Lateral Diffusion of Surface Molecules in Animal Cells and Tissues

Abstract. *When bound to cell surfaces, certain lectins such as concanavalin A induce a drop in the average diffusion coefficients* (D) *of a number of cell surface molecules. To find whether such anchorage modulation occurs naturally,* D *of surface antigens on different cell and tissue types were measured by fluorescence photobleaching recovery. Values for cells of the same tissue origin under different conditions of growth and association—in tissues, in small aggregates, and as isolated cells—varied by less than twofold when polyspecific monovalent antibodies to cell surface antigens were used, a range much less than the sixfold decrease in* D *observed after lectin-induced anchorage modulation. Thus, if reversible modulation of the diffusion rate is used naturally as a means of cell signaling, it must involve only a few kinds of surface receptors not detected by the antibodies used in this study. In certain tissues, however, a significant proportion of cells showed no apparent receptor mobility. This "all or none" modulation of lateral diffusion may reflect relatively long-lasting alterations in the states of a single cell type or differentiation among the cells of the particular tissue.*

The lateral mobility of cell surface molecules can be modulated by external perturbation. For example, the binding of nonsaturating amounts of the lectin concanavalin A (Con A) to lymphocytes inhibits the normal redistribution of surface molecules by multivalent antibodies (*1*). This restriction of mobility, called anchorage modulation, has been observed for various receptors on several cell types (*2*). Lectin-mediated anchorage modulation is global and propagated; that is, binding of Con A to a local region of the surface can restrict the mobility of receptors over the whole cell (*3*, *4*). It therefore does not result from simple trapping of receptors in a meshwork of cross-linked surface glycoproteins or from external cross-linking of mobile receptors to immobile surface molecules. These observations suggest that alterations in the mobility or distribution of surface molecules in one region of a cell can generate signals that can be transmitted cytoplasmically throughout the cell and influence the mobility of other surface molecules. Anchorage modulation is thus a mechanism by means of which information about a cell's environment or interactions with other cells might influence cellular metabolism or behavior (*2*).

Quantitative confirmation of anchorage modulation by lectins has been obtained by fluorescence photobleaching recovery (*5*). In this method, surface molecules are labeled with fluorescent tags; for example, rhodamine derivatives of monovalent Fab′ fragments from specific antibodies. Some fluorophores on several square micrometers of the cell surface are irreversibly bleached with a short, intense pulse of a focused laser beam, and the recovery of fluorescence in the same area is monitored while new receptors carrying unbleached fluorophores move into that area from surrounding regions. Kinetic measurements show that receptor movement occurs mainly via simple diffusion, and an apparent diffusion coefficient (D) can be computed from the time-dependence of fluorescence recovery. For many surface antigens, recovery is not complete, suggesting that only part of the labeled molecules are mobile in the time scale of the experiment. This mobile fraction (R) can be estimated from the recovery curve.

Surface antigens on murine 3T3 fibroblasts, tagged with polyspecific antibodies, were in one of two principal states (*4*): free, with D of $2.2 \pm 0.8 \times 10^{-10}$ cm^2/sec, or immobile, with D of less than 5×10^{-12} cm^2/sec. When Con A–coated platelets were added to the cells, D of the free receptors decreased about sixfold, and R was unchanged. Occupancy of more than 4 percent of the cell surface by coated platelets induced the effect, but occupancies of larger areas did not increase its magnitude. The decrease in D was seen in all regions of the cell surface, confirming that modulation can be propagated.

Although measurements of the mobility of various surface molecules have been made on isolated cells ranging from myoblasts to fertilized ova (*5*, *6*), suitable data have not been available for determining whether anchorage modulation occurs in the absence of experimental perturbation, or whether it plays a functional role in vivo. In particular, consistent data from the same laboratory on different cell types or on cells in tissues have not been reported. In our study, we have used fluorescence photobleaching recovery to measure (*7*) the mobility of surface antigens on cells of six types under conditions where natural modulation might be observed, including different growth states, different association states, and intact tissues. The results (Table 1) indicate that for cells of the same type under these various conditions, D differs by less than twofold. The proportion of anchored receptors can vary widely, however, both with cell type and environment.

