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The Lateral Mobility of Protein and Lipid Components of the Plasma Membrane of Soybean Cells

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THE LATERAL MOBILITY OF PROTEIN AND LIPID COMPONENTS OF THE PLASMA MEMBRANE OF SOYBEAN CELLS

by

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DEDICATION

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for her patience, support and love without which this would not have been possible.

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AFC12	5-N-(dodecanoyl)-aminofluorescein
ATPase	adenosine-5'-triphosphatase
BBS	bicarbonate buffered saline
BSA	bovine serum albumin
CB	cytochalasin B
colch	colchicine
Con A	concanavalin A
D	diffusion coefficient
DEAE	diethylaminoethyl
DiI	1,1'-diacyl-3,3,3',3'-tetramethylindocarbocyanine iodide
$DiIC_{14}$	1,1'-ditetradecyl-3,3,3',3'-tetramethylindocarbocyanine iodide
$DiIC_{18}$	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine iodide
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetate
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetate
fl	fluorescence microscopy
FRAP	fluorescence redistribution after photobleaching
Ig	immunoglobulin

MGB	modified Gamborg's buffer
MGB-N_3-BSA	modified Gamborg's buffer containing 0.01 M sodium azide and 0.2% (w/v) bovine serum albumin
M_r	molecular weight
NBD	N-4-nitrobenzo-2-oxa-1,3-diazole
NBD-PC	1-acyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidylcholine
NBD-PE	N-4-nitrobenzo-2-oxa-1,3-diazolephospha-tidylethanolamine
PA	phophatidic acid
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
ph	phase microscopy
PI	phosphatidylinositol
%R	per cent recovery
SBA	soybean agglutinin
s-Con A	succinyl concanavalin A
SDS	sodium doceyl sulfate
SEM	standard error of the mean
tris	tris(hydroxymethyl)aminomethane
TX-100	triton X-100
WGA	wheat germ agglutinin

CHAPTER I

LITERATURE REVIEW

LITERATURE REVIEW

The Plasma Membrane of Plant Cells

The plasma membrane is a differentially permeable barrier which separates the cell from its external environment. However, knowledge of the composition, structure and function of this organelle in plant cells has been greatly hindered by the presence of the cell wall. Application of sufficient force to disrupt the cell wall leads to the rupture of not only the plasma membrane, but also of other cellular organelles, resulting in a well mixed population of membranes. As a first approximation, it has been assumed that plant plasma membranes are fundamentally similar to plasma membranes in animal and bacterial systems which have been studied extensively. However, the data to verify this assumption is still lacking. The recent development of plant tissue culture techniques and the enzymatic preparation of protoplasts has helped to advance knowledge in this field.

Initial demonstration for the presence of a plasma membrane in plants was obtained by plasmolysis (1). This leads to the retraction of the plasma membrane away from the cell wall and allows it to be visualized by light microscopy. Further characterization of cross-sections of plant tissue by electron microscopy has demonstrated the plasma

membrane to be a tripartite structure, approximately 10 nm thick (2, 3) similar to plasma membranes from other systems (4). Freeze fracture electron microscopy has shown this membrane to consist of an amorphous matrix in which particles are randomly embedded (5, 6). Moreover, Williamson (7) and Burgess and Linstead (8) have demonstrated, using the lectin concanavalin A (Con A) as a probe, that membrane glycoconjugates are mobile in the plane of the plasma membrane. The plasma membrane emerges as a membrane comprised of an amorphous lipid matrix in which proteins are embedded. It seems reasonable to describe the structure of the plasma membrane of plants in terms of the fluid mosaic model of Singer and Nicolson (9). However, a satisfactory understanding of the function and structure of this membrane requires a detailed analysis of its chemical composition.

Isolation of the Plasma Membrane

The procedures used in the purification of plasma membrane from plant cells and protoplasts have been reviewed (10, 11). In general, the tissue is homgenized, and subjected to differential and density gradient centrifugation. The peak equilibrium density of plasma membrane vesicles in sucrose is 1.14-1.17 g/ml (12). Using these techniques, plasma membrane preparations of 60-80% purity have been obtained (1, 12). However, there have been few studies on the characterization of isolated plasma membrane vesicles with respect to "sidedness" or chemical composition (13, 14), due to the lack of an accepted set of markers for the

plasma membrane in plants (10). Currently, the commonly used markers for plant plasma membranes are: (a) phosphotungstate-chromate (12, 15) or $LaCl_3$ (16) for selective staining of the plasma membrane; (b) K^+-ATPase activity (12, 15, 17, 18); (c) glucan synthetase II activity (19, 20); (d) N-1-naphthylphthalamic acid binding (21, 22); and (e) use of surface labels (16, 17, 24-28). However, there is no unequivocal enzymatic activity which is used as a marker for the plasma membrane (1, 10, 16). Nevertheless, when both morphological and enzymatic markers are used in conjunction, they can provide for reasonable identification of the plasma membrane in membrane mixtures. To date, the data obtained with these various markers and approaches appears to be consistent.

The purification of the plasma membrane from soybean protoplasts has been reported (25). The plasma membrane was specifically labeled with [^{35}S]diazotized sulphanilic acid prior to lysis of the protoplasts. Previous work had demonstrated that this reagent specifically labeled cell surface lipids and protein in erythrocytes (29). Following homogenization the majority (97%) of the radioactivity incorporated in the protoplasts was associated with the particulate fraction. In contrast, 50% of the total protein was soluble. The labeled membranes were fractioned by continuous isopycnic sucrose gradient centrifugation. After centrifugation, the [^{35}S] radioactivity peak was found to be associated with K^+-ATPase activity plasma membrane marker

(12, 15, 17, 18). The same fraction was devoid of enzymatic activities associated with mitochondria, nuclear envelope or the endoplasmic reticulum.

Perlin and Spanswick have also used this technique to label the cell surface of corn protoplasts (26). However, the diazosulphanate method will not label all plant plasma membranes. Instead, Schibeci et al. (27) used the iodogen method (30) to surface label the plasma membrane of ryegrass protoplasts. Because the reagent is bound to the reaction vessel, very low levels of intracellular labeling occur by this technique.

To facilitate the separation of the plasma membrane from other cellular organelles, Schibeci et al. (27) made use of a monoclonal antibody which bound to galactose residues. After lysis of the protoplasts, the plasma membranes form large sheet-like structures which are easily separated from other membrane vesicles by centrifugation. However, the authors did not provide any independent identification for the putative plasma membrane fractions through the use of biochemical or morphological markers (10).

Overall, there have been relatively few reports on the purification of plasma membrane from plant cells. Moreover the identification of this membrane is hampered by the lack of unambiguous markers. The combined use of cell surface labeling (24-28) with K^+-ATPase activity (15, 17, 18) and phosphotungstate-chromate (12, 15) or $LaCl_3$ (16) staining

may provide for the best identification of purified plasma membrane fractions.

Chemical Composition of the Plasma Membrane

In general, the plasma membrane of plant cells consists of (on a weight basis) 40-50% lipid (31) and 35-40% protein (31, 32). The remaining mass of the plasma membrane is comprised of carbohydrates in the form of glycolipids, glycoproteins and cell wall polysaccharides (1). Since highly purified preparations of plasma membranes from plant cells are not available, the chemical composition of this membrane cannot be detailed exactly. Although analyses have been done by various groups, the validity of these reports is questioned since they lack rigorous verification. Therefore, only a brief, general description of the chemical components will be given.

Lipids represent the major constituent of the plasma membrane (31, 33). Not only do these compounds provide the structural basis of the bilayer membrane, but they also play an important role in controlling the fluidity of the membrane (34, 35) and in the activity of membrane proteins (34). The major classes of lipids in plants are: phospholipids, glycosylglycerides, and steroids (both free and acetylated steroyl glycosides) (33). Of these compounds, phospholipids are the major lipid component present in the plasma membrane (33). The primary forms of phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylglycerol (PG) (33,

36). Together, PC and PE account for 50-70% of the total phospholipids (31, 36) in the plasma membrane. It is noteworthy that the levels of PG and PI are much higher in the plasma membrane of plants (33, 36) than are found in animal plasma membrane (37). The phospholipid composition of plasma membrane from soybean cells (36) is shown in Table 1.

A second major lipid component of the plasma membrane is sterols. These compounds are present both as the free sterol and as glycosterols which can be acetylated with a fatty acid (33). Preparations from soybean root have shown that the major sterol in the plasma membrane is sitosterol (74%) (38). The other sterols which are present are campesterol (15%) and stigmasterol (10%). In contrast to animal membranes, cholesterol was present at very low levels (0.03%). The ratio of sterols to phospholipids is much higher in plasma membrane of plants (17, 39) than in that of animals (37, 40). This high sterol content of plant membrane has important consequences in terms of lipid ordering (41) and membrane fluidity (42, 43).

Also present in plant membranes are various forms of glycolipids, primarily galactolipids (33). These compounds are found primarily in the chloroplast membrane (33). However, autoradiographic evidence for the incorporation of [^{3}H]galactose into plasma membrane lipids suggests that galactolipids are also present in this membrane (44). These data support previous findings of galactolipids in the plasma membrane (31).

Table 1. Phospholipid Composition of Plasma Membrane from Soybean Hypocotyls (36).

Phosphatidylethanolamine	26
Phosphatidylcholine	24
Phosphotidylinositol	10
Phosphatidylserine	--
Phosphatidylglycerol	26
Phosphatidic acid	15

Data are expressed as % of total membrane phospholipid.

Further investigations are required in order to ascertain the lipid compositon of the plasma membrane. The lack of purified membrane preparations is the primary limiting factor in this research. With the recent development of plant tissue culture technology, cultured cells have been used more frequently in investigations of lipid composition of various cellular organelles. It is noteworthy that cells grown in tissue culture retain not only their original lipid composition (45, 46) but also their response to diverse environmental stimuli such as light and temperature (47).

Proteins of the plasma membrane have been identified (a) through the use of cell surface labeling with radioactive reagents (25-28), (b) localization of auxin receptor (21, 22, 48-50) and (c) identification of various enzymatic activities (12, 15, 17-20).

The cell surface components of intact protoplasts have been labeled with radioactive iodine or sulfur (25-27). The incorporated radioactivity can be then precipitated with trichloroacetic acid. Furthermore, the labeling reaction with [^{35}S]sulphanilic acid shows saturable incorporation of radioactivity into trichloroacetic acid precipitates (27). These experiments demonstrate that cell surface proteins are accessible to external probes. Finally, one and two dimensional polyacrylamide gel electrophoresis of plasma membrane preparations have shown a large number of protein bands are present in these preparations (13, 14, 39).

A few specific protein molecules have been identified at the plasma membrane of plant cells. The high affinity auxin binding sites have been localized to the plasma membrane (21, 22, 48, 49) using N-1-naphthylphthalamic acid, an inhibitor of auxin transport. The receptor is believed to play a role in the polar transport of auxins. Support for this hypothesis was obtained recently; a monoclonal antibody has been used to identify auxin transport carrier in pea (Piscum sativum) stem cells (50). Immunofluorescence with this antibody localized the carrier system to the plasma membrane of this tissue. The lectin, wheat germ agglutinin (WGA), has also been identified in situ by Mishkind et al. (51). Using ferritin labeled second antibody technique, WGA has been shown to be present in cells that establish direct contact with the soil. Within these cells, the lectin is localized at the plasma membrane-cell wall interface and in the protein bodies.

A number of enzymes have been localized to the plasma membrane of plant cells (12, 15, 17-20). Glucan synthetase (β 1-3) II activity has been reported to be associated with phosphotungstate-chromate stained (morphological evidence for plasma membrane) fractions (10, 49). Preliminary evidence has also indicated that there is a β 1-4 glucan synthetase activity associated with the plasma membrane and Golgi apparatus (50). However, attempts to correlate the distribution of glucan synthetase II activity with plasma membrane K^+-ATPase activity have yielded mixed results (10).

Therefore the localization of the glucan synthetase activity to the plasma membrane relies heavily on the specificity of the morphological staining.

Both cytochemical and biochemical evidence support the localization of a K^+-ATPase activity to the plasma membrane (10, 19, 20). The exact function of this enzyme is not presently understood. It is believed that this activity is involved in coupling metabolic energy to the transport of ions across the plasma membrane (1). However, ATPase activity in subcellular fractions is not solely restricted to the plasma membrane (10), thus this activity is not an unequivocal marker for the plasma membrane.

Carbohydrates are the third and final major component of the plasma membrane. It is estimated that about 20% of the mass of the plasma membrane is carbohydrate in nature (1). The primary form of the plasma membrane carbohydrates is probably cell wall polysaccharides, but glycoproteins and glycolipids are also present (1). The presence of glycoconjugates has been demonstrated by the binding of radioactive lectins to either protoplasts (24, 28) or membrane fractions (52). Moreover, using ferritin labeled Con A, the binding of the lectin to protoplasts has been visualized by electron microscopy (7, 8).

The prescence of carbohydrate residues on the plasma membrane of ryegrass protoplasts has been demonstrated by Schibeci et al. (27). Using the myeloma protein J539 that binds to galactosyl residues they were able to agglutinate

these protoplasts. This agglutination could be completely inhibited by galactose.

Various lipids of the plasma membrane are in the form of glycolipids. These include sterols, galactolipids and phytoglycolipids (33). Furthermore, there appear to be associated with the plasma membrane glycosyl transferase activities specific for sterols (33). However, the exact role of these activities in glycolipid metabolism is not clear. Moreover, other organelles contain the same activities, so the possibility of contamination is raised once more.

Overall, the plasma membrane of plant cells is a very poorly described biochemical entity. Elucidation of the chemical components and localization of enzymatic activities specific to this organelle will require a reliable method for preparation of purified plasma membranes.

Plasma Membrane Receptor Mobility

The plasma membrane has often been viewed as a static structure whose function is to act as a barrier between the cytoplasm and the external environment. This idea of a rigid membrane was also reflected in the models proposed to describe membrane structure (53). However, phenomenological observations such as membrane ruffling or pseudopod formation suggested that the plasma membrane was fluid in nature, and that some of the components of the membrane move about within the boundaries of the membrane.

Dynamic evidence of translational mobility of proteins in the plane of the plasma membrane was obtained by Fyre and Edidin (54). Human and mouse cells in culture were induced to fuse with each other. The movement of mouse and human cell surface antigens was detected by immunofluorescence, and a different fluorescent probe was used for each species. Immediately after fusion, two distinct and unmixed regions of red and green fluorescence could be identified on the surface of a single heterokaryon cell. However, after 40 min incubation at 37°C, there was a mosaic of red and green fluorescence evenly distributed over the entire cell surface of the heterokaryon. The intermixing was not sensitive to inhibitors of protein synthesis or metabolic energy. However, low temperature (<15°C) inhibited redistribution of the fluorescent probes. The most probable explanation of these data was that the mixing of fluorescence, and hence species-specific antigens, occured by diffusion of these molecules in the plane of the membrane.

Two other experiments, in cells with unmodified membranes, provided further evidence for the dynamic nature of the components of the plasma membrane. In the first experiment (55), incubation of mouse lymphocytes, in the cold, with fluorescently derivatized rabbit anti-mouse immunoglobulins resulted in ring staining of the cells. When the cells were warmed to 37°C, there was rapid redistribution of the fluorescence from ring stain into numerous patches and then into a "cap" structure at one pole of the cell.

Furthermore, the formation of patches, but not caps, was independent of metabolic energy and thus suggestive of a diffusion event. In contrast, when the experiment was repeated using monovalent Fab fragments, no redistribution of fluorescence was seen. The aggregation of cross-linked surface receptors could only occur if these molecules were free to diffuse in the membrane. Further research has since demonstrated that lectins can also induce patch formation of cross-linked receptors (56, 57).

In the second experiment, a patch of fluorescence was created on a long cultured muscle fiber using fluorescent monovalent Fab fragments (58). The size of this fluorescent patch increased with time. However, this movement of fluorescence could be inhibited by low temperatures or fixation of the cell. The diffusion coefficient (D) of the labeled complex was estimated to be 1×10^{-9} cm^2/s.

Other evidence indicating that proteins integrated into the lipid bilayer could undergo diffusion in the membrane was obtained by electron microscopy (59, 60). Intramembranous particles were seen in freeze fractures of membranes, but not in liposomes. Proteolytic or physical treatments could induce clustering of these particles. These observations, coupled with the ability of membrane components to undergo lateral diffusion, lead to the formulation of a new model for membrane structure - the fluid mosaic model (9).

The Fluid Mosaic Model of Membrane Structure

Although this model was based originally on thermodynamic considerations of membranes and membrane components (61), the model also incorporated results from dynamic and structural experiments (54-60). Some of the key points of this model are detailed below. The biomembrane, according to the fluid mosaic model, is visualized as a two-dimensional "liquid" mosaic, consisting of the lipid bilayer, in which integral membrane proteins float. These proteins, which represent a heterogenous group, are arranged in an amphipatic manner (61, 62) in the membrane. Thus, the charged, polar portions are extended out into the aqueous environment, while the non-polar portions of the protein molecules are sequestered away in the hydrophobic core of the lipid bilayer. This structure maintains the free energy of the membrane at a minimum by reducing interactions between non-polar components and the aqueous environment. The membrane lipids are organized as a discontinuous fluid bilayer and form the matrix of the bilayer. Although there is no long range ordering of the lipid (9), specific, short range interactions with integral proteins are possible. As a consequence, most integral membrane proteins have a large degree of freedom in terms of rotational and lateral mobility, and in turn are randomly distributed throughout the membrane. Furthermore, a protein of sufficient size can span the entire lipid bilayer and be in contact with both the cytoplasmic and external environments simultaneously

(62). Therefore, this model of membrane structure is formally analogous to a two-dimensional oriented solution of proteins in a viscous phospholipid bilayer. The rate at which a protein diffuses in the plane of the membrane would in turn be controlled by the viscosity of the lipid bilayer as well as by any specific interactions of the protein with other soluble or membrane proteins.

Receptor-Cytoskeletal Structure Interactions in Eukaryotic Cells

The fluid mosaic model of membrane structure was incomplete in many aspects of its description of the dynamic alterations that occur in membranes. Particularly significant were the observations of directed motion (e.g. cap formation of specific probes and the global restriction of receptor mobility when a single agent is applied locally to a small portion of the cell surface. As a result of several independent lines of experimentation from various laboratories, a new view of the cell surface has been put forth in which surface receptors are hypothesized to interact with cytoplasmic assemblies of macromolecular structures (57, 64, 65). This assembly is thought to consist of a metastable, tripartite structure: (a) certain surface receptors that bind to external ligands; (b) microfilaments and associated proteins that are responsible for systematic movement of receptors; and (c) microtubules that are responsible for reversible anchorage of receptors (Fig. 1).

Figure 1. A model for the organization of various components in the cell surface membrane complex. The model assumes that microfilaments (MF) interact with receptors, possibly via vinculin, and that microfilaments in turn interact with microtubules (MT). The interactions among the various components are assumed to involve reversible association-dissociation reactions. It is assumed that the intramembraneous particles (IMP) are not involved in these equiliberia.

Most of the early evidence suggesting the existence of this macromolecular assembly was derived from perturbation experiments using external ligands to reveal certain cellular states (for a review, see reference 64). These experiments can be arbitrarily classified into the following categories: (a) the induction of receptor redistribution (patch and cap formation) on lymphocytes and other cell types and the inhibition of receptor movement by cytochalasin B which disrupts microfilaments (55, 66); (b) the modulation of receptor mobility at the level of individual receptors by the binding of the lectin Con A (67) and the reversal of this effect of Con A by colchicine which dissociates the microtubules (68); (c) the selective and directed movement of certain surface transport sites on polymorphonuclear leukocytes during the process of phagocytosis and the loss of the selective effect after treatment with colchicine (69, 70); and (d) the co-redistribution of cell surface receptors and the microfilament and microtubule components as revealed by fluorescence and electron microscopy (56, 71, 72).

More recently, however, there has been an intensive accumulation of structural evidence in support of the receptor-cytoplasmic assembly interaction hypothesis. The key experiments that have to bear on this hypothesis are summarized below. First, it has been shown that certain cell surface receptors penetrate the lipid bilayer and are exposed to both the cytoplasmic and external environment simultaneously (73, 74). This is particularly important as

it immediately suggests a means by which receptor-cytoplasmic interactions may take place. Second, the existence of microfilaments and microtubules has been documented in a survey of many different animal cell types including non-muscle cells (75-77). Moreover, there is now strong evidence to indicate that these microfilament and microtubule proteins interact with the cell surface membrane (78-80). Third, there is also recent evidence for direct molecular interactions between cell surface receptors and the actin subunit of microfilaments (81, 82) as well as between the microtubule and microfilament proteins themselves within the assembly (83). The dynamic evidence, which served as the basis of the receptor-cytoplasmic interaction hypothesis (Fig. 1) is supported, therefore, by the presently available structural information.

Fluorescence Redistribution after Photobleaching

With the accumulation of dynamic evidence of protein lateral mobility (54-58), research emphasis shifted to the development of techniques which would enable quantitative analysis of protein diffusion to be done. Three such methods have since been developed: two measure rotational diffusion (and will not be discussed here) and the third measures lateral diffusion.

The technique of photobleaching was initially used to measure the lateral movement or, diffusion coefficient (D), of rhodopsin molecules in the plasma membrane of rod outer segments (84). Using a brief pulse of intense light, one

half of the molecules were irreversibly bleached, thus creating an asymmetric distribution of rhodopsin absorbance. The rate of diffusion of unbleached rhodopsin molecules into the bleached region of the membrane was measured spectrophotometrically. The D value calculated for rhodopsin was 4 x 10^{-9} cm^2/s.

This work was possible because of the spectral properties of the rhodopsin molecule. The photobleaching technique has subsequently been used to measure the lateral mobility of proteins and lipids through the use of fluorescent probes. An advantage of this technique of fluorescence redistribution after photobleaching (FRAP) is its high sensitivity, imparted to it by the fluorescence emission. Since its first use (85), the technique has been elaborated upon and extensively used in various systems (for reviews, see 86-88).

