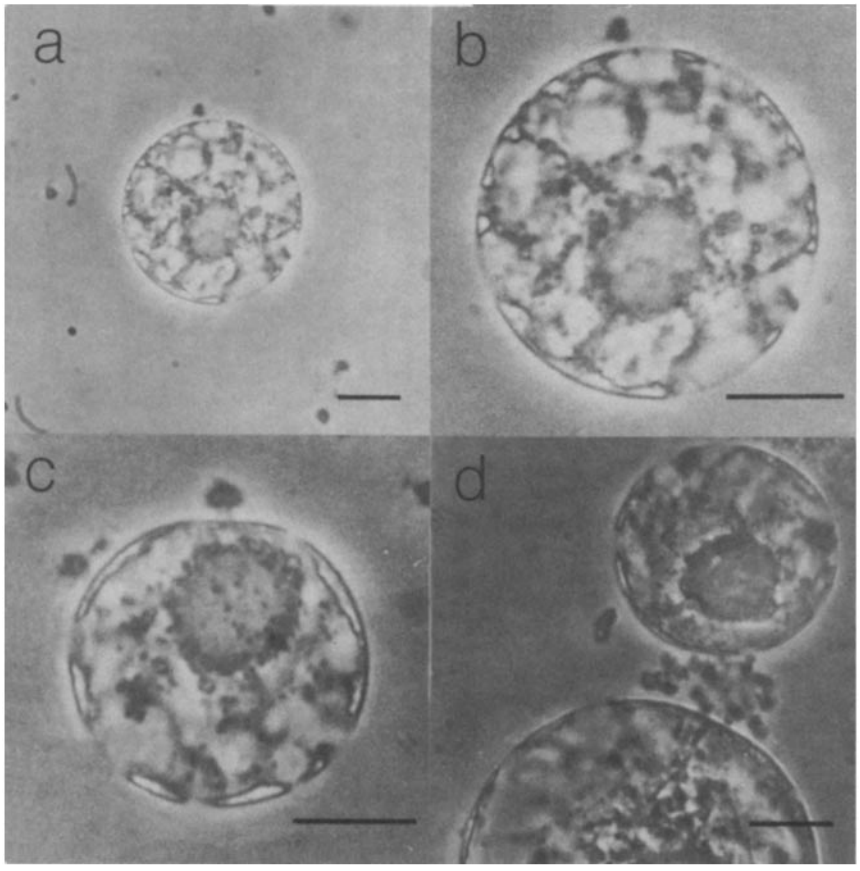

Figure 4. Phase contrast micrographs of SBA-coated platelets bound on SB-1 protoplasts. *b* is an enlargement of *a* with the plane of focus adjusted to display the platelets more clearly. Bar, 25 μm.

Table IV. Effect of Drugs on SBA-induced Modulation of the Lateral Diffusion Coefficients of Antibody MVS-1 Bound to the Plasma Membrane of Soybean Protoplasts at 20°C

Probe	Concentration	Treatment	D*	% Recovery*
	μg/ml		$cm^2/s \times 10^{+10}$	
Ig	100	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
		1 μM COL,	2.7 ± 0.8	63 ± 16
		10 μg/ml CB	6.0 ± 3.2	58 ± 18
		1 μM COL, 10 μg/ml CB	4.0 ± 2.6	61 ± 16
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

* Values are expressed as mean ± standard deviation.

antibody MVS-1 in the presence and absence of COL (1 μM) were comparable (Tables I and IV). Therefore, COL had no effect on the lateral mobility of antibody MVS-1 itself. Similarly, COL also had no effect on the values of the diffusion coefficient of SBA and WGA (see Table IV and reference 32).

In a parallel series of experiments, we found similar results with CB. Preincubation of the protoplasts with CB (10 μg/ml) also reversed the modulatory effect of SBA on antibody MVS-1 mobility (Table IV). Moreover, the simultaneous treatment of protoplasts with both COL (1 μM) and CB (10 μg/ml) completely reversed the effect of SBA (Table IV). The D values obtained from antibody MVS-1 under these conditions were the same as those obtained with the immunoglobulin alone ($D = 4 \times 10^{-10}$ cm^2/s), without either the drugs or SBA. Finally, neither CB nor the combination of CB and COL had any effect on the mobility of antibody MVS-1 itself (Tables I and IV).