Because similar cytoskeletal elements are thought to be involved in anchorage modulation and in positive and negative regulation of the propagation of responses to mitogens as well as in cell growth (*2*), we first examined whether receptor mobility is modulated by growth state. Monovalent Fab′ fragments of polyspecific rabbit antibodies against murine EL4 lymphoid tumor cells were used to label murine 3T3 fibroblasts in various growth states; similar results were obtained with rabbit antiserums against OTT6050 teratocarcinoma cells and against mouse spleen cells. Each of these serums showed immunoprecipitation with more than 15 major components from membrane extracts of fibroblasts but showed no significant activity against fibronectin, which is immobile on the cell surface (*8*).

To study the effect of growth state, we made use of the earlier observation (*9*) that, if a confluent monolayer of 3T3 cells is wounded by mechanically scraping cells from a small region of the tissue culture dish, cells in the region of the wound flatten and begin to divide, whereas those farther from the wound do not. Measurements of receptor mobility on cells across a wound in a single culture dish revealed no significant differences in D or R (Table 1, experiment 1). We found no differences in mobility between growing and nongrowing (contact-inhibited confluent or serum-starved sparse) 3T3 fibroblasts (Table 1, experiment 2), or between 3T3 cells and primary mouse embryo fibroblasts (Table 1, experiment 3), although significant differences in growth control between cells from primary cultures and from established tissue culture lines have been observed (*10*).

Taken together, these results indicate that changes in growth state lead to much smaller changes in average receptor mobility than does lectin-induced anchorage modulation. This conclusion applies only to those receptors marked by the polyspecific antiserums used; our studies do not exclude control by modulation of particular receptors not marked by our antiserums. It has been reported that D of membrane proteins can vary

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about twofold during the cell cycle of murine C1300 neuroblastoma cells (*11*); these differences, however, in part reflect changes in lipid mobility and are far less than the sixfold differences observed on external modulation.

To determine the effects of cell-cell interaction on receptor mobility, we have examined several tissues from the chicken embryo, together with cells after dissociation from these tissues and during reassociation in tissue culture. Individual cells at the surface of a tissue slice were selected visually for measurement. The Fab′ fragments of antibodies, obtained by immunizing rabbits with a membrane fraction from chick embryo brain cells and cross-reactive with several tissues from 10-day embryos, were used to label surface receptors; reference measurements on chicken embryo fibroblasts gave results (Table 1, experiment 4) similar to those obtained with other serums on mouse fibroblasts.

The average D of mobile receptors on cells in liver slices (Table 1, experiment 5) was not significantly different from that in cells isolated from the tissue by mild trypsinization and cultured for 2 hours or for 24 hours, when many colonies containing 35 to 50 cells had formed. No difference in D was seen between the flatter, more spread cells at the edge of these colonies and the rounder, tightly packed cells at the center (Table 1, experiment 5).

A significant difference between dissociated cells and intact tissue was observed, however, in the proportion of cells that showed recovery of fluorescence after photobleaching. More than 95 percent of measurements on dissociated liver cells showed recovery, but of measurements on intact liver tissue, only 25 percent showed recovery. Fifty-six percent of cells in the tissue showed no apparent recovery (under conditions where D in the range 5×10^{-12} cm^2/sec to 1×10^{-9} cm^2/sec and R greater than 10 percent would have been detectable). The remaining 19 percent of measurements were uninterpretable, usually because the shape of the recovery curve was irregular. The "uninterpretable" measurements probably result from technical problems in making measurements on tissues, such as mechanical instability in support of the tissue slices or difficulty in focusing the measuring spot.

The low proportion of tissue cells showing recovery may reflect either such technical difficulties or true differences in the anchorage state of surface receptors. No difference in the apparent mobility of the lipid probe 3,3′-dioctadecylindocarbocyanine (*12*) between cells in tissue slices and dissociated cells was seen, and the most likely interpretation is that the surface receptors stained with the antiserums used were, in fact, immobile within the time scale of the experiment. When mobility was observed, both D and R were comparable to those measured on dissociated cells, suggesting that the modulation did not result from mechanical restriction of mobility by the extracellular matrix of the tissue. Furthermore, the differences in morphology within colonies of cultured cells did not affect mobility, suggesting that the variation in morphology among the complex cell types in liver could not explain the lack of recovery. Additional experiments, in which mobility measurements on an individual cell in the tissue can be correlated with its histological type, will be necessary to exclude this possibility.