The method of FRAP involves uniform labeling of the membrane with a fluorescent probe. In studies of protein mobility, fluorescein or rhodamine derivatives are commonly used (86-88). A number of fluorescent lipid analogs have been used to measure lipid mobility including (a) fluorescein or nitrobenzodiazole (NBD) derivatives of fatty acids (89), (b) NBD-phospholipids (86-88) or sterols (90, 91), and (c) carbocyanine dyes with long hydrocarbon tails (87-89, 92). A fluorescence microscope is used to observe a single cell and collect the resulting fluorescence for analysis (86, 87, 93). A small area of the cell surface (<10 um^2) is

briefly illuminated with an intense beam of light from a laser. This results in the irreversible photobleaching of the fluorophores located in this spot, and therefore a significant decrease in the fluorescence. The diffusion of fluoresence from the unbleached surrounding membrane into the bleached spot is then monitored with respect to time. From this recovery process, the diffusion coefficient may be calculated (86, 87, 93).

Studies on the mobility of membrane components have provided information about (a) membrane organization and the control of membrane mobility, (b) functional significance of diffusional processes in membranes. Tables 2 and 3 summarize diffusion coefficients for various protein and lipid probes in both artificial bilayers and plasma membranes. In general, diffusion coefficients of both lipids and proteins are $1\text{-}5 \times 10^{-8}$ cm^2/s in artificial lipid bilayers (86, 87). The analysis of lipid diffusion in cell membranes shows a decrease in the D value to 5×10^{-9} cm^2/s (86, 87). In contrast, the lateral diffusion of proteins in membranes is greatly restricted, giving diffusion coefficients of 5×10^{-10} cm^2/s or slower (86-88). Of particular interest is the fact that a particular diffusing species of protein could have an immobile component (87).

The restriction of lipid mobility in membranes has been ascribed to the presence of both proteins and steroids in the membrane (86-88). In fact, incorporation of digitonin into the inner mitochondrial membrane creates two diffusing

Table 2. Lateral Diffusion Coefficients of Lipid Probes in Lipid Bilayers and Plasma Membranes of Cells.

Probe	Membrane	D* ($cm^2/s \times 10^{+9}$)	%Recovery	Temp (°C)	Ref
NBD-Chol	Liposome	0.17 ± 0.06	77	<25	91
		16 ± 3	91	>25	91
	RBC Ghost	1.5 ± 0.1	75	21	90
NBD-PE	Liposome	0.21 ± 0.02	79	<25	91
		1.8 ± 0.2	91	>25	91
	RBC Ghost	1.6 ± 0.4	77	21	90
	V 79 Lung Fibroblast	3.1 ± 0.6	75	22	94
$DiIC_{18}$	3T3 Mouse Fibroblast	6.0 ± 3.0	82	--	92
	V 79 Lung Fibroblast	6.2 ± 3.1	89	--	94
AFC12	A-6 Epithelial	13.3 ± 4.6	--	--	95

NBD, N-4-nitrobenzo-2-oxa-1,3-diazole; Chol, cholesterol; PE, phosphatidylethanolamine; $DiIC_{18}$, 1,1'-dioctyldecyl-3,3,3',3'-tetramethylindocarbocyanine iodide; AFC12, 5-(N-dodecanoyl)aminofluorescein.

* data are expressed as mean ± standard deviation.

Table 3. Lateral Diffusion Coefficients of Proteins in Lipid Bilayers and Bound to the Plasma Membrane of Cells.

Probe	Membrane	D* ($cm^2/s \times 10^{+9}$)	%Recovery	Temp (°C)	Ref
NBD-Gram	Liposome	350 ± 50	--	22	96
Con A	3T3 Mouse Fibroblast	1.3 ± 0.9	17	23	97
s-Con A	3T3 Mouse Fibroblast	5.1 ± 1.7	74	23	97
	Mouse Macrophage	4.9 ± 1.3	40	22	98
WGA	NIL-8M Human Fibroblast	4.0 ± 0.5	50	25	99
Fab	3T3 Mouse Fibroblast	2.4 ± 0.5	47	--	97
NGF	PC-12 Pheochromocytoma	6.8 ± 1.3	35	--	100

NBD-Gram, N-4-nitrobenzo-2-oxa-1,3-diazole derivatized gramicidin; Con A, concanavalin A; s-Con A, succinyl-concanavalin A; WGA, wheat germ agglutinin; Fab, monovalent Fab fragments of rabbit antibodies directed against mouse leukemic cell line P388;NGF, nerve growth factor.

* values are expressed as mean ± standard deviation.

species of lipid (101). In contrast, the basis for the large decrease (>100 fold) in protein mobility is not clearly understood. It has been postulated that interactions of membrane proteins with either the cytoskeletal elements of the cell or the extracellular matrix may be responsible in part (86-88, 102).

Lectin-Induced Modulation of Lateral Mobility of Cell Surface Receptors

Several groups have reported a quantitative analysis, at the molecular level, of lectin-induced modulation of mobility of cell surface receptors (98, 103-105). Previously, Con A has been shown to inhibit the formation of patches by various cross-linked membrane proteins (67). This effect could also be induced by localized binding of Con A to the cell membrane (57). The technique of FRAP was used to directly monitor the lateral mobility of surface bound fluorescent monvalent Fab fragments directed against leukemic mouse cell line P 388 on 3T3 fibroblasts (103) and monovalent Fab fragments of rabbit immunoglobulin G directed against mouse immunoglobulin on mouse lymphocytes (98, 104, 105).

Schlessinger et al. (103) observed a D value of 2.6 x 10^{-10} cm^2/s for anti p388 Fab fragments bound to 3T3 fibroblasts. Incubation of Con A-labeled platelets with the fibroblasts induced a 10 fold reduction in mobility of the Fab fragments (D = 3.6 x 10^{-11} cm^2/s). However, there was no change in the percent recoveries, as compared to control

values, indicating that the Con A did not induce the immobilization of the antibody-antigen complex in the membrane. Finally, no modulation of mobility was seen if less than 4% of the cell surface were covered by Con A-platelets. Treatment of the cells with microtubule poisons, Colcemid or vinblastine, resulted in a slight reversal of modulation ($D = 6 \times 10^{-11}$ cm^2/s).

A more complete study was subsequently reported by Henis and Elson (104). The lateral mobility of surface immunoglobulins of lymphocytes was reduced 7 fold in the prescence of Con A-platelets. A simultaneous decrease in the mobile fraction was seen, indicating that approximately 90% of the fluorescent probes were unable to diffuse. Pretreatment of the cells with colchicine, which dissociates microtubules, or cytochalasin B, which disrupts microfilaments, resulted in partial reversal of the Con A effect as seen by an increase in the diffusion coefficient ($D = 2.4 \times 10^{-10}$ cm^2/s) and in the mobile fraction (30%). Moreover, these drugs had a synergistic effect. When used together there was complete inhibition of the Con A induced modulation ($D = 5.6 \times 10^{-10}$ cm^2/s). It should be noted that there was no effect of the drugs on mobility of the surface bound fluorescent probe in the absence of the platelets. The binding of Con A, above the threshold level, is postulated to induce a propagated, long range rearrangement of the lymphocyte membrane such that mobility of the receptors is restricted. The involvement of the cytoskeletal macro-

molecules in this effect was demonstrated by partial reversal of the Con A modulation through pretreatment of the cells with either colchicine or cytochalasin B. The exact mechanism by which the modulation of mobility is achieved is still not understood. There are at least three possible mechanisms which can be considered: (a) enhancement of the interaction between receptor and cytoskeleton without a significant change in structure or stability of the binding; (b) stabilization of the cytoskeleton in a state with a higher binding affinity for the transmembrane protein (this could occur through a linker protein); and (c) an overall increase in the polymerization state of membrane associated cytoskeleton such that it forms a general barrier to lateral motion. Further experiments are required to elucidate the exact mechanism of modulation.

Recently it has been demonstrated that the Con A induced modulation of mobility is selective. When the mobility of the histocompatibility antigen, H-2, on mouse lymphocytes was examined by FRAP (105) no difference in the diffusion coefficient was observed in the presence or absence of Con A-platelets. Because Con A induced modulation is known to involve the cytoskeleton, these results suggest that selectivity exists in the interactions between the cytoskeleton and transmembrane proteins, such as the H-2 antigen. These results are in contrast to previous reports that in the P815 cell line, derived from murine mastocytoma,

there is a stable, possibly direct, interaction between H-2 and actin (81, 82).

Finally, previous studies have demonstrated that lectins can induce association of membrane proteins with the cytoskeleton (57, 106). Biochemical evidence for such interactions has been recently obtained by Painter *et al.* (107, 108). Brief treatment of platelets with Con A results in the rapid association of two integral membrane glycoproteins with the Triton X-100 insoluble cytoskeleton. These glycoproteins have been shown to form a complex with actin *in vitro* without any additional linking protein.

Microfilament Proteins in Plant Cells

The belief that all eukaryotic cells contain the same general apparatus for a variety of movements such as cytoplasmic streaming, amoeboid movement, and phagocytosis has prompted an extensive search for muscle-like structures. Observations using electron microscopy have shown that filamentous structures (5-7 nm in diameter) similar to muscle thin filaments exist in many types of eukaryotic cells, including a variety of non-muscle cells of animal origin, vascular plants, slime mold, algae, amoeba and fungi (for reviews, see references 65, 109, 110). These fibers can be characteristically identified as actin filaments by reversible binding of heavy meromyosin, which yields arrowhead decoration of the structures as observed by electron microscopy.

Actin, the principal protein component of microfilaments, has been found in all eukaryotic cells, from fungi to mammals. In this wide variety of cell types, the basic molecular structure of actin has been highly conserved in a polypeptide of molecular weight 45,000. Three classes of actin have been established on the basis of isoelectric points: α, β and γ. The most acidic form, α, is found predominently in striated muscle, while the other two forms are cytoplasmic species.

Actin has been identified and partially purified from wheat germ (111), tomato (112) and in soybean seedlings (113). The molecular weight of the polypeptide chain (M_r 45,000), immunochemical reactivity with antibodies directed against calf thymus actin, and the formation of heavy meromyosin decorated filaments (5-7 nm in diameter) all indicate that soybean actin has the basic structural and functional features reported for animal cell actin (113, 114). Meagher and co-workers (115-117) have cloned the multigene family encoding for actin-like proteins in soybean and have reported the complete nucleotide sequence of these actin genes. The deduced amino acid sequence of plant actin resembles both cytoplasmic and muscle specific actins.

A large number of studies of actin microfilaments in plant cells have focused on the subject of ctyoplasmic streaming, chromosome movement and chloroplast movement (for review see 118). Cytoplasmic streaming has been analyzed extensively in the Characean algae; the streaming follows a

spiral path along the length of the cell. Microfilament bundles which bind heavy meromyosin (119) and antibodies to actin (120) occur at the boundary between stationary cortex and streaming cytoplasm (subcortical actin bundles). The experiments of Kamiya and colleagues have led to the view that force was generated at the interface between the stationary cortex and streaming cytoplasm (121). In addition, the drugs cytochalasin B (122, 123) and phallodin (124) inhibit cytoplasmic streaming. The use of the latter drug is particularly significant in view of the highly specific interaction between the drug and filamentous actin and the stabilization of filamentous actin against depolymerization by cytochalasins and DNase. Thus, fluorescent phallodin has been used to observe filamentous actin structures in algae cells and in conifer root (125, 124).

Microtubules in Plant Cells

The major structural unit of microtubules is the protein tubulin, a dimer (M_r = 110,000) composed of α and β subunits of almost equal molecular weight (for review see 127). Initial work on isolation of tubulin from animal sources was facilitated by the fact that colchicine, which binds to the protein, can be used to monitor the course of purification. Unfortunately, colchicine binding by plant tubulin is less stable (128), although it has been detected in a range of plant species and tissues (128, 129). From higher plants, tubulin has been isolated from fern (130), Vigna seedlings (131), and from cultured cells of tobacco

(132) and Paul's Scarlet Rose (133). The latter study provided unequivocal identification by demonstrating the ability to self-assemble *in vitro* to form structures morphologically characteristic of microtubules. More recently, Fosket and co-workers (134) have shown that tubulins from different higher plants are immunologically non-identical and bind colchicine differentially.

The visualization of cytoplasmic microtubules in cells of higher plants and protoplasts by immunofluorescence after permeabilization of the cell has been recently achieved (135-137). In multicellular root apices, antibodies to porcine brain tubulin reacted with all types of microtubule arrays, namely, interphase cortical microtubules, preprophase bands, the mitotic spindle, and phragmoplast microtubules.

In addition to colchicine, other drugs such as the *Vinca* alkaloids and phenyl carbamates and benzimidazole carbamates affect microtulular systems. Isopropyl N-phenyl carbamate and chloroprophane are antimicrotubular in algae (138) and induced multipolar spindles and other abnormalities of cell division and differentiation in higher plant cells (139). One other compound, taxol, deserves mention because it shifts assembly-disassembly equilibrium toward assembly, unlike all the other drugs (140, 141). Finally, it has been recently reported that gibberellins stabilize microtubules from disruption by colchicine (142).

It is clear that microtubules play many different roles in plant cells; these include: (a) arrays of cytoplasmic microtubules enable many wall-less plant cells to deviate from a spherical shape; (b) microtubules mediate movements in establishing polarities, such as preprophase bands and planes of division; (c) microtubules mediate movements and anchorage of organelles; and (d) microtubules mediate movements in cell cortex during cell wall deposition. The data of Marchant and many other workers have shown that microtubules exert an effect on shaping walled cells by influencing the orientation of cellulose deposition and imposing directionality upon otherwise isotropic turgor forces acting to expand the cell (138, 143). Cortical microtubules regulate the orientation in which wall microfibrils are deposited, as deduced from numerous observations of microtubule-microfibril coalignment and colchicine-induced abnormalities. For example, the zone of secondary wall formation in root hair cells showed short microtubules at various angular deviations from the longitudinal axis of the hair, similar to the deviation pattern of the wall microfibrils (144).

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CHAPTER II

LECTIN RECEPTORS ON THE PLASMA MEMBRANE OF SOYBEAN CELLS. BINDING AND LATERAL DIFFUSION OF LECTINS.

ABSTRACT

Protoplasts prepared from suspension cultures of roots cells of Glycine max (SB-1 cell line) bound soybean agglutinin (SBA), concanavalin A (Con A), and wheat germ agglutinin (WGA). Binding studies carried out with ^{125}I-labeled SBA, Con A and WGA showed that these interactions were saturable and specific. Fluorescence microscopy demonstrated uniform membrane labeling. The mobility of the lectin-receptor complex was measured by fluorescence redistribution after photobleaching. The diffusion coefficients (D) for SBA and Con A were 5×10^{-11} and 7×10^{-11} cm^2/s, respectively. In contrast, WGA yielded a diffusion coefficient of 3×10^{-10} cm^2/s. Pretreatment of the protoplasts with either SBA or Con A resulted in a 6 fold reduction in the mobility of WGA ($D = 5 \times 10^{-11}$ cm^2/s). These results suggest that the binding of SBA or Con A may lead to alterations of the soybean plasma membrane which, in turn, may restrict the mobility of other receptors.

INTRODUCTION

The measurement of the dynamics of plasma membrane receptors has catalyzed research into the role of lateral mobility as an important component of transmembrane signaling mechanisms (1-3). From these investigations, two different schemes have emerged for the motion membrane components: (a) lateral mobility as a consequence of Brownian movement (4-6) and (b) directional flow on the cell surface (7, 8). Lateral mobilitiy based on diffusional fluxes in a two-dimensional continuum has been characterized for a great number of membrane proteins (3), and to a lesser extent for a few varieties of lipid (3) and glycolipid (9, 10). In general, these measurements have been performed by using the technique of fluorescence redistribution after photobleaching (FRAP) (3, 11) and have yielded values of from 10^{-8}-10^{-12} cm^2/s for membrane proteins to 10^{-8}-10^{-9} cm^2/s for phospholipids and glycolipids in the same membranes. Of particular interest was the observation that a particular diffusing species of protein could have an immobile component (3). A number of theories have been presented to explain this type of

protein mobility in the context of intraplasma membrane and cytoskeletal interactions with the diffusing molecule (10, 12, 13).

The other type of movement observed for cell membrane components was a directional flow ultimately leading to cap formation on lymphoid cells (7) and the movement of a concanavalin A (Con A)-receptor complex during late anaphase or telophase to the developing furrow in J7742 mouse macrophages (8). This type of protein mobility has been ascribed to cross-linking of membrane recpetors that normally occurs only when exogenously added ligands bind to the receptors. One receptor movement mechanism need not exclude the other, since receptors cross-linked by specific ligands and undergoing directed motion, e.g. capping, should not interfere with other receptors randomly diffusing through the plane of the membrane.

Although the use of reagents, such as the lectin Con A, to induce and modulate receptor redistribution provided much of the intial information on receptor mobility of both types, relatively little attention has been focused on whether the plant-derived lectins have similar interactions with receptors of plant cells. In the present study, we have prepared protoplasts from a suspension culture of soybean (Glycine max) cells and investigated the binding and lateral diffusion of lectins bound to the plasma membrane of these cells. FRAP measurements performed on a series of soybean protoplasts

demonstrated a slower mobility and higher recovery for Con A and soybean agglutinin (SBA) than observed in animal cells. Moreover, a significant decrease was observed for the mobility of wheat germ agglutinin (WGA) receptors in the presence of SBA or Con A.

MATERIALS AND METHODS

Cell Culture and Protoplast Isolation.

The SB-1 cell line of soybean (Glycine max) was kindly provided by Dr. F. Constabel (Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada) and was grown in the dark. Protoplasts were prepared by a modified procedure of Constabel (14). Actively growing SB-1 cells (24-48 hours after transfer) were digested with an equal volume (20 ml) of enzyme solution containing 400 mg Cellulysin (Calbiochem, La Jolla, CA), 200 mg pectinase (Sigma, St. Louis, MO), and 2 g D-sorbitol (Sigma), pH 5.5. After 2 hours, the protoplast suspension was filtered through a 48-um nylon filter and pelleted by centrifugation in a clinical centrifuge for 4 min at 460 x g. The pelleted protoplasts were washed by gentle resuspension and centrifugation by using 5 ml of protoplast medium (14), which was modified by substituting 30 g of D-sorbitol for glucose (modified Gamborg buffer, MGB). After three

(N-acetyl-D-galactosamine for SBA, α-methyl-D-mannoside for Con A and N-acetyl-D-glucosamine for WGA).

Binding of ^{125}I-Labeled Lectins.

Con A was labeled with radioactive iodine (^{125}I; Amersham, Arlington Heights, IL) by using the iodogen 1,3,4,6-tetrachloro-3a,6a-diphenyglycouril (17). SBA and WGA were iodinated according to the method of Greenwood et al. (18). Protoplasts (5 x 10^5/ml) were incubated with ^{125}I-labeled lectins for 60 min at room temperature. After three washes by centrifugation and resuspension of the cells in MGB-N_3-BSA, the protoplasts were transfered to gamma counting vials and the radioactivity was determined. Nonspecific binding was determined by incubating the radioactive lectin in the presence of the competitive saccharide. The values for specifically bound lectin were obtained by subtracting the amount of nonspecifically bound lectin from the total bound.

Fluorescence Redistibution after Photobleaching.

Lectins directly conjugated with fluorescein or rhodamine were obtained from Vector Laboratories (Burlingame, CA) and were used in all the fluorescence photobleaching experiments. Protoplasts were prepared for photobleaching by the following procedure: (a) protoplasts (5 x 10^5/ml) were incubated with fluorescently derivatized lectin at 5, 50 or 250 ug/ml

for 20 min at 23°C; (b) the protoplasts were washed by centrifugation and resuspension in 1 ml of MGB-N_3-BSA; (c) after the protoplasts were washed, they were suspended in a final volume of 50 ul of MGB-N_3-BSA. For experiments involving the inhibitor of cell wall regeneration, coumarin (Aldrich, Milwaukee, WI), MGB containing 1.4 mM coumarin was used instead of MGB-N_3-BSA.

The sequential labeling of protoplasts with two different lectins was done by pretreatment of the protoplasts (5 x 10^5/ml) with 50 ug/ml of SBA or Con A for 20 min at 23°C. After the cells were washed by centrifugation and resuspension in MGB, they were labeled with 50 ug/ml rhodamine-derivatized WGA as described above.

In experiments where the effect of drugs was examined, cells (5 x 10^5) were preincubated with 1 uM colchicine (Sigma) or lumicolchicine (prepared as described (19)) in MGB for 30 min at room temperature. After washing, the protoplasts were treated as described above, except that the concentration of the drug was maintained throughout the experiment.

Prewashed glass slides were placed in phosphate buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 2.3 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, pH 7.4) for at least one min, rinsed with distilled water, and dried prior to use. Cover slips were rinsed in distilled water and

dried. A drop of the protoplast suspension was placed on a washed slide, mounted with a coverslip, sealed with warm paraffin wax, and subjected to FRAP.

The lateral diffusion coefficients of fluorescently labeled lectins bound to the plasma membrane of protoplasts were measured by the FRAP method as previously described (20). The experimental optics and electronics have been described in detail elsewhere (11). Briefly, a Leitz Ortholux II fluorescence microscope, equipped for incident illumination, is used to focus an argon laser beam (diameter~1 um) onto the sample and collect the fluorescence for detection and subsquent processing by photon-counting electronics. The angular orientation of the incident laser beam, and hence the location on the sample of the focused spot along the scan axis, is controlled by a servo-activated galvanometric optical scanning mirror. Fluorescein emission 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 emission, the incident wavelength was 514.0 nm, and a Leitz TK580 dichroic mirror and a K570 barrier filter were used. All measurements were done with a X40/0.65 NA dry objective. The redistribution of the fluorescence, following a localized photobleaching pulse, was analyzed with a normal-mode analysis, following the approach of Koppel et al. (20).

Scanning Electron Microscopy

Freshly isolated protoplasts were prepared for scanning electron microscopy by fixation in 2% (v/v) glutaraldehyde as described by Williamson et al. (21). The cells were post-fixed in 2% (w/v) osmium tetroxide at 4°C for 30 min. After washing, the cells were applied to a glass cover slip coated with poly-L-lysine (1% (w/v) in PBS; Miles), dehydrated in alcohol, critical point-dried and coated with carbon, followed by gold. The specimens were examined on a JEOL JSM 35C at an accleration voltage of 15kV. Photographs were taken using Polaroid 665 film.