Therefore, these results suggest that the binding of certain ligands, such as SBA, to the plasma membrane of soybean cells results in alterations of other components of the soybean plasma membrane in such a way as to restrict the mobility of other receptors, such as those for antibody MVS-1 and for WGA.

Lateral Mobility of Endogenous SBA

Recently, we have demonstrated that SBA is present on the plasma membrane of SB-1 protoplasts.[2] We were interested, therefore, in determining the lateral mobility of the endogenous SBA. Fluorescently derivatized antibodies directed against SBA were used in FRAP experiments to determine the mobility of endogenous SBA. The D value obtained, 2.5×10^{-10} cm^2/s, showed that the endogenous SBA also belongs to the class of "fast" receptors along with the antigenic target of MVS-1 and the receptors for WGA. This result was in direct contrast to exogenously added SBA, which exhibited D values of 4.1×10^{-11} cm^2/s (19).

Discussion

The present experiments document: (*a*) Antibody MVS-1, which binds to a defined target on the surface of SB-1 protoplasts, exhibited diffusional mobility with a D value of 3×10^{-10} cm^2/s; (*b*) the binding of exogenously added SBA to the protoplasts resulted in a 10-fold reduction of the diffusion coefficient of antibody MVS-1 bound on the same cells ($D = 4 \times 10^{-11}$ cm^2/s); and (*c*) COL and CB each reversed, at least partially, the effect of SBA on the lateral mobility of surface-bound antibody MVS-1.

In previous experiments, we have reported values of diffusion coefficients, on SB-1 protoplasts, for several exogenously added lectins including WGA, concanavalin A (Con A), and SBA (19). We found that the D values for the various lectins could be separated (arbitrarily) into two classes: a relatively "fast" group exemplified by WGA ($D = 3 \times 10^{-10}$ cm^2/s) and a relatively "slow" group exemplified by SBA ($D = 5 \times 10^{-11}$ cm^2/s). Our present results indicate that the mobility of antibody MVS-1 bound on the protoplast surface belongs to the relatively "fast" class of protein mobilities. It should be noted, however, that lectins bind to a heterogeneous population of glycoconjugates at the cell surface and therefore, the D values previously determined for these lectins most probably reflect ensemble averages rather than the behavior of any single diffusing species in the membrane.

In contrast, the determination of the D values in the present study used a monoclonal antibody (MVS-1; M_r 150,000) whose target is a defined species (M_r ~400,000).[3] Therefore, the D value of antibody MVS-1 bound to its target reflects the lateral mobility in the plasma membrane of a very high molecular weight complex. Nevertheless, the value of the diffusion coefficient is still of the order of 10^{-10} cm^2/s, a value comparable to those found for many protein ligand–receptor complexes. We also found similar D values for antibody MVS-1 (M_r 150,000) and its univalent Fab fragment (M_r 48,000). These results are consistent with the prediction of the theory of Saffman and Delbruck (25), which suggests that diffusion in a two-dimensional continuum would be rather insensitive to the size of the diffusing entity.

In animal cells, diffusion coefficients have been determined for several defined ligand–receptor complexes: (*a*) rabbit anti-mouse IgG bound on surface immunoglobulin of lymphocytes (9); (*b*) rabbit anti-IgE bound to IgE on mast cells (27); (*c*) α-bungarotoxin bound to acetylcholine receptors on myotubes (2); (*d*) anti-Thy-1 bound to the Thy-1 antigen of mouse spleen cells (5); (*e*) growth factors bound to their specific receptors (14, 30); (*f*) cell adhesion molecules and specific antibodies on embryo fibroblasts (8). More recently, monoclonal antibodies against specific proteins such as histocompatibility antigen H-2 (7), as well as partially characterized antigens (10, 22), have been used to monitor the lateral diffusion of the resulting antibody–antigen complexes.