Using the same antiserum, we made comparisons between chick embryo brain tissue and dissociated cells (Table 1, experiment 6) and between chick embryo retina and dissociated cells (Table 1, experiment 7). In contrast to the results obtained with liver, about 80 percent of the measurements on brain and retina tissue slices showed recovery, whereas only 6 percent showed no apparent recovery. As with liver, when recovery was observed, no difference was detected between D in the intact tissue and in the corresponding dissociated cells. After only 4 hours in culture, the R of brain cells was slightly decreased, however, possibly reflecting the loss of some surface molecules during the dissociation of the tissues. Otherwise, R was not significantly different. In

Table 1. Diffusion coefficients (D) and mobile fractions (R) of cell surface antigens. Results are presented as the mean ± standard deviation of the number of measurements given in parentheses.

Experiment	Cell type	Condition	$D \times 10^{10}$ (cm^2/sec)	R
		Antibody to EL-4		
1	3T3 fibroblasts	Near wound	12.6 ± 5.8 (8)	0.66 ± .08 (7)
		Far from wound	12.6 ± 3.9 (13)	0.74 ± .07 (13)
2	3T3 fibroblasts	Sparse, growing	7.6 ± 2.7 (22)	0.69 ± .11 (16)
		Confluent	6.7 ± 2.2 (13)	0.70 ± .14 (10)
		Serum-starved	10.1 ± 2.9 (20)	0.73 ± .09 (20)
3	Mouse embryo fibroblasts	Sparse	12.1 ± 5.3 (11)	0.67 ± .15 (11)
		Confluent	7.8 ± 3.8 (13)	0.72 ± .15 (13)
		Antibody to brain membrane		
4	Chick embryo fibroblasts	Sparse	6.4 ± 3.6 (33)	0.72 ± .12 (33)
5	Chick embryo liver	Tissue slice*	5.3 ± 3.8 (15)	0.50 ± .16 (15)
		Dissociated cells, 2 hours in culture	7.5 ± 2.4 (15)	0.53 ± .10 (12)
		Cells in colonies, 24 hours in culture: edge of colony	5.1 ± 2.2 (8)	0.58 ± .11 (8)
		Center of colony	3.7 ± 1.1 (7)	0.58 ± .16 (7)
6	Chick embryo brain	Tissue slice†	5.0 ± 2.0 (15)	0.52 ± .10 (13)
		Dissociated cells, 4 hours in culture	6.2 ± 4.1 (10)	0.40 ± .08 (9)
		Dissociated cells, 24 hours in culture	6.6 ± 3.7 (24)	0.57 ± .08 (22)
		Antibody to N-CAM		
		Dissociated cells, 24 hours in culture	5.7 ± 2.5 (27)	0.50 ± .07 (22)
		Antibody to brain membrane		
7	Chick embryo retina	Tissue slice‡	7.0 ± 3.7 (17)	0.50 ± .11 (15)
		Dissociated cells, 24 hours in culture	9.6 ± 4.7 (24)	0.54 ± .11 (23)
		Antibody to N-CAM		
		Dissociated cells, 24 hours in culture	7.5 ± 3.2 (33)	0.50 ± .14 (30)

*Of 59 independent measurements, 15 showed fluorescence recovery, 33 showed no recovery, and 11 were uninterpretable. †Of 17 independent measurements, 13 showed recovery, one showed no recovery, and three were uninterpretable. ‡Of 18 independent measurements, 15 showed recovery, one showed no recovery, and two were uninterpretable.

general, the average R of neural and liver tissues was somewhat lower than that of fibroblasts.

The mobility of a single surface molecule, the neural cell adhesion molecule (N-CAM), was also measured on chick brain and retina cells (Table 1, experiments 6 and 7). This cell surface molecule has been identified with the use of highly specific antiserums, and its role in cell-cell adhesion and development of neural tissue has been intensively studied in our laboratory (*13*). Despite its role in cell-cell adhesion, the mobility of this specific receptor was similar to that of the more general population of receptors measured using polyspecific anti-brain membrane serum.

Our results indicate that the average D's of a wide variety of surface receptors (but not necessarily all) fall within a narrow range, varying less than twofold under different conditions of cell growth and interaction. This variation is much less than the sixfold decrease in D seen on lectin-induced anchorage modulation (*4*). We conclude that if reversible modulation of receptor mobility is a significant mechanism for signaling cell-cell interactions, it must take place by the specific modulation of a small set of particular individual receptors rather than by general modulation of surface properties.