RESULTS

The Binding of Lectins to Soybean Protoplasts

Protoplasts prepared from suspension cultures of soybean root cells bound the lectins SBA, Con A and WGA. Typical fluorescence microscopy results, obtained after incubation for 20 min at 23°C, are shown in Fig. 1. The general labeling patterns of the cells showed a diffuse ring-like stain, suggesting that most of the receptors were uniformly distributed in the cell membrane (Fig. 1). However, in a number of protoplasts (accounting for about 15% of the population), the fluorescence was localized in patches spread over one hemisphere of the cell or completely segregated over one pole of the cell (Fig. 1, B top, C bottom). Both of these patterns were observed for all lectins tested in our study. The proportion of cells showing patches or polar distribution of fluorescence did not change with different times of incubation. Finally, the binding of the lectins to the protoplasts resulted in agglutination of some of these cells (Fig. 1, A bottom, B top).

Figure 1: Fluorescence staining patterns of soybean protoplasts treated for 20 minutes at 23 °C with various lectins. (A) Soybean agglutinin (50 ug/ml); (B) concanavalin A (50 ug/ml); (C) wheat germ agglutinin (50 ug/ml). ph, phase contrast microscopy; fl, fluorescence microscopy; magnification 260x; bar = 25 um.

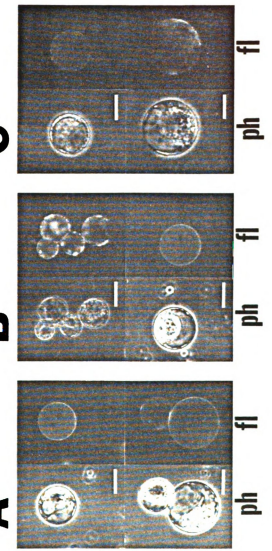

Figure 1

The results of control experiments demonstrated that the observed fluorescence patterns were due to binding of the lectin to carbohydrate structures on the plasma membrane. First, protoplasts prepared from the SB-1 cell line did not exhibit autofluorescence. Second, the majority (95 %) of the protoplasts used in our studies did not stain with the fluorescent dye, Calcofluor, which is commonly used to demonstrate the presence of cell wall material (22). Fig. 2 A is an example of the fluorescent staining due to the binding of Calcofluor to the cell wall of intact soybean cells. In marked contrast, a similarly stained protoplast, shown in Fig. 2 B, exhibits no fluorescence. Third, when freshly prepared protoplasts were fixed in glutaraldehyde, followed by osmium tetroxide and examined by scanning electron microscopy, the plasma membrane was devoid of cellulose microfibrils, as shown in Fig. 3 C, D. The cellulose microfibrils of the cell wall are readily visualized on untreated cells (Fig. 3 A, B).

We have also found that the binding of SBA, Con A and WGA was inhibited by the monosaccharides N-acetyl-galactosamine, α-methyl-D-mannoside, and N-acetyl-glucosamine, respectively. Finally, incubation of protoplasts with fluorescein-labeled or rhodamine labeled goat antibodies directed against rabbit immuno-globulin in the absence of lectins did not show any fluorescence. All of these results strongly suggest that

Figure 2: Fluorescence pattern of soybean cells and soybean protoplast treated with Calcofluor (0.05% w/v). (A) Undigested cells (magnification 200x, bar = 40 um); (B) protoplast (magnification 320x, bar = 25 um). ph phase contrast microscopy; fl, fluorescence microscopy.

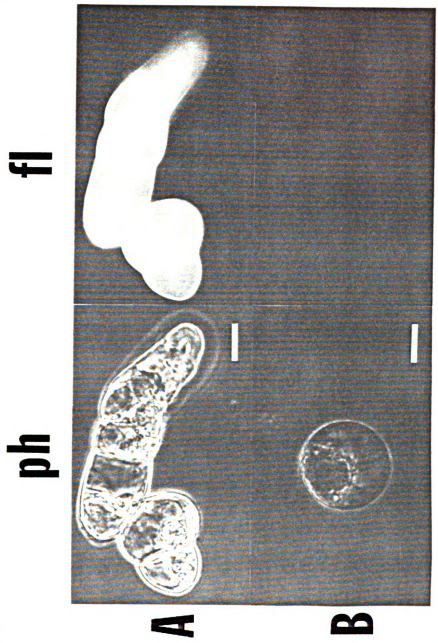

Figure 2

Figure 3: Scanning electron micrographs of undigested soybean cells and protoplast. (A) Undigested soybean cells showing cell wall (magnification 1500x; bar = 5 um); (B) undigested soybean cell showing cellulose microfibrils of cell wall (magnification 11,500x; bar = 1 um); (C) freshly isolated protoplast (magnification 2960x; bar = 4 um); (d) high magnification of freshly isolated protoplast showing no cellulose microfibrils on plasma membrane (magnification 11,250x; bar = 1 um).

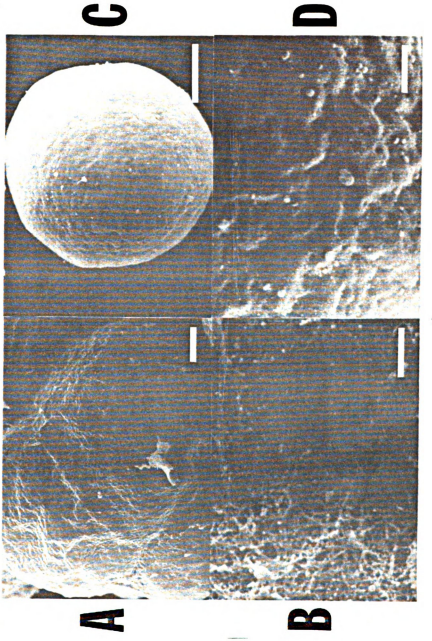

Figure 3

the observed staining patterns for each of the lectins could be ascribed to the binding of the ligand to heterosaccharide structures of the plasma membrane.

The binding of the lectins, SBA, Con A, and WGA to soybean protoplasts was dependent on the concentration of the ligand added (Fig. 4). This was demonstrated by incubating ^{125}I-labeled lectin with soybean protoplasts for 1 h at 23°C. Under these conditions, the binding of each one of the lectins can be saturated. At 50 ug/ml, the concentration used for many of the FRAP and fluorescence microscopy studies, there are approximately 5×10^7, 2×10^8, and 4×10^7 subunits of SBA, Con A, and WGA bound per cell, respectively.

Lateral Mobility of Lectins Bound to Soybean Protoplasts

The mobility of fluorescently-labeled lectins bound on protoplasts at 23°C was determined using the FRAP method. Photobleaching experiments were done on individual, non-agglutinated cells. Representative data from an experiment using SBA (50 ug/ml) are shown in Fig. 5. This graph shows a semilogarithmic plot of the time-course of the first normal mode of fluorophore distribution (20) after a photobleaching pulse. Each point represents a complete fluorescence scan across the protoplast. The inset presents a typical scan across the protoplast prior to the photobleaching pulse. The peaks indicate that the fluorescent lectin is associated predominantly with the membrane, giving more intense

Figure 4: Dose response curve of the binding of ^{125}I-labeled lectin to soybean protoplasts. ^{125}I-labeled lectin was incubated with 5 x 10^5 protoplasts/ml for 1 hour at 23°C in duplicate. Non-specific binding of ^{125}I-labeled lectin was determined by incubating [^{125}I]lectin with the protoplasts in the presence of the competing sugar, and substracted from total [^{125}I]lectin bound for each concentration. The subunit molecular weights used in the calculations were 3 x 10^4, 2.6 x 10^4 and 1.8 x 10^4 for soybean agglutinin, and concanavalin A and wheat germ agglutinin, respectively.

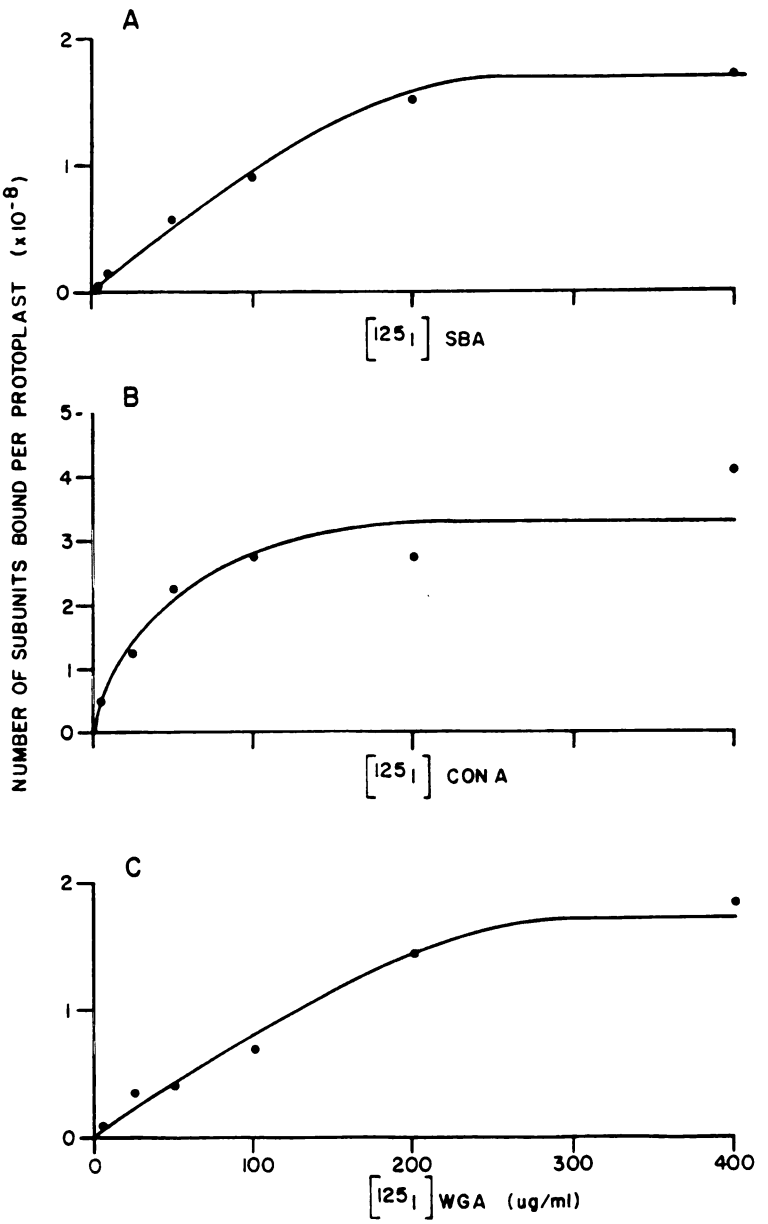
A
B
C
NUMBER OF SUBUNITS BOUND PER PROTOPLAST (x10⁻⁸)
[125I] SBA
[125I] CON A
[125I] WGA (ug/ml)
0
1
2
3
4
5
100
200
300
400

Figure 4

Figure 5: (A) Semilogarithmic plot of $\hat{u}_1(t)$ (the experimental estimate of the normalized first moment of fluorophore concentration distribution) as a function of time after photobleaching on a soybean protoplast labeled with fluorescein-derivatized soybean agglutinin. D = 3.6 x 10^{-11} cm^2/s. (B) A typcial scan across the protoplast membrane prior to the photobleaching pulse.

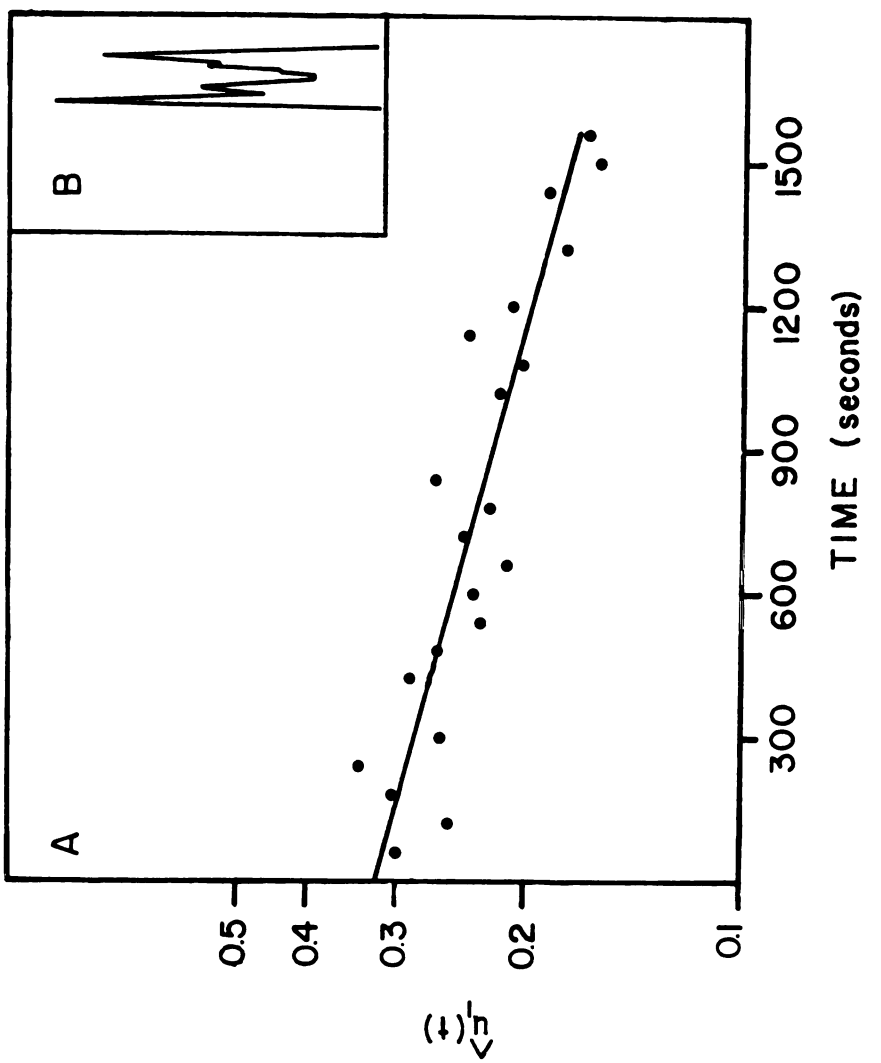

Figure 5

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, diffusion coefficients were determined from the initial slopes of the semilogarithmic plots and correspond to the ensemble average D values.

The experiment shown in Fig. 5 yielded a value of 3.6 x 10^{-11} cm^2/s for the diffusion coefficient of SBA (50 ug/ml) at 23 ^{o}C . D values of this magnitude are indicative that the component, though mobile in the membrane, has very slow lateral mobility. Furthermore, we did not obtain any significant change in the diffusion coefficient (Table I) when the photobleaching experiments were repeated using either very low (5 ug/ml) or very high (250 ug/ml) concentrations of SBA (see Fig. 4). The molecular weight of SBA under these conditions was estimated to be 120,000 by gel filtration studies. Therefore, the aggregation of SBA into high molecular weight complexes (23) is not involved in the slow diffusion constant.

Two further experiments were conducted to ascertain the validity of the D values obtained for SBA. First, the presence of sodium azide (NaN_3-BSA) in the protoplast medium was shown to have no effect on the mobility of SBA bound to the protoplasts (Table 1). Second, in order to prevent the deposition of newly

Table 1. Lateral Diffusion Coefficients of Soybean Agglutinin Bound to Soybean Protoplasts at 23 °C.

Soybean Agglutinin Concentration (ug/ml)	Treatment	D^a ($cm^2/s \times 10^{+11}$)	$\%R^{a,b}$
5	- -	5.8 ± 4.6	83 ± 11
50	- -	4.6 ± 2.1	91 ± 6
250	- -	6.2 ± 2.6	84 ± 8
50	no Na_3-BSA	6.9 ± 0.4	83 ± 8
50	1.4 mM Coumarin	2.1 ± 1.8	79 ± 10

[a] values are expressed as mean ± standard deviation.
[b] %R, % recovery. This value represents the fraction of membrane bound fluorophore which is mobile and able to diffuse on the membrane.

synthesized cell wall material on the surface of the protoplast plasma membrane, a series of photobleaching experiments were conducted in the presence of coumarin, an inhibitor of cell wall synthesis (24). The resulting diffusion coefficient (2.1 x 10^{-11} cm^2/s) showed no significant difference from previous data. Thus, the slow lateral mobility of SBA bound to protoplasts is not due to the regeneration of cell wall components and is not affected by the lack of metabolic energy.

The diffusion constants of other lectins bound on their receptors of the soybean protoplast are summarized in Tables 2 and 3. At a concentration of 50 ug/ml, Con A yielded a diffusion constant of 7.2 x 10^{-11} cm^2/s, similar to that obtained for SBA (see Table 1). The diffusion constant for succinyl-Con A, a chemical derivative of Con A (25), was approximately 1 x 10^{-10} cm^2/s (Table 2). In contrast, WGA gave D values of ~3 x 10^{-10} cm^2/s (Table 3). Therefore, it was apparent that lectins bound to soybean membranes exhibited two (rather arbitrary) classes of lateral mobility: (a) relatively fast ($D \cong 3 \times 10^{-10}$ cm^2/s) and (b) relatively slow ($D \cong 5 \times 10^{-11}$ cm^2/s).

Modulation of WGA-Receptor Mobility by SBA and Con A

The effect of the binding of unlabeled SBA or unlabeled Con A on the mobility of the more rapidly diffusing rhodamine-conjugated WGA was studied. In both cases, the D values of the labeled lectin were decreased

Table 2. Lateral Diffusion Coefficients of Concanavalin A and Its Succinyl Derivative Bound to Soybean Protoplasts at 23°C.

Probe	Concentration (ug/ml)	D^a ($cm^2/s \times 10^{+10}$)	$\%R^{a,b}$
Con A	5	0.41 ± 0.09	86 ± 8
	50	0.72 ± 0.52	78 ± 11
	250	1.4 ± 0.09	83 ± 16
Succinyl-Con A	5	1.5 ± 1.4	93 ± 4
	50	1.0 ± 0.6	90 ± 9
	250	3.8 ± 2.8	92 ± 7

[a] values are expressed as mean ± standard deviation.
[b] %R, % recovery. This value represents the fraction of membrane bound fluorophore which is mobile and able to diffuse on the membrane.

Table 3. Lateral Diffusion Coefficients of Wheat Germ Agglutinin Bound to Soybean Protoplasts at 23 °C.

Concentration (ug/ml)	Treatment	D[a] ($cm^2/s \times 10^{+10}$)	%R[a,b]
5	- -	1.5 ± 0.2	86 ± 7
50	- -	3.0 ± 1.8	84 ± 8
250	- -	4.3 ± 0.8	80 ± 9
50	Pretreat with 50 ug/ml SBA	0.46 ± 0.17	34 ± 11
50	Pretreat with 50 ug/ml Con A	0.55 ± 0.32	41 ± 14
50	Pretreat with 1 uM colchicine 50 ug/ml SBA	1.3 ± 0.9	37 ± 11
50	Pretreat with 1 uM lumicolchicine 50 ug/ml SBA	0.35 ± 0.06	36 ± 9

[a] values are expressed as mean ± standard deviation
[b] %R, % recovery. This value represents the fraction of membrane bound fluorophore which is mobile and able to diffuse on the membrane.

by six-fold (Table 3). Therefore, WGA yielded diffusion constants of 4.6×10^{-11} cm^2/s in the presence of SBA and 5.5×10^{-11} cm^2/s in the presence of Con A. Both of these values approached the diffusion coefficients of the unlabeled lectin (see Tables 1 and 2).

Using ^{125}I-labeled WGA, we have determined that pretreatment of soybean protoplasts with unlabeled SBA (50 ug/ml) did not alter the number of molecules of WGA bound to the cells. There were approximately 4×10^7 WGA subunits bound per protoplast at a concentration of 50 ug/ml [^{125}I]WGA. This value is comparable to that obtained in the absence of SBA (Fig. 4 C). Therefore, the decrease in D values of WGA in the presence of SBA most probably cannot be ascribed to changes in the number of receptors (either the disappearance of a population of rapidly diffusing receptors or the appearance of a population of relatively immobile receptors).

Because many lectins are themselves glycoproteins, it was important to establish that WGA did not interact with SBA or Con A. Using double immunodiffusion assays, we found no evidence of interaction in the two pairs of lectins. We have also carried out gel filtration studies of ^{125}I-labeled WGA in the presence and absence of unlabeled SBA. The positions of elution for ^{125}I-WGA under both conditions were essentially identical. It appears, therefore, that the modulation of the mobility of WGA bound on its receptors was not due to cross-

linking of the mobile lectin to another lectin anchored on a set of slow-moving receptors.

Effect of Colchicine on the Modulation of Mobility

When protoplasts were preincubated with colchicine (1 uM) prior to FRAP analysis, the effect of SBA on the lateral mobility of fluorescently labeled WGA was partially reversed (Table 3). The D value increased from 0.46×10^{-11} cm^2/s to 1.3×10^{-10} cm^2/s, close to the D value of WGA in the absence of SBA (Table 3). Lumicolchicine, a photo-inactivated derivative of colchicine which does not bind to tubulin (26), had no effect on the SBA modulation (Table 3).

DISCUSSION

Plant lectins have been shown to bind and agglutinate protoplasts prepared from carrot (27), grapevine (28), soybean (21), tobacco (28-30), broad bean (30) and leek (31) cells. Our present results confirm the observations of Williamson and co-workers on soybean cells (21). Calcofluor staining and scanning electron microscopy show neither the characteristic fluorescence indicative of cell wall material (22) nor cellulose microfibrils (29), respectively. Although small amounts of residual cell wall components may still be present, the specific saturable binding of lectins observed is best interpreted in the context of lectin binding to heterosaccharide structures that are plasma membrane components in a manner analogous to animal cells.

These considertions are particularly important in interpreting the results of experiments aimed at assessing the lateral mobility of lectins bound to the plasma membrane of protoplasts. The key observations of the present study include: (a) the diffusion coefficient of SBA bound on soybean membrane is 5×10^{-11} cm^2/s; this value is not significantly affected by ligand

concentration, by the presence of sodium azide, a metabolic inhibitor, or by coumarin, an inhibitor of cell wall regeneration (24); (b) the diffusion coefficient of Con A bound to protoplasts is similar to that observed for SBA ($D = 7 \times 10^{-11}$ cm^2/s); (c) in contrast, WGA showed much higher lateral mobility on the protoplasts, yielding diffusion coefficients of 3×10^{-10} cm^2/s; (d) the binding of SBA or Con A to soybean protoplasts can trigger a reduction in the mobility of WGA ($D = 5 \times 10^{-11}$ cm^2/s).