We have reported previously that pretreatment of SB-1 protoplasts with SBA resulted in the reduction in the mobility of WGA (19) and that COL partially reversed this effect of SBA on the mobility of the WGA receptors (32). Again,

because of the heterogeneous nature of WGA receptors on the cell surface, we could not ascertain whether a reduction in the D value of WGA receptors was due to: (*a*) an increase in the population of "slow" receptors; (*b*) a decrease in the population of "fast" receptors; or (*c*) a real decrease in the intrinsic value of the diffusion coefficient of all WGA receptors. The present study using a monoclonal antibody with a defined target has obviated these difficulties in interpretation. Thus, the binding of SBA, leading to a 10-fold reduction in the value of antibody MVS-1, is most simply interpreted in terms of a real decrease in the intrinsic value of the diffusion coefficient itself.

The mechanism of SBA modulation of receptor mobility is not known. The effect appears specific inasmuch as WGA, which binds to approximately the same number of receptors on the plasma membrane as does SBA (19), failed to yield the same effect. Moreover, localized binding of SBA-coated platelets, covering a small area of the cell surface, is sufficient to restrict the mobility of antibody MVS-1. The lectin binds neither antibody MVS-1 nor its target. Therefore, it does not appear likely that SBA is exerting its effect by directly cross-linking the relatively "fast" ligand–receptor complexes to a set of relatively "slow" SBA receptors. Instead, the present results may be analogous to the modulation by Con A of receptor mobility in a variety of animal cells (6). The binding of Con A to lymphocytes inhibits patch and cap formation of cell surface immunoglobulins as well as many other different receptors. It has also been shown that Con A binding results in a sevenfold reduction in the D values of surface immunoglobulin of lymphocytes (9) and a 10-fold reduction in the D values of receptor proteins on 3T3 fibroblasts (26). This modulation can be partially reversed by COL, which implicates a role for microtubules in the Con A effect.

Consistent with this proposed analogy, we have found that drugs that can disrupt cytoskeletal structures also reversed the effect of SBA on the mobility of both WGA receptors (32) and antibody MVS-1. The targets of these drugs, microtubules for COL and microfilaments for CB, have been identified in plant cells (17, 18, 21, 24, 29, 31, 33). In particular, we have previously identified an actin-like protein in soybean cells whose immunological cross-reactivity with animal actin, chemical properties (M_r 46,000), and binding and polymerization properties, paralleled those of actin (17, 18). These considerations, along with the results reported here, strongly suggest that the binding of external ligands to the plasma membrane of soybean cells can alter the cytoskeletal structures of these cells. These alterations may be similar to animal cell membrane rearrangements that lead to signal transduction across membranes.

Recently, we have obtained immunochemical evidence for the presence of a lectin similar to SBA on the plasma membrane of SB-1 protoplasts.[2] Using fluorescently derivatized antibody directed against seed SBA, we determined the diffusion coefficient of the endogenous SBA to be 2.5×10^{-10} cm^2/s, similar to the D values of WGA (19) and antibody MVS-1 (Table I). The lateral mobility of endogenous SBA is distinctly faster than that of exogenously added SBA ($D = 4.1 \times 10^{-11}$ cm^2/s) (19). The question is now raised as to why the endogenous lectin of SB-1 cells does not modulate the mobility of membrane components as was seen with exogenous SBA. It has been demonstrated that low levels of exogenous SBA (5 μg/ml) do not induce modulation (see Table II). Moreover, exogenous SBA will bind to a large number of glycoconjugates present at the soybean plasma membrane. Therefore, it seems reasonable to propose that the endogenous lectin is present in low numbers such that it cannot induce restricted mobility of other membrane components. In addition, the physicochemical properties of the endogenous lectin remain to be studied. Although this lectin cannot be eluted from the plasma membrane by D-galactose or *N*-acetyl-D-galactosamine,[2] competitive sugars of the seed lectin (15), the occupancy of the saccharide binding sites, as well as their specificity, need to be determined. It is possible that the endogenous lectin is an integral membrane protein whose binding sites are occupied and cannot cross-link other integral membrane components to induce modulation of lateral mobility.