In attempting to analyze our results in the context of animal cell membrane models, which have been formulated based on a larger body of experiments, it is important to examine the membrane composition of the plant cell. Recently, it was reported that purified plasma membrane fractions from soybean hypocotyl have high levels of negatively charged phospholipids (33). Furthermore, another major difference that may play some role in possible differences in lateral mobility in plants is that soybean roots contain very low levels of cholesterol (34), a membrane component implicated in a number of diffusion control mechanisms in animal cells (35, 36). This, however, is probably compensated for by high levels of β-sitosterol and stigmasterol, the major phytosterols of the plant membrane (34).

If the lipid and sterol composition of membranes were the dominant factors in the ability of intramembranous

components to diffuse, then one would, to a first approximation, expect to see lower D values for lectin receptor mobility for plant cells as compared to animal cells. Given our results for the diffusion constant of WGA ($D = 3 \times 10^{-10}$ cm^2/s), which is comparable to similar experiments in animal cells for WGA, this simple assumption may not be valid. However, a larger difference is observed for Con A and SBA diffusion constants on the soybean protoplast membrane when compared to the reported mobilities of Con A on animal cells. SBA has not been used in animal cell studies. Although the diffusion measurements for Con A have shown large variations (3), it is now generally accepted that in most animal cell systems, the mobility of Con A receptors varies between $1\text{-}5 \times 10^{-10}$ cm^2/s (37, 38). Compared to these values, the diffusion constant of Con A on soybean protoplasts ($D = 7 \times 10^{-11}$ cm^2/s) is considerably slower than reported for 3T3 fibroblast, erythrocytes and rabbit lymphocytes (37, 39, 40). The behavior of SBA was similar to that of Con A, namely $D = 5 \times 10^{-11}$ cm^2/s. Preliminary experiments indicate that SBA does not share the same receptors with Con A and WGA on the soybean protoplasts. This conclusion is based on the observation that mild trypsinization of soybean protoplasts abrogates the binding of SBA without appreciable effects on the binding of WGA and Con A (T. Metcalf, unpublished observations).

A comparison of the molecular weights and saccharide-binding valencies for the various lectins and their measured diffusion constants indicates that these two parameters have a limited role in determining the mobility of lectin receptors on soybean cells. Both Con A (41) and SBA (23) are bivalent under the conditions of the experiment, yet they both yielded considerably slower diffusion rates than tetravalent WGA (42, 43). The molecular weight of SBA in solution is approximately 120,000 while those for Con A, succinyl-Con A, and WGA are two to four times smaller. However, the identities of the soybean protoplast receptors for the various lectins are not known. Therefore, the actual molecular weights of the diffusing species in the membrane cannot be simply ascribed to the molecular sizes of the lectins.

Perhaps the most striking observation in the present study is the effect of SBA and Con A on the mobility of WGA receptors on the soybean plasma membrane. Pretreatment of the protoplast with either SBA or Con A, followed by incubation with fluorescently-derivatized WGA resulted in a six fold decrease in the diffusion coefficient of the latter lectin. This effect cannot be ascribed to the anchoring of one set of relatively mobile molecules to another set of immobile receptors through lectin-lectin interactions. This conclusion is based on double immunodiffusion and gel filtration experiments which suggest that WGA does not interact with SBA.

Similarly, it has been shown that WGA and Con A do not interact in solution (44). Moreover, the binding of SBA does not change the amount of WGA bound to the soybean cell. Therefore, it appears that the binding of SBA and Con A results in alterations of other components of the soybean plasma membrane in such a way as to restrict the mobility of WGA receptors. To the best of our knowledge this is the first report of an endogenously-produced protein, SBA, that is capable of modulating the dynamic properties of its own membrane. Work with red blood cells has demonstrated how endogenously produced low molecular weight polyanions and cations are capable of altering erythrocyte membrane dynamics (45, 46).

Although the detailed mechanism of modulation remains to be investigated, the present results may be analogous to the modulation by Con A of receptor mobility in a variety of animal cells. The binding of Con A to lymphocytes inhibits patch and cap formation of cell surface immunoglobulin as well as many other different receptors (47). It has also been shown that Con A binding results in a seven fold reduction in the D values of surface immunoglobulin of lymphocytes (38) and a ten fold reduction in the D values of receptors on 3T3 mouse fibroblasts (48). This modulation can be partially reversed by colchicine, implicating a role for microtubules in the Con A effect. Consistent with this proposed analogy, we have found that colchicine also

partially reversed the effect of SBA on the mobility of WGA receptors ($D = 1.3 \times 10^{-10}$ cm^2/s). In contrast, lumicolchicine did not effect the SBA modulation ($D = 3.5 \times 10^{-11}$ cm^2/s). These alterations may be similar to animal cell membrane rearrangements that occur in the process of ligand-receptor mediated endocytosis. The functional signficance of this type of membrane change for a plant cell may be in the binding and subsequent transport of Rhizobium across the membrane of root hair cells to start the process of nodule formation.

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CHAPTER III.

MONOCLONAL ANTIBODIES DIRECTED AGAINST PROTOPLASTS OF SOYBEAN CELLS. CHARACTERIZATON AND ANALYSIS OF THE LATERAL MOBILITY OF SURFACE BOUND ANTIBODY MVS-1.

ABSTRACT

Splenocytes derived from mice, immunized with protoplasts prepared from suspension cultures of root cells of Glycine max (SB-1 cell line), were fused with a murine myeloma cell line. The resulting hybridoma cultures were screened for the production of antibodies directed against the soybean protoplast and were then cloned. One monoclonal antibody, designated MVS-1, was shown to secrete an immunoglobulin which binds to the external surface of the plasma membrane of intact protoplasts. The antigenic target of Antibody MVS-1 was identified as an integral membrane protein (M_r 40,000). 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 resulted in a ten-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 soybean agglutinin modulation

effect. These results have thus refined our previous analysis of the effect of soybean agglutinin on receptor mobility to the level of a single receptor and suggest that the binding of soybean agglutinin 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.

INTRODUCTION

The previous chapter described the diffusion constants (D), determined by the method of fluorescence redistribution after photobleaching (FRAP), for lectin-receptor complexes on the plasma membrane of soybean protoplasts derived from the SB-1 cell line (1, 2). Of particular interest were the following observations: (a) the D values for soybean agglutinin (SBA) and concanavalin A (Con A) were $\sim 5 \times 10^{-11}$ cm^2/s; (b) wheat germ agglutinin (WGA) exhibited higher mobility ($D \cong 3 \times 10^{-10}$ cm^2/s); (c) pretreatment of the protoplast with either SBA or Con A resulted in a six-fold reduction in the mobility of WGA ($D \cong 5 \times 10^{-11}$ cm^2/s); and (d) colchicine, but not lumicolchicine, can partially reverse the effect of SBA on the mobility of the WGA receptors (2). These results suggest that the binding of SBA or Con A may lead to alterations of the soybean plasma membrane which, in turn, may restrict the mobility of other receptors.

Using immunofluorescence and immunoblotting techniques, we have recently demonstrated that the SB-1 soybean cells produce SBA, some of which can be localized

on the plasma membrane of the protoplasts (3). To the best of our knowledge, this is the first report of an endogenously produced protein, SBA, which is capable of modulating the dynamic properties of its own membrane. For this reason, it was of interest to analyze the chemical components and mechanism(s) involved in the modulation process.

Because lectins are known to bind to a heterogeneous population of glycoconjugates on the cell surface, the diffusion constants determined for lectin-receptor complexes most probably reflect ensemble averages rather than the behavior of any single diffusion species within the plane of the membrane. Thus, a decrease in the D value of the WGA receptors could reflect (a) an increase in the number of slowly-diffusing molecules, (b) a decrease in the number of rapidly-diffusing species, or (c) an actual change in the mobility of the receptors. Therefore, it would be extremely useful to have a probe of the cell surface whose target is a homogeneous, well-defined molecule. One approach to this problem is to generate a monoclonal antibody directed against a given component of the soybean plasma membrane. The use of such a probe would enable us to refine our previous analysis of the modulation of receptor mobility by SBA.

This chapter describes the generation of several hybridoma clones, each secreting a monoclonal antibody directed against the soybean protoplast. One of these

monoclonal antibodies, designated MVS-1, was characterized in terms of its cell surface binding and its antigenic target. Antibody MVS-1 and its monovalent Fab fragment were used in a more refined analysis of the lateral mobility of proteins of the plasma membrane of soybean protoplast at the level of a single, defined receptor.

MATERIALS AND METHODS

Immunization and Hybridoma Cell Cultures

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 (1, 4). SB-1 cells were metabolically labeled with $^{35}SO_4^{2-}$ (NEN, Boston, MA) by the following protocol: (a) a 15 ml suspension of cells was transfered into a 125 ml erlenmeyer flask containing 30 ml sulfate-free 1B5 media (sulfate salts were replaced with the appropriate chloride salts (4)) and 0.5 mCi of $H_2{}^{35}SO_4$ was added to the flask. The cells were labeled for 3 days and harvested.

Cellulase (Calbiochem, La Jolla, CA) and pectinase (Sigma, St. Louis, MO) were used to remove the cell wall in the preparation of protoplasts (1, 5). Following this enzymatic digestion, the protoplasts were washed by centrifugation (460 x g for 4 min) and resuspension in 5 ml of Buffer A (10 mM $CaCl_2$, 0.55 M sorbitol, 50 mM Tris-HCl, pH 7.5). Fluorescence microsocpy after Calcofluor staining (5) and scanning electron microscopy

(1) of the protoplasts, showed neither the characteristic fluorescence indicative of cell wall material (6) nor cellulose microfibrils (7), respectively.

Protoplasts (5 x 10^5/ml) were suspended in phosphate buffered saline (PBS, 8 g/l NaCl, 0.2 g/l KCl, 2.3 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, pH 7.4) and were emulsified with an equal volume of Complete Freund's adjuvant (Difco, Detroit, MI). The suspension was injected, intraperitoneally, into male Balb/c mice. Three booster injections (5 x 10^5 cells/ml) in Incomplete Freund's adjuvant (Difco) were given at one week intervals. Three days after the final booster injection, a mouse was sacrificed. The serum was tested for antibodies directed against the SB-1 cells (see below) and the splenocytes were used for fusion with the myeloma cell line NS-1-Ag 4/1 (Cell Distribution Center, Salk Institute, La Jolla, CA).

The fusion between immune spleen cells and myeloma cells (10:1 ratio) was carried out with polyethylene glycol 1500 (8). Hybridoma cultures were selected and cultured as described (8). Hybridoma cultures showing positive reaction in one of the screening assays (see below) were cloned by limiting dilution (9).

Screening Assays for Hybridoma Cultures Producing Antibodies against SB-1 Protoplasts

Two different assays, designated (a) "solid phase" binding assay and (b) "whole cell" binding assay, were developed to screen for hybridoma cultures producing antibodies directed against SB-1 protoplasts. These two assays are schematically diagrammed in Fig. 1 and will be described briefly below.

(a) "Solid phase" binding assay: The procedures for this assay were adapted from a similar radioimmunoassay for actin detailed previously (10). Soybean protoplasts (6×10^5/ml) were frozen at -80 °C, thawed, and then placed in a waterbath-sonicator for 1 h; the resulting suspension was termed cell lysate. Polyvinyl microtitre plates (96-well, Costar, Rochester Scientific, Rochester, NY) were coated in sequence with 100 ul of each of the following materials (i) poly-L-lysine (100 ug/ml in 10 mM Tris-HCl, pH 7.2; Miles Laboratories, Elkhart, IN), 3 h at room temperature; (ii) cell lysate, 4 h at 4 °C; and (iii) 3% (w/v) solution of bovine serum albumin (BSA) in PBS, 1 h at room temperature. After steps (i) and (iii), the wells were washed twice with PBS. After step (iii), the wells were incubated, at room temperature for 1 h, with 100 ul of the solution to be tested for the presence of mouse antibodies against SB-1 cells (hybridoma culture supernatant or immune serum). The binding of the mouse antibody was quantitated by ^{125}I-labeled (11) rabbit

Figure 1. Schematic diagram illustrating the key steps in the two screening assays used to detect hybridoma cultures producing antibodies directed against SB-1 protoplasts. (A) "solid phase" binding assay. In some assays, the target antigen deposited in the microtiter wells consisited of whole lysate. In other assays, specific fractions derived from whole cell lysate were used as a target antigen. (B)"whole cell" binding assay.

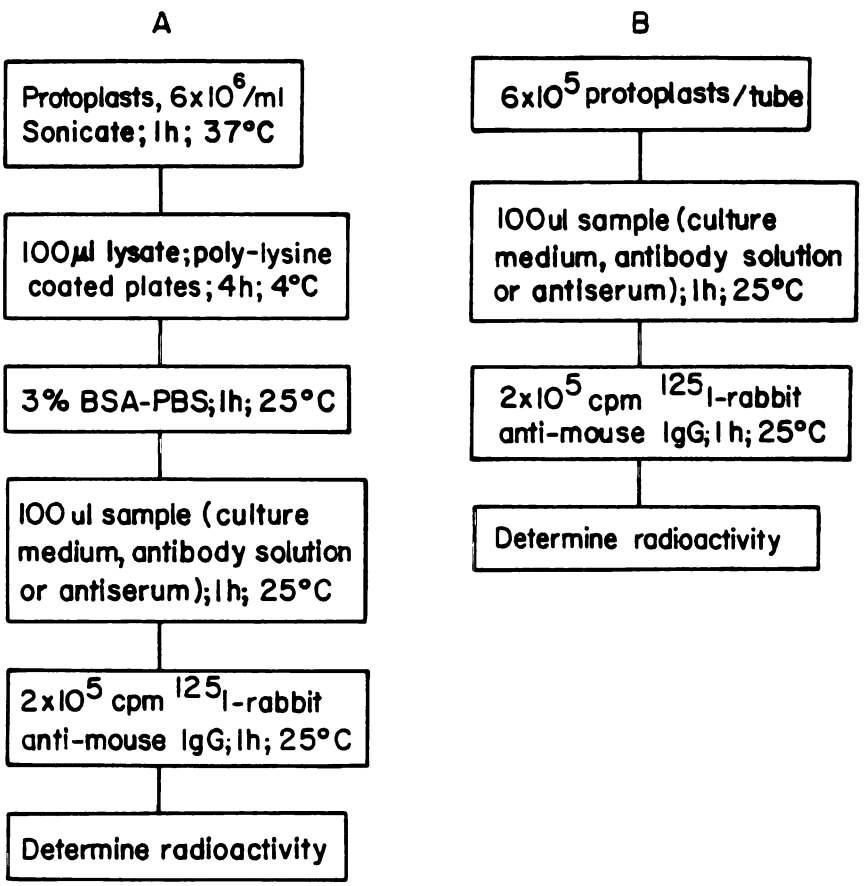
A
Protoplasts, 6x10^6/ml
Sonicate; 1h; 37°C
100µl lysate; poly-lysine coated plates; 4h; 4°C
3% BSA-PBS; 1h; 25°C
100 ul sample (culture medium, antibody solution or antiserum); 1h; 25°C
2x10^5 cpm 125I-rabbit anti-mouse IgG; 1h; 25°C
Determine radioactivity
B
6x10^5 protoplasts/tube
100ul sample (culture medium, antibody solution or antiserum); 1h; 25°C
2x10^5 cpm 125I-rabbit anti-mouse IgG; 1h; 25°C
Determine radioactivity

Figure 1

anti-mouse immunoglobulin (Miles Laboratories) (100 ul, 2 x 10^6 cpm, 1 h at room temperature). After each incubation with antibody, the wells were washed three times with 0.3% (w/v) BSA in PBS. The polyvinyl plates were cut into individual wells and the amount of radioactivity bound to each well was determined in a gamma counter.

In some experiments, various fractions derived from cell lysates were tested for the presence of the antigenic target of the monoclonal antibody by a variation of the "solid phase" binding assay described above. The fraction to be tested was deposited in wells of Immulon-2 plates (Dynatech, Alexandria, VA) and the binding of the monoclonal antibody was quantitated following the same procedure as above, starting at step (iii), the addition of BSA in PBS.

(b) "Whole cell" binding assay: Protoplasts (100 ul of a 6 x 10^5/ml suspension in Buffer A were incubated at room temperature for 1 h in glass tubes (6 x 50 mm) containing 100 ul of the solution to be tested for the presence of mouse antibodies against SB-1 cells. The cells were washed three times by centrifugation (400 x g for 4 min) and resuspension in equal volumes of 0.3% (w/v) BSA in Buffer A. The amount of mouse antibody bound to the SB-1 protoplasts was quantitated by ^{125}I-labeled rabbit anti-mouse immunoglobulin (100 ul, 2 x 10^6 cpm, 1 h at room temperature). The cells were

again washed four times by centrifugation and resuspension. The amount of radioactivity bound to the cell pellet was determined.

Isolation and Characterization of Monoclonal Antibody from Clone MVS-1

The monoclonal antibody produced by clone MVS-1 was isolated from the supernatant solution of culture medium by affinity chromatography on a column (2.1 x 1.1 cm) of agarose derivatized with goat anti-mouse immunoglobulin (Sigma). The column was equilibrated with 10 mM phosphate buffer (pH 8.0). The bound murine monoclonal antibody was eluted with 0.1 M citrate buffer (pH 3.0).

Alternatively, the monoclonal antibody from clone MVS-1 was isolated from ascites fluid obtained from mice bearing the hybridoma tumor. For ascites tumors, Balb/c mice were primed with 0.5 ml Pristine (Aldrich, Milwaukee, WI) by intraperitoneal injection. Three days after the last injection, 3 x 10^5 cells of clone MVS-1 were innoculated into the peritoneal cavity. Ascites fluid from tumor bearing mice was clarified by centrifugation (1430 x g, 10 min). The majority of the non-immunoglobulin proteins were precipitated by adding Rivanol (Sigma; 0.65 ml of a 3% (w/v) aqueous solution per 1 ml of ascites fluid) (12). After centrifugation (17,300 x g, 15 min, 4 ^{0}C), the supernatant was made 5% (w/v) in NaCl and passed through a Whatman filter (No. 2). An equal volume of saturated ammonium sulfate

solution was then added to the filtered solution, and then allowed to stand for at least 1 h at 4°C. The precipitated protein was collected by centrifugation (17,300 x g , 15 min). The pellet was dissolved in PBS and dialyzed against PBS at 4°C.

The immunoglobulin fractions were further purified by affinity chromatography on Protein A-Sepharose (Pharmacia, Piscataway, NJ) equilibrated in PBS, pH 7.4, at room temperature. The bound immunoglobulins were eluted with 0.1 M citrate buffer, pH 3.0. The purified Antibody MVS-1 was dialyzed (at 4 °C) against (a) distilled water, (b) PBS diluted 1:10 in distilled water, pH 6.4, (c) PBS, and then concentrated by ultrafiltration and stored at -20°C. The immunoglobulin fractions were identified by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) (Sigma) (13) and by the screening assay for antibodies directed against SB-1 proptoplasts as described above.

Preparation of Monovalent Fab Fragments of Antibody MVS-1

The procedure for the digestion of immunoglobulin to yield the monovalent Fab fragment was modified from that reported previously (14). All steps were at 4°C unless otherwise specified. Antibody MVS-1 (3 mg/ml) was dialyzed overnight against 0.1 M sodium phosphate, 4 mM sodium (ethylenedinitrilo)tetraacetate (EDTA)(pH 7.5). β-Mercaptoethanol (0.01 M) was added to the immunoglobulin solution, which was then digested with papain (ICN)(2%

(w/w) with respect to immunoglobulin) for 2 h at 37°C. The reaction was stopped by adding iodoacetamide to a final concentration of 0.14 M. After 30 min incubation, the sample was dialyzed against 5 mM Tris-HCl (pH 8.0). The Fab fragment was purified by DEAE cellulose chromatography. Bound material was eluted with a linear gradient of 0-0.3 M NaCl in 5 mM Tris-HCl, pH 8.0. The first eluted peak was Fab fragment of Antibody MVS-1.

Binding of Antibody MVS-1 to SB-1 Protoplasts

For agglutination assays, soybean protoplasts (3 x 10^5 cells/ml) were incubated at 4°C with 150 ug/ml of Antibody MVS-1 in glass tubes with gentle agitation. After 30 min, agglutination was scored with samples deposited on microscope slides and observed with an Olympus inverted microscope. To ascertain the specificity of the agglutination reaction, normal mouse immunoglobulin (150 ug/ml) was tested in parallel.

Antibody MVS-1 was labeled with radioactive iodine (^{125}I, ICN) by using the iodogen, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (11). The specific activity of the labeled immunoglobulin was 2.7 x 10^9 dpm/mg. Protoplasts (3 x 10^4/100 ul) were incubated at 4°C for 1 h with ^{125}I-labeled Antibody MVS-1. The cells were washed three times by centrifugation (460 x g for 4 min) and resuspension in equal volumes of 0.3% (w/v) BSA in Buffer A. After transferring to a fresh tube, the amount

of radioactivity bound to the cell pellet was determined in a gamma counter and the data was analyzed by the method of Scatchard (15).

Identification of the Antigenic Target of Antibody MVS-1

Several fractionation steps were carried out: (a) separation of whole cell lysate into soluble and pellet fractions by centrifugation at 15,000 x g for 15 min; (b) extraction of the pellet fraction of the cell lysate with 50 mM potassium phosphate, 0.13 M KCl, pH 7.5 (Buffer B) containing 0.5% (v/v) Triton X-100 (TX-100) (Research Products Int., Elk Grove, IL) with sonication at 25°C for 15 min, followed by centrifugation at 15,000 x g for 15 min, to yield TX-100 soluble and TX-100 insoluble fractions; and (c) extraction of the TX-100 soluble fraction with 5 volumes of isoamyl alcohol to yield the aqueous and organic phases (16). All fractions were tested for Antibody MVS-1 binding activity by the "solid phase" binding assay. The organic phase contained the detergent and was discarded. The aqueous phase contained the antigenic target of Antibody MVS-1 and is designated as the "antigen-enriched" fraction.