We thank Dr. G. Lark for generous gifts of the SB-1 cell line and Mrs. Teresa Vollmer for her help in the preparation of the manuscript.

This work was supported by Grant 83-CRCR-1-1288 from the U.S. Department of Agriculture, Grant PCM-8011736 from the National Science Foundation, and a McKnight Award for Individual Research Projects in Plant Biology to M. Schindler. M. A. Villanueva was supported by a scholarship from CONACYT (National Council of Science and Technology, Mexico), and J. L. Wang was supported by Faculty Research Award FRA-221 from The American Cancer Society. Publication No. 11842 from the Michigan Agricultural Experiment Station.

Received for publication 15 July 1985, and in revised form 3 January 1986.

References

1. Allen, A. K., and A. Neuberger. 1975. A simple method for the preparation of an affinity absorbent for soybean agglutinin using galactosamine and CH-Sepharose. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 50:362–364.

2. Axelrod, D., P. Ravdin, D. E. Koppel, J. Schlessinger, W. W. Webb, E. L. Elson, and T. R. Podleski. 1976. Lateral motion of fluorescently labeled acetylcholine receptors in membranes of developing muscle fibers. *Proc. Natl. Acad. Sci. USA.* 73:4594–4598.

3. Burgess, J., P. J. Linstead, and V. E. L. Fisher. 1977. Studies on higher plant protoplasts by scanning electron microscopy. *Micron.* 8:113–122.

4. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. Derivatizations of agarose and polyacrylamide beads. *J. Biol. Chem.* 245:3059–3065.

5. Dragsten, P., P. Henkart, R. Blumenthal, J. Weinstein, and J. Schlessinger. 1979. Lateral diffusion of surface immunoglobulin, Thy-1 antigen, and a lipid probe in lymphocyte plasma membrane. *Proc. Natl. Acad. Sci. USA.* 76:5163–5167.

6. Edelman, G. M., J. L. Wang, and I. Yahara. 1976. Surface modulating assemblies in mammalian cells. *In* Cell Motility. T. Pollard, R. Goldman, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. 305–321.

7. Edidin, M., and T. Wei. 1982. Lateral diffusion of H-2 antigens on mouse fibroblasts. *J. Cell Biol.* 95:458–462.

8. Gall, W. E., and G. M. Edelman. 1981. Lateral diffusion of surface molecules in animal cells and tissues. *Science (Wash. DC).* 213:903–905.

9. Henis, Y. I., and E. L. Elson. 1981. Inhibition of the mobility of lymphocyte surface immunoglobulins by locally bound concanavalin A. *Proc. Natl. Acad. Sci. USA.* 78:1072–1076.

10. Jacobson, K., D. O'Dell, and J. T. August. 1984. Lateral diffusion of an 80,000 dalton glycoprotein in the plasma membrane of murine fibroblasts: relationships to cell structure and function. *J. Cell Biol.* 99:1624–1633.

11. Koppel, D. E. 1979. Fluorescence redistribution after photobleaching. A new multipoint analysis of membrane translational dynamics. *Biophys. J.* 28:281–292.

12. Koppel, D. E., M. P. Sheetz, and M. Schindler. 1980. Lateral diffusion in biological membranes. A normal mode analysis of diffusion on a spherical surface. *Biophys. J.* 30:187–192.

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

14. Levi, A., Y. Shechter, E. J. Neufeld, and J. Schlessinger. 1980. Mobility, clustering and transport of nerve growth factor in embryonal sensory cells and in a sympathetic neuronal cell line. *Proc. Natl. Acad. Sci. USA.* 77:3469–3473.

15. Lis, H., B.-A. Sela, N. Sharon, and L. Sachs. 1970. Specific inhibition by N-acetyl-D-galactosamine of the interaction between soybean agglutinin and animal cell surfaces. *Biochim. Biophys. Acta.* 211:582–585.

16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

17. Metcalf, T. N., III, L. J. Szabo, K. R. Schubert, and J. L. Wang. 1980. Immunochemical identification of an actin-like protein from soybean seedlings. *Nature (Lond.).* 285:171–172.