To identify the molecule bound by Antibody MVS-1, protoplasts were prepared from [^{35}S]sulfate labeled SB-1 cells, lysed and fractionated as described above to yield a ^{35}S-labeled preparation of the "antigen-enriched" fraction. This fraction was then subjected to immunoaffinity purification. The immunoaffinity

isolation procedure was modified from that reported previously (17). Antibody MVS-1 or normal mouse immunoglobulin was added (12 ug in 100 ml) to individual wells of an Immulon-2 plate and incubated at 4°C for 24 h. The wells were then flooded with a solution of 3% (w/v) BSA in Buffer B for 2 h at room temperature, followed by washing three times with 0.3% (w/v) BSA in Buffer B. The ^{35}S-labeled "antigen enriched" fraction (100 ul) was added and incubated overnight at 4°C. After washing seven times with 0.3% (w/v) BSA in Buffer B, the radioactivity bound to the individual Immunlon-2 wells was extracted with 100 ul of buffer containing 0.1% (w/v) SDS and subjected to polyacrylamide gel electrophoresis. Alternatively, the "antigen-enriched" fraction was chromatographed on a Biogel A 1.5 column (Bio-Rad Laboratories, Richmond, CA; 64 x 1 cm) equilibrated at 4°C with: (a) Buffer B; (b) Buffer B containing 0.5% (v/v) TX-100; and (c) Buffer B containing 10 mM β-mercapto-ethanol. Individual fractions derived from the columns were tested for binding of Antibody MVS-1 by the "solid phase" binding assay.

Fluorescent Derivatives of Cell Surface Probes

Fluorescein-derivatized SBA or wheat germ agglutinin (WGA) were obtained from Vector Laboratories (Burlingame, CA). The immunochemical probes - Antibody MVS-1, its monovalent Fab fragment and monospecific rabbit anti-SBA immunoglobulin (3) - were labeled with morpholino-

rhodamine isothiocyanate (Research Organics, Cleveland, OH) using the following procedure (18). All steps were done at 4°C. The protein solution was dialyzed against (a) 0.15 M NaCl overnight; (b) 10% (w/v) bicarbonate buffered saline (BBS) (8.8 g NaCl, 2.66 g $NaHCO_3$, 1.96 g Na_2CO_3) pH 8.5, four hours; (c) BBS, pH 9.5, 2 hours. A 30 fold molar excess of dye (5 mg/ml in dimethylsulfoxide (DMSO)) with respect to the protein was then added to the protein sample and incubated for 16 h in the dark with gentle shaking. The reaction was terminated by adding glycine to a final concentration of 0.1 M, followed by dialysis against BBS pH 9.2, with one change of buffer. Unincorporated free dye was removed by gel filtration on a Sephadex G-25 column (35 x 1.2 cm) equilibrated in BBS, pH 8.5. The fluorescently derivatized material was concentrated by ultrafiltration and stored at -20°C.

Fluorescence Redistribution After Photobleaching

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

The sequential labeling of protoplasts with SBA and fluorescent antibody was done by pretreatment of the protoplasts (5 x 10^5/0.5 ml) with SBA (5,50 or 250 ug/ml) or WGA (250 ug/ml) for 1 h at room temperature. The protoplasts were washed by centrifugation (460 x g for 4 min), resuspended in Buffer A and labeled with the fluorescent probe as described above.

In photobleaching experiments where the effect of drugs was examined, cells (5 x 10^5) were preincubated with 1 uM colchicine (CH, Sigma) or lumicolchicine (prepared as described (19)), or 10 ug/ml cytochalasin B (CB, Sigma) in DMSO, for 30 min at room temperature. After washing, the protoplasts were treated as described above, except that the concentration of each drug was maintained throughout.

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

Glass microscope slides were prepared as previously described (1). A drop of protoplast suspension was placed on a washed slide, mounted with a coverslip and sealed with warm paraffin wax, and the lateral diffusion was analyzed by FRAP (20).

The experimental optics and electronics have been described elsewhere (1, 21). Fluorescence emissions for NBD 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 fliter. For rhodamine derivatized probes, the incident wavelength was 514 nm and a Leitz TK580 dichroic mirror and K570 barrier filter were used. The redistribution of fluorescence, following a localized photobleaching pulse, was analyzed using a normal-mode analysis, according to the approach of Koppel et al. (20).

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. ^{125}I labeled Antibody MVS-1 (100 ug, 8 x 10^6 cpm) was analyzed on a column of Sepharose 4B (Pharmacia; 50 x 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 ug, 8 x 10^6 cpm) was incubated with 250 ug SBA in 1 ml of 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5 for 30 min prior to loading onto the column.

The second approach was to pass [^{125}I]MVS-1 over an affinity column of SBA coupled to Sepharose beads. Sepharose 4B (5 ml) was activated with cyanogen bromide (22), washed and suspended 15 ml 0.2M galactose, 0.1 M $NaHCO_3$, pH 8.0 (Buffer C). SBA (10 mg (23)) was dissolved in 5 ml Buffer C and incubated overnight at 4°C with stirring. After washing with Buffer C, the gel suspension was stirred in 1 M ethanolamine-HCl (pH 8.0) at room temperature for 1 h. The gel was then washed with 0.2 M galactose in Buffer C, followed by PBS. ^{125}I-labeled Antibody MVS-1 (50 ug, 4.3 x 10^6 cpm in PBS) was applied to the column. After washing, bound material was eluted with 0.2 M galactose in PBS.

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

The "antigen enriched" fraction was prepared from 1 x 10^8 protoplasts. 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 previously described. Alternatively, after passing the antigen enriched fraction over the SBA-Sepharose column,

^{125}I-labeled Antibody MVS-1 was loaded onto the column and eluted. Radioactivity in the unbound and 0.2M galactose eluent was measured by gamma counting. As a control for both experiments, the same sample was applied to an underivatized column of Sepharose 4B and treated as described above.

Analytical Procedures

Protein concentration, in various samples, was determined by the method of Lowry et al. (24) using BSA as a standard. Polyacrylamide gel electrophoresis in SDS was done according to the method of Laemmli (14). The acrylamide composition of the stacking gel was 4% (w/v) and the running gels were either 10% or 5-16% (w/v) gradient. After electrophoresis, the gels were fixed in 10% (w/v) trichloroacetic acid for 30 min, stained with Coomassie Brilliant Blue, destained and dried. Alternatively, after destaining, radioactive bands were detected by fluorography (25). The fluorograms were exposed to Kodak X-Omat XAR-5 film at -80°C.

RESULTS

Hybrodoma Cultures and Monoclonal Antibodies Reactive with Soybean Protoplasts

Mice were immunized *in vivo* with protoplasts derived from SB-1 cells and their sera were tested for antibodies directed against the immunogen using the two assays outlined in Fig. 1. The serum collected from the mouse, whose spleen cells were subsequently used for fusion, showed strong reactivity in the screening assay using the "whole cell" binding assay. The spleen cells of this particular immune responsive mouse were fused with NS-1 myeloma cells and hybridoma cultures which produced antibodies reacting with SB-1 protoplasts were detected by the same primary screening assay. Representative results from the "whole cell" binding assay, carried out on the supernatant from several cloned cultures, are shown in Fig. 2. Several positive clones were found; these exhibited 1.6-2.0 fold greater binding activity as compared to that seen with the supernatant from the parental myeloma NS-1 cell line. Essentially the same results were obtained using the "solid phase" binding assay except that in this case, clone 21 showed

Figure 2. Representative results obtained using the "whole cell" binding assay to screen hybridoma cultures producing antibodies directed against SB-1 protoplasts. The supernatant from the parental myeloma line, NS-1, was used as a control. The data represent the averages of triplicate determinations ($\pm$ SEM).

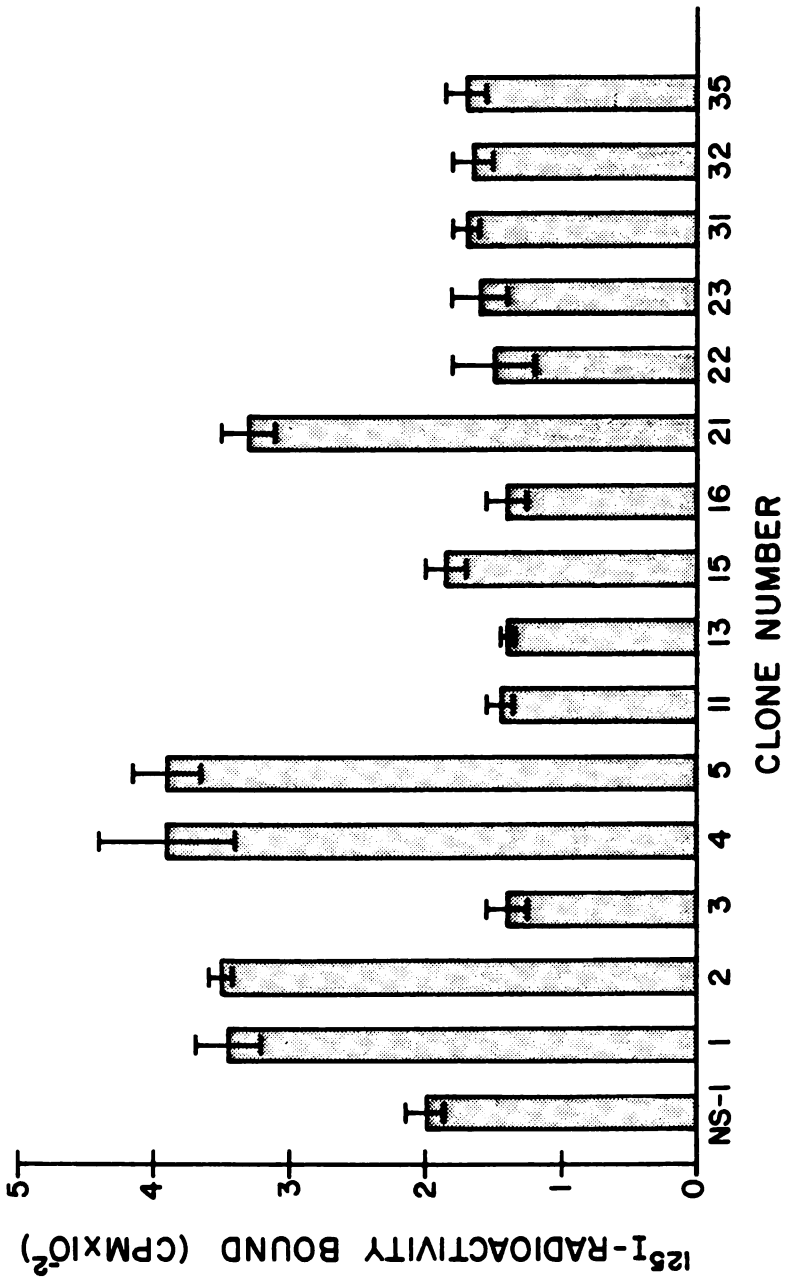
^{125}I-RADIOACTIVITY BOUND (CPMx10^{-2})
0
1
2
3
4
5
NS-1
1
2
3
4
5
11
13
15
16
21
22
23
31
32
35
CLONE NUMBER

Figure 2

no binding activity compared to the NS-1 controls and was therefore a negative clone by this assay.

Isolation and Characterization of Antibody MVS-1 and Monovalent Fab Fragments

Clone 1 was one of the earliest clones established in the monoclonal antibody screening program and several lines of evidence indicated that it secreted an antibody reactive with the outer surface of the protoplast membrane (see below). This culture was recloned by limiting dilution and one subclone, designated MVS-1, was used for all subsequent experiments.

The purified immunoglobulin (Ig) from clone MVS-1, hereafter designated Antibody MVS-1, was analyzed by polyacrylamide gel electrophoresis. Under non-reduced conditions, Antibody MVS-1 yielded a single band (M_r 150,000; Fig. 3, lane d). Upon reduction, this material yielded two bands of 55,000 and 23,000 molecular weight which correspond to the heavy and light chains of IgG (Fig. 3, lane a). Thus, the immunoglobulin produced by clone MVS-1 is an IgG molecule.

Antibody MVS-1 was digested with papain and the digestion mixture was fractionated to yield Fab fragments. Polyacrylamide gel electrophoresis yielded a single band (M_r 48,000) under non-reducing conditions and a doublet (M_r 23,000, 25,000) in the presence of β-mercaptoethanol (Fig. 3, lanes b, e). Compared with the

Figure 3. Polyacrylamide gel electrophoretic analysis of Antibody MVS-1 immunoglobulin and Fab fragment under reducing and non-reducing conditions. Lane a - Antibody MVS-1 immunoglobulin (reduced); lane b - Fab fragment of Antibody MVS-1 (reduced); lane c - Fc fragment of Antibody MVS-1 (reduced); lane d - Antibody MVS-1 (non-reduced); lane e - Fab fragment of Antibody MVS-1 (non-reduced); lane f - Fc fragment of Antibody MVS-1 (non-reduced). The arrows indicate the position of migration of the molecular weight markers : β -galactosidase (130,000), bovine serum albumin (68,000), glutamic dehydrogenase (55,000), aldolase (40,000), carbonic anhydrase (29,000), and myoglobin (17,500). Approximately 40 - 50 ug of protein was loaded in each lane.

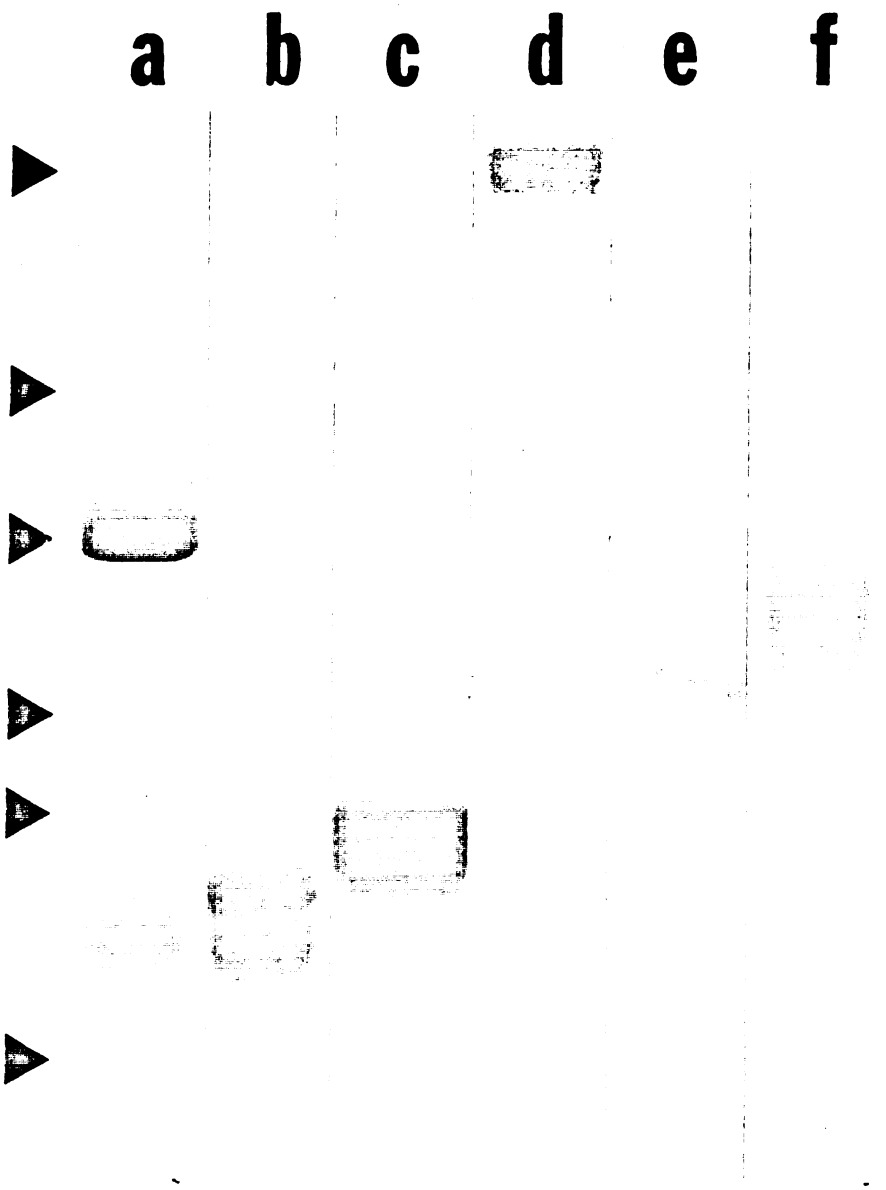

Figure 3

patterns obtained for the intact Antibody MVS-1, these results indicate that we have obtained a highly purified preparation of Fab fragments.

Binding of Antibody MVS-1 to SB-1 Protoplasts

Two lines of evidence indicated that Antibody MVS-1 had, as its antigenic target, a component located on the external face of the plasma membrane of SB-1 protoplasts. First, purified Antibody MVS-1 can agglutinate protoplasts (Fig. 4a). In contrast, normal mouse immunoglobulin failed to agglutinate the same cells (Fig. 4b). Second, Antibody MVS-1, derivatized with ^{125}I, bound to intact protoplasts with a dose response curve which showed saturation (Fig. 5). Analysis of the binding data, using the method of Scatchard (15), indicated there were approximately 1.5×10^6 antigenic sites per protoplast exposed at the plasma membrane (inset, Fig. 5).

Fractionation and Enrichment of the Antigen in Lysates of SB-1 Protoplasts

Several fractionation steps were carried out on the SB-1 cell lysate to enrich the antigenic target of Antibody MVS-1. At each step of the fractionation, the presence of the antigenic target was tested using the "solid phase" binding assay (Fig. 1a). When the cell lysate of SB-1 protoplasts was centrifuged to yield supernatant and precipitate fractions, Antibody MVS-1 bound only to the precipitate (Table 1). The precipitate

Table 1. Binding of Antibody MVS-1 to various Fractions of Lysates of SB-1 Protoplasts.

Sample	Specific Binding* (cpm/ug protein)
Cell Lysate	308
Lysate Pellet	1963
Lysate Supernatent	17
TX-100 Insoluble Fraction	285
TX-100 Soluble Fraction	559
Aqueous Phase of the Isoamyl Alcohol Extract	1343

* The samples were deposited in wells of microtiter plates and the binding of Antibody MVS-1 was quantitated by the "solid phase" binding assay describer in Materials and Methods. The data are expressed as specific binding (cpm of Antibody MVS-1 bound minus cpm of normal mouse immunoglobulin bound) and are normalized to the amount of protein in the samples which was bound in the microtiter wells. The data represent the average of of triplicate determinations.

Figure 4. Agglutination of SB-1 protoplasts by monoclonal Antibody MVS-1. (A) Antibody MVS-1 (150 ug/ml); (B) control incubation containing normal mouse immunoglobulin (150 ug/ml). Protoplasts (3×10^4 cells/ml) were incubated with the antibody for 30 min at room temperature in 50 mM Tris-HCl, 0.5 mM $CaCl_2$, 0.55 M sorbitol, pH 7.5.

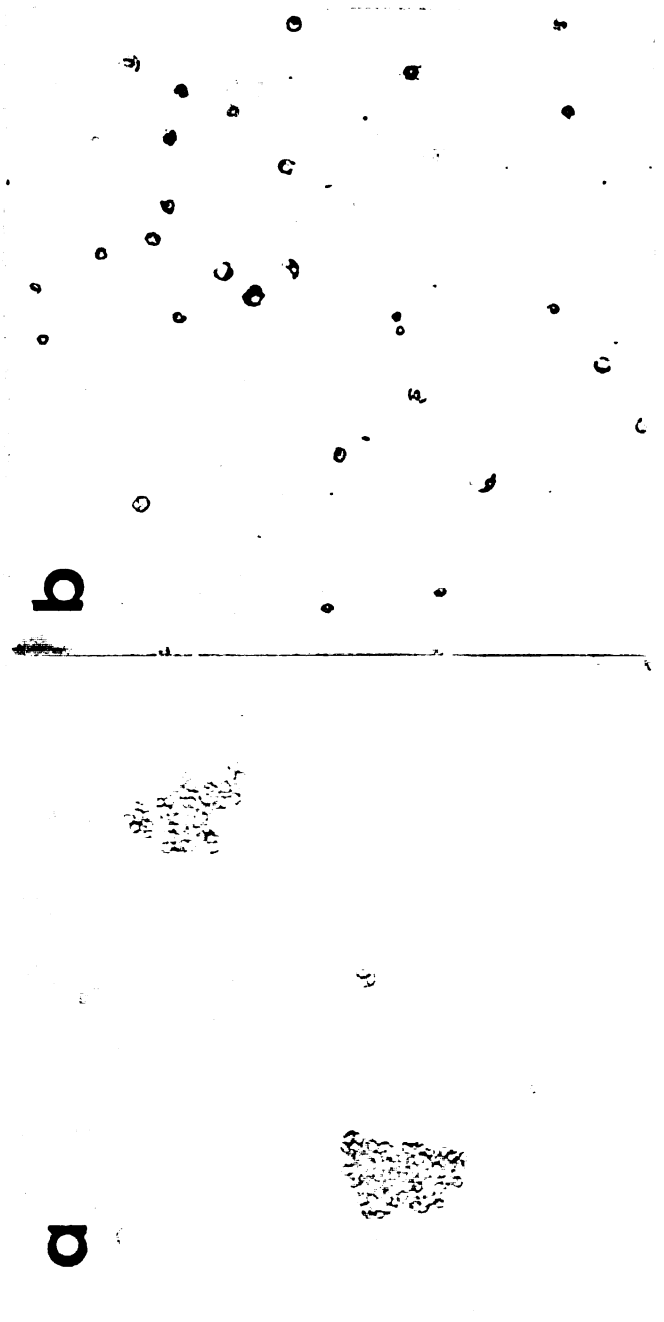

Figure 4

Figure 5. Concentration dependence of the binding of ^{125}I-labeled Antibody MVS-1 (2.7 x 10^{9} dpm/mg) to SB-1 protoplasts. The experiment was carried out in 50 mM Tris-HCl, 0.5 mM $CaCl_2$, 0.55 M sorbitol, pH 7.5 at 4 °C for one hour. The data represent the averages of triplicate determinations (± SEM). The inset represents the analysis of the binding data by the method of Scatchard (15).

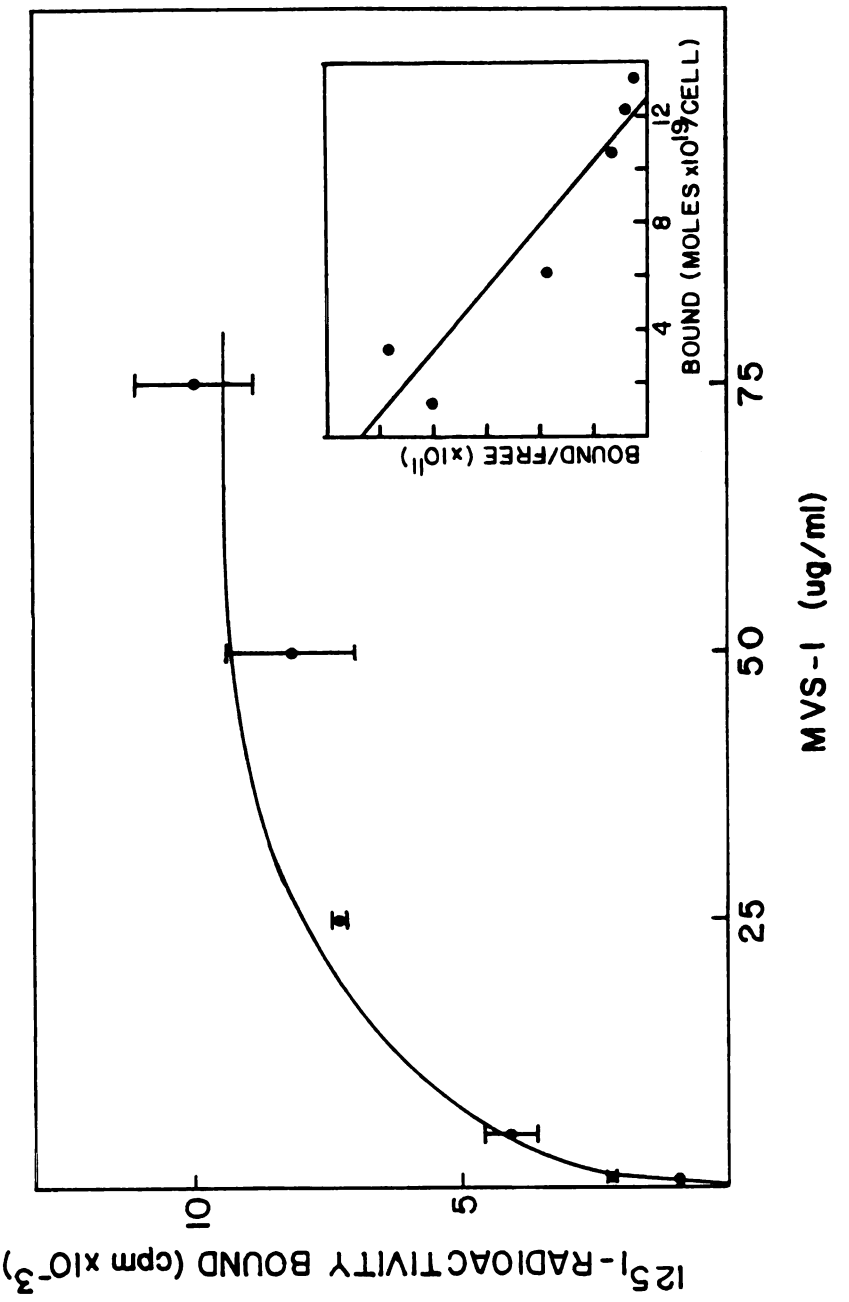
^{125}I-RADIOACTIVITY BOUND (cpm x10^{-3})
10
5
25
50
75
MVS-I (ug/ml)
BOUND/FREE (x10^{11})
BOUND (MOLES x10^{19}/CELL)
4
8
12

Figure 5

fraction of the cell lysate was further partitioned by extraction with TX-100. Both the material solubilized by TX-100 and the TX-100 insoluble material contained some antigenic activity. When the TX-100 soluble fraction was further extracted with isoamyl alcohol to remove the detergent, the aqueous phase showed an enrichment of 2.3-2.4 fold in terms of specific binding activity (Table 1). These results are consistent with the notion that the antigenic target of Antibody MVS-1 contained a polypeptide component derived from the membrane fraction of SB-1 protoplasts.

Identification of the Antigenic Target of Antibody MVS-1

To identify the molecule bound by Antibody MVS-1, SB-1 cells were cultured in the presence of $^{35}SO_4^{2-}$ and protoplasts were prepared, lysed, and fractionated to yield a ^{35}S-labeled preparation of "antigen-enriched" fraction (aqueous phase after isoamyl alcohol extraction). When this labeled fraction was subjected to immunoaffinity purification and SDS gel electrophoresis, one predominant polypeptide band (M_r 40,000) was observed on fluorograms (Fig. 6, Table 2). These results suggest that the antigenic target recognized by Antibody MVS-1 was a protein (M_r 40,000).

The "antigen-enriched" aqueous phase derived from unlabeled SB-1 protoplasts was subjected to gel filtration on a Biogel A-1.5 column. When the presence of the antigenic target in each of the individual

Figure 6. Polyacrylamide gel electrophoectic analysis of the antigenic target of Antibody MVS-1 after immunoaffinity isolation. SB-1 cells were cultured in $^{35}SO_4^{2-}$ to label cellular components. Following the removal of the cell wall, the protoplasts were lysed and fractionated; the aqueous phase of the isoamyl alcohol extract was subjected to immunoaffinity isolation with Antibody MVS-1 bound to Immulon-2 wells. The radioactive material bound on the wells was analyzed on polyacrylamide gels (5-16% (w/v) acrylamide) in the presence of β-mercaptoethanol. The arrows indicate the positions of migration of molecular weight markers. (a) ^{35}S-labeled material bound and extracted from Immulon-2 wells coated with normal mouse immunoglobulin. (b) ^{35}S-labeled material bound and extracted from Immulon-2 wells coated with Antibody MVS-1. Approximately 900 cpms were electrophoresed in each lane.

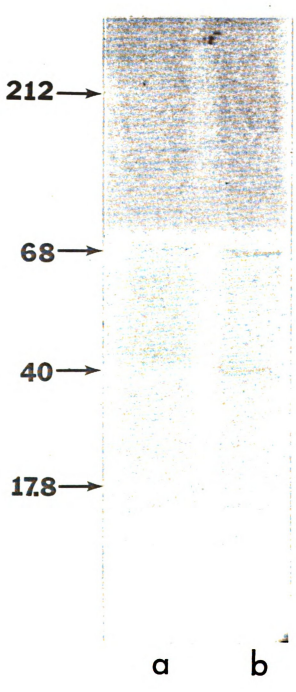

Figure 6

Table 2. Molecular weight of the Antigenic Target of Antibody MVS-1 under Different Conditions.

Detergent	Reducing Agent	M_r
SDS	β-mercaptoethanol	40,000
none	β-mercaptoethanol	400,000 + 40,000
TX-100	none	400,000
none	none	400,000

fractions eluting from the column was quantitated by the "solid phase" binding assay, we found a single peak of activity. The position of elution of this material, in the absence of any detergent or reducing agent, corresponded to a M_r of 400,000 (Table 2). Detergent (TX-100) alone did not produce any change as only a 400,000 dalton peak was observed. When 10 mM β-mercaptoethanol was added in the buffer, a small peak (M_r = 40,000) was found in addition to the 400,000 dalton peak (Table 2). These results strongly suggest that, in the absence of SDS, the antigenic target of Antibody MVS-1 formed high moleculer weight aggregates, either with itself or with other types of molecules.

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

The mobility of fluorescently labeled Antibody MVS-1 bound on protoplasts at 20°C was determined by using the FRAP method. Photobleaching experiments were carried out 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 ug/ml Antibody MVS-1 are shown in Fig. 7.

The experiment shown in Fig. 7 yielded a value of 2.7 x 10^{-10} cm^2/s for the diffusion coeficient (D) of Antibody MVS-1 (100 ug/ml). Using the dose-response curve of ^{125}I-labeled Antibody MVS-1 (Fig. 5) as a guide, I

Figure 7. Semilogarithimic plot of $\hat{u}_1(t)$ (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 ug/ml of morpholinorhodamine derivatized Antibody MVS-1. $D = 1.1 \times 10^{-10}$ cm^2/s. The inset shows a scan across the protoplast membrane prior to the photobleaching pulse.

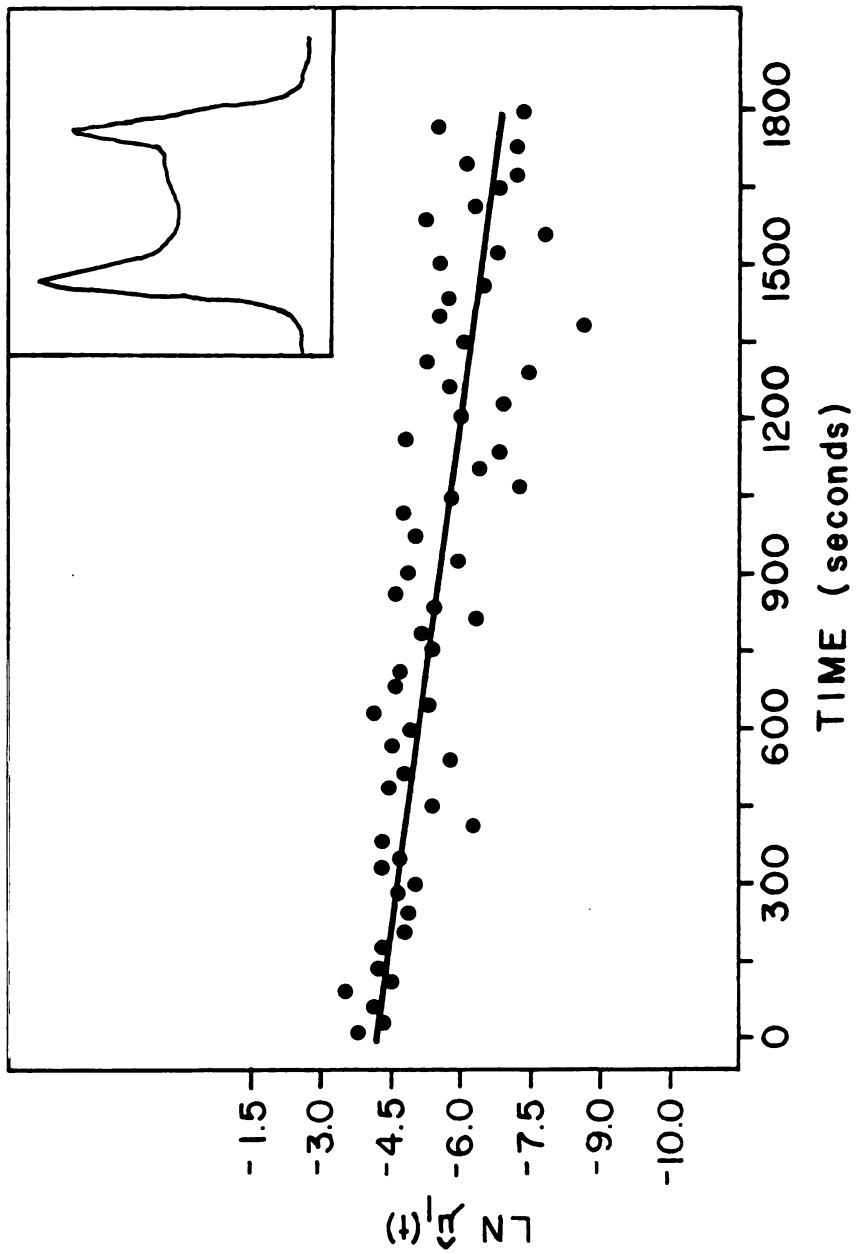
-1.5
-3.0
-4.5
-6.0
-7.5
-9.0
-10.0
LN $P_1(t)$
0
300
600
900
1200
1500
1800
TIME (seconds)

Figure 7

have carried out photobleaching experiments below (50 ug/ml) and above (100, 250 ug/ml) saturation concentrations. As shown in Table 3, there was no significant variations in the D values. The 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 (1). The "relatively fast" group was exemplified by WGA with a D value of 3×10^{-10} cm^2/s (2).

The lack of variation in D and recovery values with concentration (Table 3) also suggests that crosslinking by Antibody MVS-1, at non-saturating concentrations, does not lead to the formation of large immobile patches. This conclusion is further supported by the results of photobleaching experiments carried out with monovalent Fab fragments which yielded D values very similar to those obtained with the intact immunoglobulin (Table 3).

Modulation of Antibody MVS-1 Mobility by SBA

In previous experiments, I have 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 six-fold reduction in the D values of rhodamine-conjugated WGA (1). Since the mobility of Antibody MVS-1 on the soybean protoplast belonged to the "relatively fast" group, similar to WGA,

Table 3. 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 (ug/ml)	D[a] ($cm^2/s \times 10^{+10}$)	%R[a,b]
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

[a] values are expressed as mean ± standard deviation.
[b] %R, % recovery. This value represents the fraction of membrane bound fluorophore which is mobile and able to diffuse on the membrane.

it was of interest to investigate whether SBA can exert its modulatory effect on a single, defined, diffusing component. The results showed that SBA (250 ug/ml) reduced the mobility of MVS-1 approximately ten-fold (D = 0.41×10^{-10} cm^2/s) (Table 4).

This effect of SBA was concentration dependent. High concentrations of SBA (50-250 ug/ml) showed the modulatory effect on the lateral mobility of Antibody MVS-1. Low concentrations of SBA (5 ug/ml) were ineffective in reducing the D value of the same fluorescent antibody (Table 4). 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 4). Finally, the presence of SBA (250 ug/ml) had no effect on the lateral mobility of a phospholipid probe, NBD-PC, which yielded a D value of $\sim 6 \times 10^{-9}$ cm^2/s (Table 4).

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 M_r 150,000, see Fig. 8). It appears,

Table 4. Effect of Soybean Agglutinin 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	Conc. (ug/ml)	Treatment	D[a] ($cm^2/s \times 10^{+10}$)	%R[a,b]
Ig	100	5 ug/ml SBA	3.5 ± 1.4	57 ± 16
		50 ug/ml SBA	0.42 ± 0.17	30 ± 19
		250 ug/ml SBA	0.41 ± 0.18	29 ± 7
Ig	100	250 ug/ml WGA	2.9 ± 1.1	58 ± 23
Fab	450	5 ug/ml SBA	4.1 ± 1.7	59 ± 17
		250 ug/ml SBA	0.40 ± 0.10	28 ± 10
NBD-PC	40	- -	65 ± 9	68 ± 24
		250 ug/ml SBA	47 ± 14	62 ± 28

[a] values are expressed as mean ± standard deviation.
[b] %R, % recovery. This value represents the fraction of membrane bound fluorophore which is mobile and able to diffuse on the membrane.

Figure 8. Elution profile of ^{125}I-labeled Antibody MVS-1 and unlabeled SBA fractionated on a column of Sepharose 4B. The column was equilibrated in 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5, at room temperature. ^{125}I-labeled Antibody MVS-1 (100 ug, 8 x 10^6 cpm) was incubated with 250 ug SBA for 30 min in 1 ml of 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5, prior to loading onto the column. The arrows indicate the elution of the molecular weight markers: blue dextran (void volume), apoferritin (480,000), catalase (270,000), ^{125}I-labeled Antibody MVS-1 (150,000) and total volume of the column.

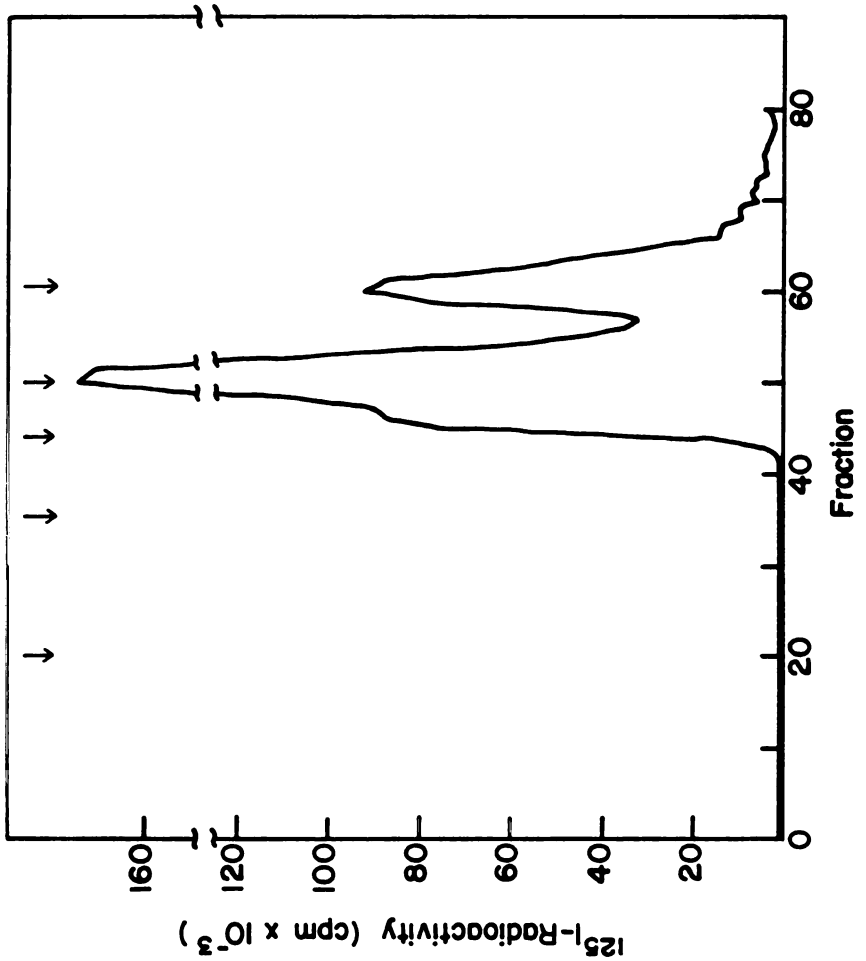
125I-Radioactivity (cpm x 10^-3)
160
120
100
80
60
40
20
0
20
40
60
80
Fraction

Figure 8

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 4).

In addition, we have also carried out experiments to ascertain that the antigenic target of Antibody MVS-1 did not interact with SBA. As described previously the target of Antibody MVS-1 can be partially purified by extraction with TX-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 various fractions was assayed by the solid phase binding assay. As shown in Table 5, the material which did not bind to the SBA-Sepharose column showed an enrichment of MVS-1 binding activity. This indicates that the antigenic target did not bind to SBA. Therefore, the decrease in the lateral mobility of Antibody MVS-1 on the presence of SBA does not come about by cross-linking of the antigenic target of MVS-1 by SBA to an immobile component.

Table 5. Test for the Binding of the Antigenic Target of Antibody MVS-1 to SBA-Sepharose.

Sample	Specific Binding* (cpm/ug protein)
SBA-Sepharose Column:	
Unbound Material	193
Galactose Elution	0
Underivatized Sepharose Column:	
Unbound Material	134

* 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 in Materials and Methods. The data are expressed as specific binding (cpm of Antibody MVS-1 bound minus cpm of normal mouse immunoglobulin bound) and are normalized to the amount of protein in the samples bound on the microtiter wells. The data represent the average of triplicate determinations.

Effect of Colchicine and Cytochalasin B on the Modulation of Mobility by SBA

When the protoplasts were preincubated with colchicine (1 uM) prior to FRAP analysis, the effect of SBA on the lateral mobility of fluorescently labeled Antibody MVS-1 was partially reversed (Table 6); the D value increased from 4×10^{-11} cm^2/s to 1.3×10^{-10} cm^2/s. This value is close to that obtained from Antibody MVS-1 monitored in the absence of SBA (Table 3). Lumicolchicine, a photo-inactivated derivative of colchicine that does not bind to tubulin (26), failed to yield this reversal of the SBA effect. The D values obtained for surface-bound Antibody MVS-1 in the presence and absence of colchicine (1 uM) were comparable. Therefore, colchicine had no effect on the lateral mobility of Antibody MVS-1 itself. Similarly, colchicine also had no effect on the values of the diffusion coefficient of SBA and WGA (Table 6).

In a parallel series of experiments, we found similar results with cytochalasin B. Preincubation of the protoplasts with cytochalasin B (10 ug/ml) also reversed the modulatory effect of SBA on Antibody MVS-1 mobility (Table 6). Moreover, the simultaneous treatment of protoplasts with both colchicine (1 uM) and cytochalasin B (10 ug/ml), completely reversed the effect of SBA (Table 6). The D values obtained from Antibody MVS-1 under these conditions were the same as those obtained

Table 6. Effect of Drugs on Soybean Agglutinin Induced Modulation of the Lateral Diffusion Coefficients of Antibody MVS-1 Bound to the Plasma Membrane of Soybean Protoplasts at 20 °C.

Probe	Conc. (ug/ml)	Treatment	D[a] ($cm^2/s \times 10^{+10}$)	%R[a,b]
Ig	100	1 uM colch.	1.3 ± 0.1	39 ± 21
		250 ug/ml SBA		
		1 uM lumicolch.	0.50 ± 0.17	24 ± 6
		250 ug/ml SBA		
		10 ug/ml CB	1.3 ± 0.4	54 ± 11
		250 ug/ml SBA		
		1 uM colch.	4.2 ± 1.5	46 ± 18
		10 ug/ml CB		
		250 ug/ml SBA		
		1 uM colch.	2.7 ± 0.8	63 ± 16
		10 ug/ml CB	6.0 ± 3.2	58 ± 18
		1 uM colch.	4.0 ± 2.6	61 ± 16
		10 ug/ml CB		
SBA	100	1 uM colch.	0.70 ± 0.19	81 ± 20
		10 ug/ml CB	0.49 ± 0.11	78 ± 18
		1 uM colch.	0.64 ± 0.23	73 ± 12
		10 ug/ml CB		
WGA	100	1 uM colch.	3.7 ± 0.9	75 ± 19
		10 ug/ml CB	4.0 ± 1.2	80 ± 15
		1 uM colch.	3.1 ± 0.7	72 ± 11
		10 ug/ml CB		

[a] values are expressed as mean ± standard deviation.
[b] %R, % recovery. This value represents the fraction of membrane bound fluorophore which is mobile and able to diffuse on the membrane.
colch., colchicine; lumicolch., lumicolchicine; CB, cytochalasin B.

with the immunoglobulin alone (D = 4 x 10^{-10} cm^2/s), without either the drugs or SBA. Finally, neither cytochalasin B nor the combination of cytochalasin B and colchicine had any effect on the mobility of Antibody MVS-1 itself (Table 6).