18. Metcalf, T. N, III, L. J. Szabo, K. R. Schubert, and J. L. Wang. 1984. Ultrastructural and immunochemical analyses of the distribution of microfilaments in seedlings and plants of *Glycine max. Protoplasma.* 120:91–99.

19. Metcalf, T. N., III, J. L. Wang, K. R. Schubert, and M. Schindler. 1983. Lectin receptors on the plasma membrane of soybean cells. Binding and lateral diffusion of lectins. *Biochemistry.* 22:3969–3975.

20. Mishell, B. B., and S. M. Shiigi, editors. 1980. *In* Selected Methods in Cellular Immunology. W. H. Freeman, San Francisco. 486 pp.

21. Morejohn, L. C., and D. E. Fosket. 1982. Higher plant tubulin identified by self-assembly into microtubules *in vitro. Nature (Lond.).* 297:426–428.

22. Myles, D. G., P. Primakoff, and D. E. Koppel. 1984. A localized surface protein of guinea pig sperm exhibits free diffusion in its domain. *J. Cell Biol.* 98:1905–1909.

23. Nagata, T., and I. Takebe. 1970. Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. *Plant.* 92:301–308.

24. Pesacreta, T. C., W. W. Carley, W. W. Webb, and M. V. Parthasarathy. 1982. F-actin in conifer roots. *Proc. Natl. Acad. Sci. USA.* 79:2898–2901.

25. Saffman, P. G., and M. Delbruck. 1975. Brownian motion in biological membranes. *Proc. Natl. Acad. Sci. USA.* 72:3111–3113.

26. Schlessinger, J., E. L. Elson, W. W. Webb, I. Yahara, U. Rutishauser, and G. M. Edelman. 1977. Receptor diffusion on cell surfaces modulated by locally bound concanavalin A. *Proc. Natl. Acad. Sci. USA.* 74:1110–1114.

27. Schlessinger, J., W. W. Webb, E. L. Elson, and H. Metzger. 1976. Lateral motion and valence of Fc receptors on rat peritoneal mast cells. *Nature (Lond.).* 264:550–552.

28. Sela, B.-A., J. L. Wang, and G. M. Edelman. 1975. Isolation of lectins of different specificities on a single affinity adsorbent. *J. Biol. Chem.* 250:7535–7538.

29. Shah, D. M., R. C. Hightower, and R. B. Meagher. 1982. Complete nucleotide sequence of a soybean actin gene. *Proc. Natl. Acad. Sci. USA.* 79:1022–1026.

30. Shechter, Y., J. Schlessinger, S. Jacobs, K.-J. Chang, and P. Cuatrecasas. 1978. Fluorescent labeling of hormone receptors in viable cells: preparation and properties of highly fluorescent derivatives of epidermal growth factor and insulin. *Proc. Natl. Acad. Sci. USA.* 75:2135–2139.

31. Vahey, M., and S. P. Scordilis. 1980. Contractile proteins from the tomato. *Can. J. Bot.* 58:797–801.

32. Wang, J. L., T. N. Metcalf III, and M. Schindler. 1983. Lateral diffusion of lectin receptors on the plasma membrane of soybean cells. *In* Chemical Taxonomy, Molecular Biology and Function of Plant Lectins. I. J. Goldstein and M. E. Etzler, editors. Alan R. Liss, Inc., New York. 273–276.

33. Wick, S. W., R. B. Seagull, M. Osborn, K. Weber, and B. E. S. Gunning. 1981. Immunofluorescence microscopy of organized microtubule arrays in structurally stabilized meristemic plant cells. *J. Cell Biol.* 89:685–690.

34. Wilson, L., and M. Friedkin. 1967. The biochemical events of mitosis. II. The *in vivo* and *in vitro* binding of colchicine in grasshopper embryos and its possible relation to inhibition of mitosis. *Biochemistry.* 6:3126–3135.

35. Yahara, I., and G. M. Edelman. 1975. Modulation of lymphocyte receptor mobility by locally bound concanavalin A. *Proc. Natl. Acad. Sci. USA.* 72:1579–1583.