Taken together, these results suggest that the binding of external ligands, such as SBA, to the plasma membrane of plant cells may alter the cytoskeletal structures of these cells. The cytoskeletal alterations may in turn affect the mobilities of other receptors, such as those for Antibody MVS-1 (and for wheat germ agglutinin).

Lateral Mobility of Endogenous SBA

Previous work in our laboratory has demonstrated that SBA is present on the plasma membrane of SB-1 protoplasts (3). We were interested, therefore, in determining the lateral mobility of the endogenous SBA. Fluorescently derivatized antibodies directed against SBA (3) were used in FRAP experiments. The D value obtained, 2.5 x 10^{-10} cm^2/s, shows 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 is in direct contrast to exogenously labeled SBA, which exhibited D values of 4.1 x 10^{-11} cm^2/s (1).

DISCUSSION

The key results of this chapter are (a) Antibody MVS-1 binds to a single, defined target present on the exterior of the plasma membrane of soybean protoplasts; (b) the antibody-antigen complex exhibits diffusional mobility similar to that of WGA receptors ($D \sim 3 \times 10^{-10}$ cm^2/s)(1); (c) the binding of exogenously added lectin, SBA, to the protoplasts, resulted in a ten fold reduction of the diffusion coefficient of Antibody MVS-1 bound on the same cells ($D = 4 \times 10^{-11}$ cm^2/s); and (d) both colchicine and cytochalasin B reversed, at least partially, the effect of SBA on the lateral mobility of surface bound Antibody MVS-1.

We have generated several different hybridoma clones, each of which secretes an immunoglobulin directed against protoplasts from SB-1 cells. One of these clones, MVS-1, secreted an antibody that binds to the plasma membrane of intact SB-1 protoplasts. This conclusion is based on three independent sets of experiments: (a) agglutination of SB-1 protoplasts; (b) saturable binding of

^{125}I-labeled Antibody MVS-1 to the protoplasts; (c) fluorescence labeling by rhodamine-derivatized Antibody MVS-1 of the cell, showing distinct edge peaks.

The antigenic target of Antibody MVS-1 was identified, by immunoaffinity isolation techniques, as a polypeptide (M_r 40,000). This polypeptide is probably membrane-derived, a conclusion which is based on the pattern of distribution in a series of preliminary fractionations. The antigen was found in the pellet fraction of SB-1 protoplast lysates and was extractable from this pellet with the detergent Triton X-100. In contrast, the antigen was not soluble in organic solvents such as chloroform-methanol, isopropanol-acetone (unpublished obeservations) or isoamyl alcohol.

The molecular weight of the antigenic target of Antbody MVS-1 varied, depending on the presence or absence of detergent and/or reducing agent (see Table 2). In the presence of SDS and β -mercaptoethanol, the molecular weight is 40,000. In contrast, in the absence of any detergent or reducing agent, the antigenic molecule was found as a high molecular weight complex (M_r 400,000). Whether this antigenic target forms high

molecular weight aggregates with itself or with other types of molecules (e.g. polysaccharides) remains to be determined.

In previous experiments, we had reported values of diffusion coefficients, on SB-1 protoplasts, for several exogenously added lectins including WGA, succinyl concanavalin A, concanavalin A, (Con A) and SBA (1). We found that the D values for the various lectins would be (arbitrarily) separated into two classes: a relatively "fast" group exemplifed by WGA and a relatively "slow" group exemplifed by SBA. Our present results indicate that the mobility Antibody MVS-1 bound on the protoplast surface belong 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 D values obtained in the present study utilized a monoclonal antibody (MVS-1; M_r 150,000) whose target is a single, well-defined species (M_r 40,000). The D value determined for Antibody MVS-1 would thus reflect the behavior of the defined ligand-receptor complex. In animal cells, diffusion coefficients have been determined for defined ligand-receptor complexes: (a) rabbit anti-IgE bound to

IgE on mast cells (27); (b)α-bungarotoxin bound to acetylcholine receptors on myotubes (28); (c) anti-Thy-1 on mouse spleen cells (29); (d) growth factors bound to their specific receptors (30, 31); (e) cell adhesion molecules and specific antibodies on embryo fibroblasts (32). More recently, monoclonal antibodies against specific proteins, such as histocompatibility antigen H-2 (33, 34), as well as partially characterized antigens (35, 36), have been used to monitor the lateral diffusion of the resulting antibody-antigen complexes.

We had also reported previously that pretreatment of SB-1 protoplasts with SBA resulted in the reduction in the mobility of WGA (1) and that colchicine partially reversed this effect of SBA on the mobility of the WGA receptors (2). 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 utilizing a monoclonal antibody with a defined target has obviated these difficulties in interpretation. Thus, the binding of SBA, leading to a ten-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 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 recetor mobility in a variety of animal cells (37). 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 7-fold reduction in the D values of surface immunoglobulin of lymophocytes (38) and a 10-fold reduction in the D values of receptor proteins on 3T3 fibroblasts (39). This modulation can be partially reversed by colchicine, implicating a role for microtubules in the Con A effect.

Consistent with this proposed analogy, we have found that drugs capable of disrupting cytoskeletal structures also reversed the effect of SBA on the mobility of both WGA receptors (2) and Antibody MVS-1. The targets of these drugs, microtubules for colchicine and microfilaments for cytochalasin B, have been identified in plant cells (40-44). In particular, we have previously identified an actin-like protein in soybean cells whose immunological cross-reactivity with animal

cell actin, chemical properties (M_r 45,000) and binding and polymerization properties, paralleled that of actin (10, 45).

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 which lead to signal transduction across membranes.

Recently, we have obtained immunochemical evidence for the presence of a lectin similar to SBA at the plasma membrane of SB-1 protoplasts (3). Using fluorescently dervatized 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 (1) and Antibody MVS-1 (Table 3). The lateral mobility of endogenous SBA is distinctly faster than that of exogenously-added SBA ($D = 4.1 \times 10^{-11}$ cm^2/s) (1). 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 ug/ml) do not induce modulation (see Table 4). Moreover, 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. Finally, the physiochemical properties of the endogenous lectin remain to be studied. Atlthough this lectin cannot be eluted from the plasma membrane by D-galactose or N-acetyl-D-galactosamine (3), competitive sugars of the seed lectin (46), the occupancy of the saccharide binding sites, as well as their specificity, need to be determined. It is possible that the binding sites are occupied, and thus unable to cross-link other integral membrane components and thus induce modulation of lateral mobility.

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CHAPTER IV

LATERAL MOBILITY OF PHOSPHOLIPIDS IN THE PLASMA MEMBRANE OF SOYBEAN PROTOPLASTS

ABSTRACT

Fluorescent lipid and phospholipid probes were incorporated at 4°C into soybean protoplasts prepared from cultured soybean (SB-1) cells. Fluorescence microscopy demonstrated that the plasma membrane as well as the nucleus were labeled. Fluorescence redistribution after photobleaching (FRAP) analysis was performed on these cells at 18°C to monitor the lateral mobility of the incorporated probes. At low concentrations (40 ug/ml) of phosphatidylethanolamine (NBD-PE), a single mobile component was observed with a diffusion coefficient of $\sim 3 \times 10^{-9}$ cm^2/s. At higher probe concentrations (>100 ug/ml), two diffusing species were observed, with diffusion coefficients of $\sim 3 \times 10^{-9}$ cm^2/s ("fast") and $\sim 5 \times 10^{-10}$ cm^2/s ("slow"). Similar results were observed with fluorescent derivatives of phosphatidylcholine and fatty acid probes. In contrast to these results, parallel analysis on 3T3 fibroblasts, using the same probes and conditions, yielded only a single diffusion component. These data suggest that the soybean plasma membrane may contain two distinct lipid domains in terms of lipid mobility.

Consistent with this notion, experiments with soybean protoplasts yielded a single diffusion component under the following conditions: (a) NBD-PE (100 ug/ml) at 37 °C, $D = 1.1 \times 10^{-8}$ cm^2/s; (b) NBD-PE (100 ug/ml), 18 °C, 2mM EGTA, $D = 4.2 \times 10^{-9}$ cm^2/s; and (c) 5-(N-dodecanoyl)aminofluorescein, a short chain lipid probe, $D = 2.5 \times 10^{-8}$ cm^2/s under all conditions. These results suggest that the plasma membrane of soybean cells may contain stable immiscible domains of fluid and gel-like lipids.

INTRODUCTION

A number of biophysical measurements using electron spin resonance (1), fluorescence depolarization (2), and differential scanning calorimetry (3), have provided evidence that the biological membrane may contain a patchwork of immiscible gel and fluid lipid domains. Such domains could be significant as regulators of the enzyme activity of integral and peripheral membrane proteins, and may also serve as topological organizers for cell membrane receptors. Although small (a few lipid molecule diameters) (4) and large (>300 nm in diameter) (4,5) lipid domains have been observed in model liposome systems, only small lipid patches of a particular phase have been demonstrated to exist in natural biological systems (6, 7). Using the technique of fluorescence redistribution after photobleaching (FRAP), a number of investigators have shown that in model binary phospholipid systems, distinct large lipid domains could be diagnosed by the variation in diffusion components and bleaching recoveries of a fluorescent probe capable of partitioning into the fluid and gel phases (8, 9). These

measurements, however, failed to demonstrate any major differences in the diffusion coefficient of a lipid probe in mammalian plasma membranes (10).

We have previously reported diffusion coefficients (D) for lectin-receptor complexes on the plasma membrane of soybean protoplasts derived from the SB-1 cell line (11). In this chapter, we have extended the analysis of the lateral mobility of the components of the soybean cell plasma membrane by studying the diffusion of fluorescent lipid analogs. The data suggest that the soybean membrane may be a composite of large scale immiscible gel and fluid lipid domains. This suggestion is particularly intriguing in light of the recent biochemical analysis of soybean membrane, showing high amounts of negatively charged phospholipid species in plant plasma membranes (12), particularly phosphatidyl-glycerol, when compared to mammalian cell plasma membrane (13). In this context, reports by Verkleij et al. (14) and Ohnishi and Ito (15) have demonstrated phase separation and the formation of negatively charged phospholipid domains following calcium ion addition.

MATERIALS AND METHODS

Fluorescent Probes

The fluorescent probes used in this study were 1-acyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole)aminocaproyl phosphatidylcholine (NBD-PC, Avanti Polar Lipids, Birmingham, AL), N-4- nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE, Avanti Polar Lipids), 1,1'-dioctadecyl-3,3,3', 3'-tetramethylindocarbocyanine iodide ($diIC_{18}$, Molecular Probes, Plano, TX), 1,1'-ditetradecyl- 3,3,3',3'tetramethylindocarbocyanine iodide ($diIC_{14}$, Molecular Probes) and 5-N-(dodecanoyl)-aminofluorescein (AFC12, Molecular Probes). The NBD probes, originally in chloroform, were blown dry under nitrogen. All probes were dissolved in ethanol at a concentration of 1 or 5 mg/ml.

Cell Culture and Protoplast Isolation

Suspension cultures of the soybean (Glycine max) cell line SB-1 were grown as described (11) in 1B5C medium (16). Soybean protoplasts were prepared by enzymatic digestion of the cell wall using cellulase (Calbiochem, La Jolla, CA) and pectinase (Sigma, St. Louis, MO) as described (11, 17). Following the removal of the cell

wall material, the protoplasts were washed by centrifugation (460 x g for 4 min) and resuspension in 5 ml of protoplast medium which was modified by (a) substituting 30 g D-sorbitol for glucose; (b) addition of 0.01 M NaN_3 and 2 % (w/v) bovine serum albumin ($MGB-N_3-BSA$).

Swiss 3T3 fibroblasts (American Tissue Culture Center, Rockville, MD) were cultured in Delbecco's Modified Media as previously described (20). Cultures near saturation density (4×10^4 cells/cm^2) were trypsinized (0.05% (w/v) in Versene, (20)), and washed three times in Hank's buffered salt solution containing 12.5 mM HEPES (pH 7.2) by centrifugation (1050 x g for 3 min) and resuspension in 10 ml of cold buffer.

Incorporation of Fluorescent Lipid Analogs

Cells (5×10^5/0.5 ml) were labeled with fluorescent lipid (1 ug/ml - 1 mg/ml) at 4 °C for 15 min. After washing to remove free lipid, the cells were either resuspended in 100 ul of cold buffer and analyzed, or extracted to determine the amount of incorporated lipid. Fluorescence microscopy was done using a Leitz fluorescence microscope equipped with a Leitz KP470-490 excitation filter, RKP510 dichroic mirror and a LP515 barrier filter. Micrographs were taken with Kodak Tri-X film (Rochester, NY), which was pushed to ASA 3200.

The amount of fluorescent lipid incorporated was determined by lipid extraction (18), fluorescence

spectroscopy, and phosphate analysis (19). Cells labeled with fluorescent lipid were solubilized in 1 ml of 2% (v/v) Triton X-100 in phosphate buffered saline (8 g/l NaCl, 2.3 g/l Na_2HPO_4, 0.2 g/l KCl, 0.2 KH_2PO_4, pH 7.4). Triton insoluble material was removed by centrifugation (15,000 x g for 5 min) in a Brinkman Microfuge (Westbury, NY). The fluorescence in the clarified supernatent was measured using a Perkin-Elmer 650-40 fluorescence spectrophotometer. For NBD derivatives, the excitation and emission wavelengths were 468 nm and 534 nm respectively. For the diI derivatives, the respective wavelengths were 556 nm and 571 nm.

Lipids were extracted from fibroblasts or protoplasts as previously described (18) and amount of extracted lipid phosphate was determined (19).

Fluorescence Redistribution after Photobleaching

The procedure for FRAP analysis has been described in detail (11). Fluorescence emission for NBD and fluorescein derivatives was monitored with an incident wavelength of 476.5 nm in combination with a Leitz TK510 dichroic mirror and K530 barrier filter. For diI derivatives, the incident wavelength was 514 nm, and a Leitz TK580 dichroic mirror and K570 barrier filter were used. The redistribution of fluorescence, following a localized photobleaching pulse, was subjected to a

normal-mode analysis, following the approach described by Koppel et al. (21), using two exponentials to analyze the data.

Experiments at 37°C were done using a Sage Instruments Air Curtain Incubator. Samples were incubated at 37°C for 5 min prior to FRAP analysis.

To test the effect of Ca^{2+} on lipid mobility, protoplasts were labeled with fluorescent lipid as previously described. The cells were then washed twice with calcium free MGB-N_3-BSA (11) which contained 2mM sodium ethylenebis(oxyethylenenitrilo)tetraacetate (EGTA). Following an incubation period of 5 min, the cells were analyzed.

RESULTS

Incorporation of Fluorescent Lipid Probes into Protoplast Membranes

Soybean protoplasts were incubated with NBD-PE (1 ug/ml - 1 mg/ml) for 15 min at 4°C. At concentrations of 20 ug/ml or below, there was little or no staining observable by fluorescence microscopy. Above 40 ug/ml, the lipid uniformly labeled the plasma membrane; a representative photograph of a cell stained with NBD-PE (100 ug/ml) is shown in Fig. 1A. Besides the characteristic fluorescent ring around the protoplast indicating cell surface labeling, there was also significant fluorescence observed from the center of the cell, indicating labeling of internal membranes. This staining of intracellular membranes was also noticed when the cells were labeled for 1 minute. This redistribution of fluorescence from the plasma membrane to the intracellular membrane compartment occurred within 10 minutes. Such redistribution of fluorescent lipid to internal membrane systems has also been observed in lung fibroblasts labeled at 4°C and warmed to 37°C (22) albeit requiring significantly longer times of incubation,

Figure 1. Fluorescence staining patterns of soybean protoplasts labeled with fluorescent lipids. A - NBD-PE, B - AFC12. ph - phase contrast microscopy, fl - fluorescence microscopy. Protoplasts were labeled with 100 ug/ml of fluorescent lipid for 15 min on ice. The cells were washed with cold buffer, and examined by fluorescence microscopy.

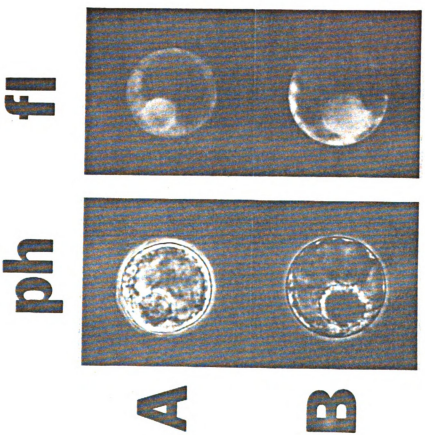

Figure 1

routinely 1-2 hours. Similar labeling patterns were also observed when protoplasts were treated with AFC12 (Fig. 1B), NBD-PC, $diIC_{14}$, and $diIC_{18}$. Transmission electron micrographs (23) of soybean protoplasts, following lipid incorporation, demonstrated that the plasma membrane of the protoplasts was devoid of adhering lipid vesicles (data not shown). Therefore, it could be assumed that most of the fluorescent lipid analog was incorporated into the protoplast membranes.

The amount of lipid incorporated into the protoplasts was quantitated by fluorescence spectroscopy. At 40 ug/ml NBD-PE, approximately 3 mmoles of fluorescent lipid was incorporated per mole of endogenous lipid phosphate; at 100 ug/ml NBD-PE, approximately 6 mmoles of fluorescent label was incorporated per mole of lipid phosphate (Table 1). The incorporation of the fluorescent lipid were saturable above 200 ug/ml. Similar results were obtained for $diIC_{18}$ (Table 1), as well as for $diIC_{14}$ and NBD-PC, except that in the latter case the incorporation of fluorescent lipid failed to saturate within the concentration range examined.

In general, SB-1 cells incorporated much less fluorescent lipid than mouse 3T3 fibroblasts (Table 1). In the case of NBD-PE, the concentration of fluorescent lipid required to yield an incorporation of 6 mmoles per mole of endogenous lipid phosphate was 100 ug/ml and 2 ug/ml, respectively, for SB-1 cells and 3T3 cells. At a

Table 1. Lateral Diffusion Coefficient as a Function of Lipid Probe Concentration at 18°C.

Cell	Fluorescent Lipid	Conc. (ug/ml)	mmoles incor.	First Component D^a ($cm^2/s \times 10^{+9}$)	First Component $\%R^{a,b}$	Second Component D^a ($cm^2/s \times 10^{+9}$)	Second Component $\%R^{a,b}$	Total %R
SB-1	NBD-PE	40	3.3	2.6 ± 2.7	45 ± 35	- NONE -		45
		100	6.3	2.8 ± 1.3	35 ± 9	0.47 ± 0.41	21 ± 16	56
		200	10.7	3.3 ± 0.6	30 ± 10	0.46 ± 0.22	32 ± 22	62
SB-1	NBD-PC	100	7.4	1.3 ± 0.6	27 ± 16	0.45 ± 0.20	33 ± 12	60
SB-1	$diIC_{18}$	40	3.1	15 ± 24	90 ± 10	- NONE -		90
		100	7.0	3.4 ± 1.9	42 ± 24	0.26 ± 0.27	32 ± 16	74
		200	13.2	3.8 ± 2.6	44 ± 14	0.27 ± 0.23	32 ± 10	76
SB-1	$diIC_{14}$	100	6.5	5.7 ± 3.0	39 ± 4	0.33 ± 0.20	26 ± 8	65
SB-1	AFC12	40	ND	22 ± 1.5	71 ± 36	- NONE -		71
		100	ND	25 ± 1.4	69 ± 24	- NONE -		69
3T3	NBD-PE	2	6.2	3.4 ± 2.0	52 ± 25	- NONE -		52
		20	73	14 ± 13	68 ± 24	- NONE -		68
3T3	$diIC_{18}$	2	ND	6.8 ± 5.1	52 ± 28	- NONE -		52
		20	ND	11 ± 9	47 ± 24	- NONE -		47

[a] values are expressed as mean ± standard deviation.
[b] %R, %recovery. This value represents the fraction of membrane bound fluorophore which is mobile and able to diffuse in the membrane.
mmoles incor. - mmoles of lipid label incorporated per mole of endogenous lipid phosphate.
ND - not determined.

concentration of 20 ug/ml, previously used in FRAP studies on animal cell membranes (10), there was insufficient fluorescence incorporated into the SB-1 cells for either fluorescence microscopy or FRAP studies. Therefore, all subsequent experiments were carried out at 40 ug/ml fluorescent lipid or above.

Lateral Mobility of Lipids in the Soybean Plasma Membrane

The technique of FRAP was used to determine the lateral mobility at 18°C of protoplasts labeled with each of the fluorescent lipids. Representative data from an experiment using diIC_{18} is shown in Fig. 2. A fluorescence excitation scan across a single protoplast is shown in Fig. 2B. Assuming similar probe environments, the two outer peaks indicate that the majority (70%) of the fluorescent lipid is localized in the plasma membrane, although some is also present in the cytoplasmic membranes, as demonstrated by the inner peak. This confirms the results obtained by fluorescence microscopy. Data from a representative FRAP experiment is plotted in Fig. 2A. This graph shows a semi-logarithmic plot of the time course of the first normal mode of fluorophore distribution after a photobleaching pulse (21). Each point represents a complete fluorescence scan across the protoplast. The solid line represents data obtained with soybean protoplasts, while the dashed line shows a corresponding experiment on 3T3 fibroblasts.

Figure 2. A) Semilogarthmic plot of $\hat{u}_1(t)$, the experimental estimate of the normalized first moment of the fluorophore concentration distribution, as a function of time after the photobleaching pulse on a) a soybean protoplast labeled with $diIC_{18}$ (———), b) a 3T3 fibroblast labeled with NBD-PE (----). Analysis of the data was done according to Koppel et al. (21) using two exponentials to describe $\hat{u}_1(t)$. The lines shown represent a plot of the data points from typical experiments. Each point represents a complete scan across the cell, which was done once every second. The diffusion coefficients are: soybean protoplast - fast (1-20 seconds) $D = 4.1 \times 10^{-9}$ cm^2/s; slow (200-900 seconds) $D = 2.0 \times 10^{-10}$ cm^2/s; 3T3 fibroblast $D = 1.6 \times 10^{-9}$ cm^2/s. B) A typical fluorescence excitation scan across the soybean protoplast membrane prior to the photobleaching pulse.

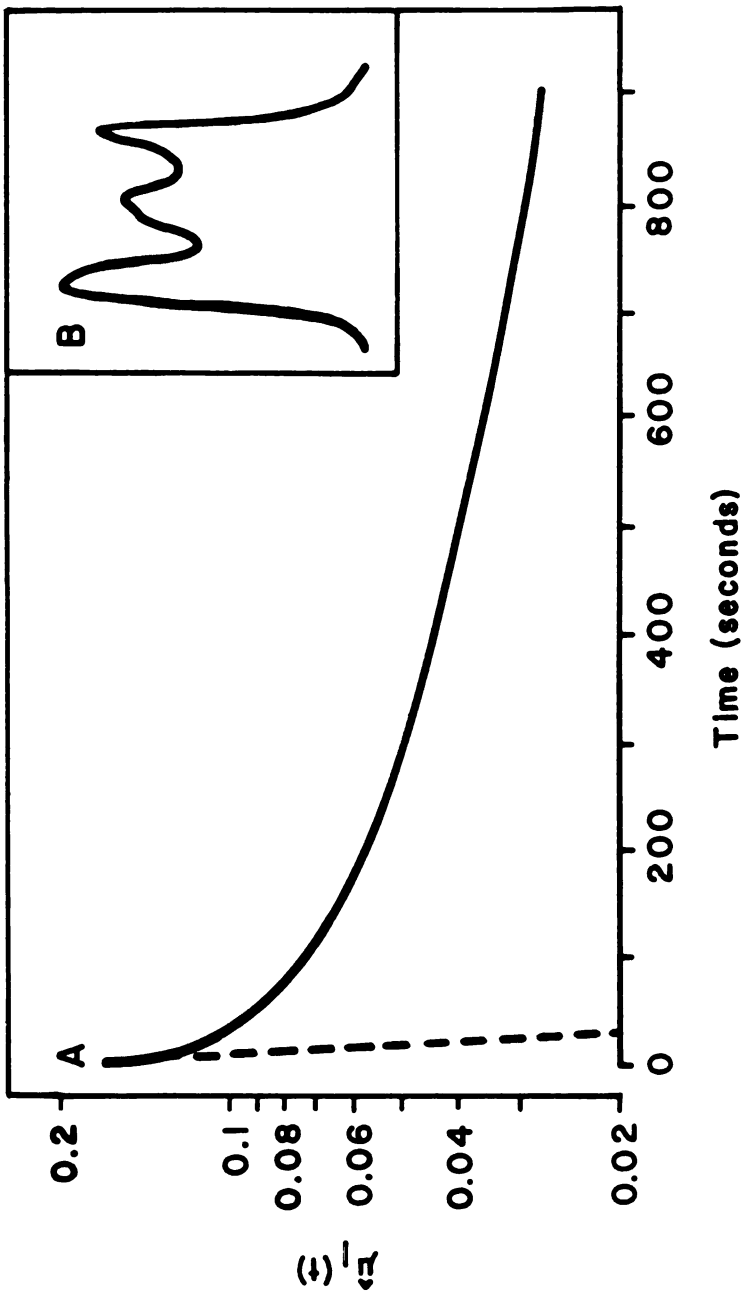

Figure 2

The diffusion coefficient of the various lipid analogs are summarized in Table 1. At low concentrations (40 ug/ml), the data are best fit by a single exponential component and yielded a D value of 4 x 10^{-8} cm^2/s for $diIC_{18}$. At higher concentrations (>100 ug/ml), however, two exponential components were required to fit the data (Fig. 2A). A "fast" component, with a D value of ~3 x 10^{-9} cm^2/s, accounted for approximately 40% of the total initial fluorescence (% Recovery in Table 1). A "slow" component, with a D value of ~3 x 10^{-10} cm^2/s, gave 30% recovery. Finally, approximately 30% of the probe was found to be immobile. These results, described and illustrated in Fig. 2 for $diIC_{18}$, were also obtained when NBD-PE, NBD-PC, and $diIC_{14}$ were used as probes (Table 1).

Variation in the bleaching conditions used (intensity and/or duration of bleach) did not result in any change in the lateral mobility of lipids in the protoplasts. Moreover, rebleaching of the samples still showed two mobile component recovery processes. We have also studied the lateral mobility of $diIC_{18}$ and NBD-PE in the plasma membrane of 3T3 cells at initial concentrations such that the incorporation of fluorescent lipid per mole of endogenous lipid phosphate was comparable to those obtained with soybean cells. In all cases, only a single exponential term was required to describe the time course of redistribution of

fluorescence (Fig. 2A). The values of the diffusion coefficients for $diIC_{18}$ ($D = 10^{-8}$ cm^2/s) and NBD-PE ($D = 10^{-9} - 10^{-8}$ cm^2/s) on 3T3 fibroblasts (Table 1) were similar to those previously reported for fibroblasts (10, 22, 24). Taken all together, these results argue against the notion that the two different diffusing species seen with SB-1 protoplasts are due to artifacts of the experimental protocol and data analysis.

Effect of Temperature Alterations and Addition of EGTA

A striking change in the diffusional behavior of the lipids was observed when the FRAP experiments on NBD-PE and $diIC_{18}$ were carried out at 37 °C instead of 18 °C. For example, NBD-PE yielded a single component with a D value of 1×10^{-8} cm^2/s at 37 °C. The recovery was 60%, accounting for essentially all the mobile molecules of the probe in the membrane (Table 2). Similar results were also observed for $diIC_{18}$. An increase in mobility has been observed previously in animal cell systems, when the effect of temperature was studied (24, 25).

The addition of EGTA (2 mM) to protoplasts at 18 °C also reduced the two component diffusion system to a single mobile species (Table 2). The "fast" ($D = 2.8 \times 10^{-9}$ cm^2/s, 35% recovery) and "slow" ($D = 4.8 \times 10^{-10}$ cm^2/s, 21% recovery) components observed at 18 C in the absence of EGTA were collapsed into a single component in its presence ($D = 4.2 \times 10^{-9}$ cm^2/s, 58% recovery).

Table 2. Effect of Temperature and EGTA on Lateral Mobility of Lipids in Soybean Protoplasts

Fluorescent lipid (100 ug/ml)	Temp (°C)	First Component D[a] ($cm^2/s \times 10^{+9}$)	First Component %R[a,b]	Second Component D[a] ($cm^2/s \times 10^{+9}$)	Second Component %R[a,b]	Total %R
NBD-PE	18	2.8 ± 1.3	35 ± 9	0.47 ± 0.41	21 ± 16	56
	37	11 ± 12	62 ± 22	- NONE -		62
$diIC_{18}$	18	3.4 ± 1.9	42 ± 24	0.26 ± 0.27	32 ± 16	74
	37	12 ± 16	56 ± 25	- NONE -		56
NBD-PE + 2 mM EGTA	18	4.2 ± 2.6	58 ± 34	- NONE -		58

[a] values are expressed as mean ± standard deviation.
[b] %R, % recovery. This value represents the fraction of membrane bound fluorophore which is mobile and able to diffuse in the membrane.

It should be noted that the mmoles lipid label per mole of endogenous lipid phosphate for protoplasts incubated under the various conditions of these experiments are the same as those values reported in Table 1, since the original incorporation conditions were maintained. Therefore, differences in the characteristics of lateral diffusion cannot be ascribed to a difference in the level of lipid probe incorporated into the membrane. These effects of temperature and EGTA addition suggest the possibility that the two different diffusing species may represent the partitioning of our lipid probes into two distinct domains, one fluid-like and another gel-like.

FRAP Analysis on a Short Chain Lipid Probe

To test the hypothesis that there may be two distinct lipid domains, we analyzed the mobility of a short chain (12-carbon atoms) lipid probe, AFC12. Strikingly, this lipid probe yielded only a single "fast" diffusing species ($D = 2.5 \times 10^{-8}$ cm^2/s). The level of recovery was 70% (Table 1). Rebleaching these samples gave recoveries of >90%. This suggests that once the immobile fluorescent molecules are bleached, the mobile component represents the predominant class of molecules within the membrane bilayer.

DISCUSSION

This chapter extends the analysis of the lateral mobility of the components of the plasma membrane of cultured soybean cells, previously studied with lectin probes that bind to glycoconjugates of the cell surface (Chapter 2) and with monoclonal Antibody MVS-1 which binds to a defined polypeptide target (Chapter 3). The key observations of this study are: (a) at low concentration of probe (40 ug/ml), the diffusion of lipids in protoplasts is similar to that seen in animal membranes (D $\sim 10^{-8}$ - 10^{-9} cm^2/s); (b) at higher probe concentrations (>100 ug/ml), two recoveries are required to describe the time course of fluorescence redistribution; (c) the second recovery process is sensitive to temperature alterations and calcium concentration; and (d) a short chain lipid probe, AFC12, showed a single D value.

The most striking result is the presence of the second "slow" component in diffusion of NBD and diI probes. Two possibilities were considered to explain this: (a) it represents a consequence of membrane flow (26) between the plasma membrane and intracellular

membranes; (b) it represents a true diffusion coefficient. The results obtained with the short chain probe, AFC12, provide for the best differentiation of these two possible sources of the second component. If the second component did indeed represent membrane flow, the AFC12 probe should participate in this exchange process and one should have seen two recoveries with this probe. On the other hand, in the presence of immiscible gel and fluid lipld domains, previous reports have indicated that such a small probe should partition primarily into the fluid phase (8, 27). Thus, this probe is the least likely to be affected by the organization of membrane as suggested by Dragsten et al. (28).Therefore, one would predict a single recovery to describe the redistribution process of AFC12. The single D value obtained for AFC12 (2.5×10^{-8} cm^2/s) suggests that the second "slow" component does indeed represent diffusion of the lipid probe in a gel domain in the plasma membrane of the protoplast. Moreover, the percent recovery is equivalent to the total mobile fraction of the other lipid probes (see Table 1).

Further support for this conclusion is derived from the effects of temperature and EGTA on lipid mobility. When the photobleaching experiments were conducted at 37 C, the "fast" and "slow" components coalesced into a single "fast" component. Again, the percent recovery of this component is approximately the sum of the "fast" and

"slow" recoveries at 18 °C. Such an observation would be expected for the melting of a gel domain to a more fluid organization (25).

Finally. the EGTA results may provide the most provocative clue to the organization and stabilization of the lipid domains. A major feature of soybean protoplast membranes is the high level of negatively charged phospholipids (12), particularly phosphatidylglycerol (Table 3). A number of investigators (14, 15) have demonstrated that Ca^{2+} can aggregate negatively charged phospholipids by forming salt-linked cross-bridges between phospholipid head groups. Such cross-linked lipid blocks are capable of forming stable domains in the membrane that could be dissociated either by Ca^{2+} chelation or by high temperature. A prediction based on these results is that membranes containing a high percentage of negatively charged phospholipids would demonstrate diffusional domains. Previous work showed that Salmonella typhimurium G30 membranes displayed multiple diffusing phospholipid species (M. Schindler, unpublished data). Since the inner membrane of this gram negative organism contains 40% negatively charged phospholipids (Table 3), the results are consistant with the inner membrane containing diffusional domains. To the best of our knowledge, the case of Salmonella typhimurium G30 and the present soybean results represent

Table 3. Comparison of the Phospholipid Compositions of Various Plasma Membranes.

Plasma Membrane (ref.)	Phospholipid Composition (%) PC	PE	PI	PS	PG	PA	C	PI+PG+PA+C / Total PL
1. BG-9 Foreskin Fibroblast (24)	50	22	6	7	--	--	--	15
2. V 79 Foreskin Fibroblast (29)	53	26	1	8	--	0.3	--	11
3. Rat Liver (hepatocyte) (13)	39	24	8	3.5	--	--	<0.2	15
4. Soybean Hypocotyl (*Glycine max*) (12)	24	26	10	--	26	15	--	51
5. *S. typhimurium* G30 (O.M.) (30)	--	81	--	--	17	--	1.6	19
6. *S. typhimurium* G30 (I.M.) (30)	--	60	--	--	33	--	7	40

Abbreviations:
O.M., outer membrane; I.M., inner membrane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; C, cardiolipin; PL, phospholipid.

new evidence for stable immiscible macroscopic lipid domains in a complex biological membrane of an intact cell.

Another possible influence on lipid sequestration and domain formation may be the higher steroid/phospholipid ratio (1:1) in the plant membrane (31) compared to 0.36:1 in 3T3 cells (32, 33) and/or the substitution of stigmasterol, β-sitosterol, and campesterol for cholesterol as the major steroids in plant membranes (34). It is known that the sterol content of membranes can have a pronounced effect on the ordering (35), and as a consequence, the lateral mobility (36, 37) of lipids in membranes. The observation that cholesterol reduces the lateral mobility of phospholipids in liposomes (9) and erythrocytes (37), while digitonin incorporation into the inner mitochondrial membrane creates two lipid diffusing species (38), is suggestive of such a sterol domain inducing effect.

At present, it would appear that the high content of negatively charged phospholipids and the higher content of sterols in the plant membrane, when compared to animal membranes, may provide the unique membrane environment for phase separation and the creation of large immiscible lipid domains at room temperature. Because these conditions do not exist in mammalian cells under normal conditions, small domain diffusions are averaged in

mammalian cell membrane when analyzed with FRAP measurements, resulting in a single diffusion coefficient.

Our present results may now be viewed in the context of our previously reported observation that cell surface glycoconjugates that bind to wheat germ agglutinin diffuse in soybean protoplast membranes with $D = 3.0 \times 10^{-10}$ cm^2/s, while glycoconjugates that bind either soybean agglutinin or concanavalin A diffuse approximately seven times slower, $D = 4.6 \times 10^{-11}$ cm^2/s (11). In contrast, similar studies using a variety of lectins, including wheat germ agglutinin and concancavalin A, showed that lectin receptor glycoproteins in general diffuse with approximately the same diffusion coefficients in 3T3 cell plasma membranes (10). This difference between 3T3 cells and soybean cells and our present observations demonstrating that a single lipid probe could reveal two different lipid domains raises the possibility that, in soybean cells, receptors for wheat germ agglutinin have a higher affinity for more fluid domains, while the receptors for soybean agglutinin prefer a more aggregated or organized gel lipid environment. The observation of a 6-7 fold variation in lipid mobility between the two mobile species is very suggestive of this possibility. The role that these lipid domains, both mobile and immobile, play

in the activity of the cell, and the effect of a cell wall on their distribution and development remain to be determined.

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CLOSING STATEMENT

This thesis has described an extensive analysis of the lateral mobility of the glycoconjugate, protein and lipid components of the plasma membrane of cultured soybean (Glycine max) cells. The technique of fluorescence redistribution after photobleaching (FRAP) was used to determine the diffusion coefficients (D), on protoplasts derived from suspension cultures (SB-1 cell line) of soybean cells, for fluorescently-derivatized (a) lectins - soybean agglutinin (SBA), concanavalin A (Con A) and wheat germ agglutinin (WGA), which bind to a heterogeneous group of glycoconjugates; (b) a monoclonal antibody (MVS-1) which has a single, defined cell surface protein target (M_r = 40,000); (c) analogs of phosphatidylethanolamine (NBD-PE) and phosphatidylcholine (NBD-PC) and (d) fatty acid analogs (carbocyanine dyes and C_{12}-aminofluorescein).

The key results derived from our study with the protein probes are:

(a) there is a class of "fast" receptors, characterized by D values of 3×10^{-10} cm^2/s and exemplified by surface molecules bound by WGA and by Antibody MVS-1;

(b) there is a class of "slow" receptors, characterized by D values of 5×10^{-11} cm^2/s and exemplified by surface

molecules bound by SBA and Con A;

(c) pretreatment of protoplasts with SBA ("slow" class) resulted in a 6-10 fold reduction in the mobility of WGA or Antibody MVS-1 ("fast" class) such that their D value approached $5x10^{-11}$ cm^2/s;

(d) colchicine (but not lumicolchicine) and cytochalasin B can each partially reverse the effect of SBA on the reduction of D values of the "fast" receptors.

These results indicate that the binding of SBA may lead to alterations of the soybean plasma membrane which, in turn, may restrict the mobility of other receptors such as those for WGA or Antibody MVS-1. These effects are most likely mediated, at least in part, by the cytoskeletal components of the plant cell. SBA has been identified on the plasma membrane of the SB-1 cells. To the best of our knowledge, this is first example of an endogeneous protein (SBA) that is capable of modulating the dynamic properties of its own membrane.

Analysis of the lateral diffusion of lipid analogs yielded the following key observations:

(a) at low concentrations of NBD-PE (40 ug/ml), a single mobile component was observed, with a D value of $3x10^{-9}$ cm^2/s corresponding to the diffusion coefficient of this lipid probe in a variety of other membrane lipid systems;

(b) at higher concentrations of NBD-PE (>100 ug/ml), two components were observed. The "fast" component yielded

a D value of $3x10^{-9}$ cm^2/s. The "slow" component had a D value of $2x10^{-10}$ cm^2/s;

(c) similar results were also obtained with fluorescent derivatives of phosphotidylcholine (NBD-PC) and fatty acid analogs ($diIC_{14}$ and $diIC_{18}$);

(d) the short fatty acid probe, C_{12}-aminofluorescein, which partitions solely into the fluid domains of a membrane, showed only one component ($D=5x10^{-9}$ cm^2/s) at both low and high concentrations.

On the basis of these results and other lines of evidence, including temperature studies, we interpret this data to suggest the presence of two lipid phases in the plasma membrane of soybean protoplasts.

A possible model of the plasma membrane of the soybean cell, which incorporates the conclusions from thses studies, is shown in Fig. 1. The key points of this model are:

(a) the lipids of the plasma membrane are organized into two distinct, immiscible domains. One of these domains is gel-like and and the second one is fluid-like in nature.

(b) there appear to be two classes of cell surface glyco-conjugates and proteins in terms of their lateral mobility: a class of "slow" molecules characterized by the receptors for SBA; and a class of "fast" molecules characterized by the receptors for WGA and the anti-genic target of Antibody MVS-1. The receptors for SBA maybe preferentially located in the gel-like lipid

Figure 1. A possible model of the structure of the plasma membrane of the soybean cell. The receptors for SBA are located preferentially in a gel-like domain of the membrane, while those for WGA and Antibody MVS-1 are in fluid-like domains. This model predicts that the receptors for SBA are associated with the cytoskeletal elements, microfilaments (MF) and microtubules (MT), of the soybean cell.

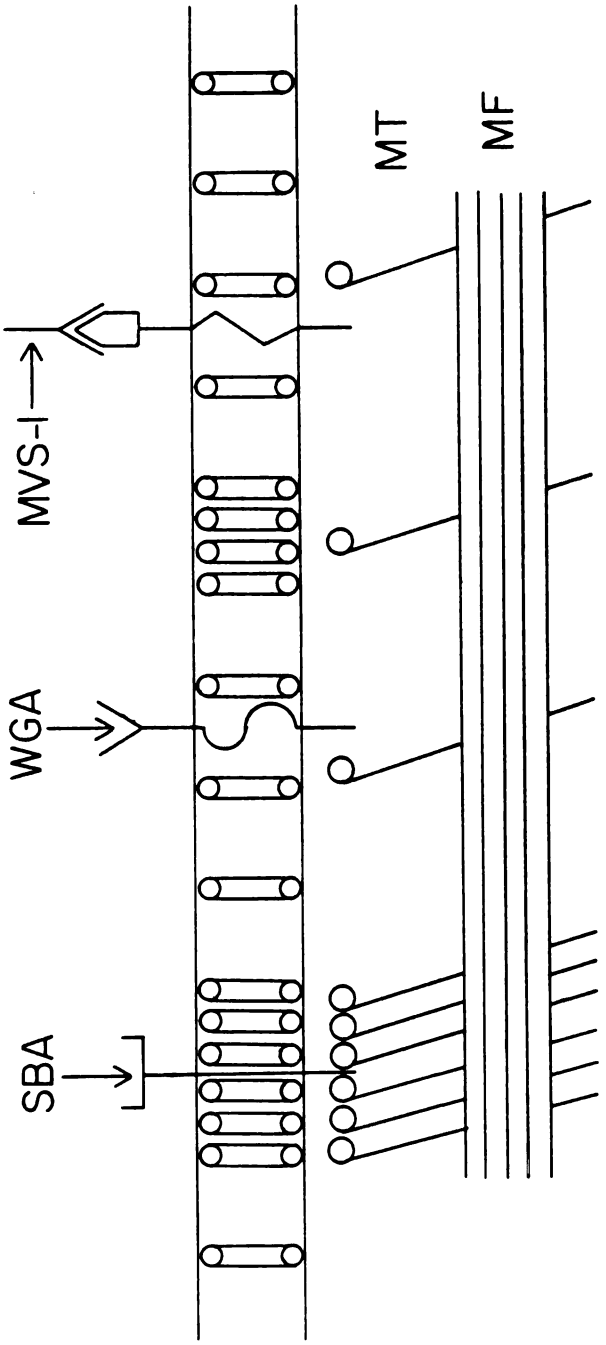

Figure 1

domains, which would provide a physical explanation for their "low" D values ($\sim 4 \times 10^{-11}$ cm^2/s).

(c) the binding of an endogenous protein, SBA, to its receptors on the plasma membrane results in a rearrangment of the cytoskeleton of the soybean protoplast such that the lateral mobility of other receptors, such as those for WGA and the antigenic target of Antibody MVS-1, is reduced.

We are now in the position to carry out a biochemical analysis of the soybean cell to demonstrate a direct connection between the SBA receptors and the cytoskeleton.

APPENDIX

LIST OF PUBLICATIONS

CHAPTER II:

1. Metcalf, T. N., III, J. L. Wang, K. R. Schubert and M. Schindler, "Lectin receptors on the plasma membrane of soybean cells. Binding and lateral diffusion of lectins", Biochemistry, 22, 3969-3975 (1983).
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CHAPTER III:

1. Villanueva, M. A., T. N. Metcalf, III and J. L. Wang, "Monoclonal antibodies directed against protoplasts of soybean cells. I. Generation of hybridomas and characterization of a monoclonal antibody reactive with the cell surface", Journal of Cell Biology, submitted for publication (1985).
2. Metcalf, T. N., III, M. A. Villanueva, M. Schindler and J. L. Wang, "Monoclonal antibodies directed against protoplasts of soybean cells. II. Analysis of the

lateral mobility of surface bound Antibody MVS-1", Journal of cell Biology, submitted for publication (1985).

CHAPTER IV:

1. Metcalf, T. N., III, J. L. Wang and M. Schindler, "Lateral diffusion of lipid domains in soybean protoplast plasma membrane", Proceedings of the National Academy of Sciences (USA), submitted for publication (1985).