The differences in the fraction of mobile receptors observed between fibroblasts and the other cells suggest that the distribution of individual receptors in the population between the anchored and free mobility states may be characteristic of differentiation states, cell types, or morphologies. Consistent with this suggestion is the observation that about half of the cells measured in liver tissue showed no apparent recovery. Also, it has been shown that half of human lymphocytes labeled uniformly with a fluorescent monoclonal antibody against HLA antigens show no detectable redistribution of fluorescence after photobleaching, while the other half show redistribution with D of 6.9×10^{-10} cm^2/sec (*14*).

In summary, while Con A binding decreases receptor mobility in a variety of cells, the presence of cells in tissues does not appear to mimic this kind of modulation. However, receptors on about half of the cells in liver tissue labeled with polyspecific antibodies recognizing at least 15 different surface antigens, they were essentially immobile ($D < 5 \times 10^{-12}$ cm^2/sec). In contrast, the same receptors on dissociated liver cells showed values for D and R comparable to other cells. This suggests that naturally occurring modulation may take place by an "all or none" change (or greater than 100-fold decrease) in the mobility of specific receptors, rather than by a sixfold decrease as induced by lectins. Further experiments with monoclonal antibodies or other antibodies of very restricted specificity will be required to test this hypothesis.

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References and Notes

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7. Measurements were made with an apparatus similar to that described by D. E. Koppel, D. Axelrod, J. Schlessinger, E. L. Elson, W. W Webb [*Biophys. J.* **16**, 1315 (1976)], connected to a DEC PDP-12 computer for on-line data collection, shutter control, and data processing. Intensity measurements were made with a beam power (at 568.2 nm) of 15 watt/cm^2 in the specimen plane; pulses about 200 msec long with intensities from 1×10^4 to 6×10^4 watt/cm^2 were used for photobleaching. The fluorescence was monitored every 200 msec during the initial recovery, then every second for the remainder of the measurement. Bleaching by the measuring beam was less than 5 percent. These conditions are in the range devoid of detectable photoinduced artifacts [D. E. Wolf, M. Edidin, P. R. Dragsten, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2043 (1980)]. The time constant of recovery and the fluorescence intensities at the start of recovery and at infinite recovery time (used to estimate the mobile fraction) were obtained from a nonlinear least-squares fit of the observed data to the theoretical equation for recovery. Control measurements of the diffusion of rhodamine-labeled Fab' fragments in glycerol solutions gave the expected coefficients (*6*). In a typical experiment, cells or tissue slices were incubated with rhodamine-labeled monovalent Fab' fragments of rabbit antibodies to cell surface antigens (50 μg/ml) for 15 minutes at room temperature in Hanks balanced salt solution (lacking phenol red) containing 50 m*M* *N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid (*p*H 7.3) and crystalline bovine serum albumin (1 mg/ml). The cells were then washed in the same buffer, and measured at room temperature (20° to 23°C), usually with a ×40 water-immersion objective (numerical aperture, 0.75), giving a spot radius ($1/e^2$) of 1.2 μm. The relative accuracy of the D's is not affected by errors in the estimation of the spot radius.
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Pesticides: Insecticides and Fungicides Are Chitin Synthesis Inhibitors

Abstract. *Several important groups of fungicides and insecticides are specific inhibitors of chitin synthesis in a* Phycomyces *enzyme system and in insect organ cultures. The recently discovered benzoylphenylurea insecticides, which prevent chitin synthesis in insect tissues, are apparently not direct-acting chitin synthetase inhibitors. These insecticides may prevent insect chitin synthesis by interfering with the proteolytic activation of the chitin synthetase zymogen.*

The biosynthesis of chitin skeletal structures is a promising molecular target for pesticide action, since chitin is restricted in its biological distribution (*1*). The effects of fungicides and insecticides on chitin synthesis have received increasing attention following reports (*2, 3*) that several agricultural chemicals affect insect chitin synthesis [also see review (*4*)].

We have developed biochemical and tissue culture methodologies that allow us to examine the mode of action of compounds that specifically interfere with chitin biosynthesis in insects and fungi. Table 1 lists compounds that are specific inhibitors of a cell-free preparation of chitin synthetase derived from the fungus *Phycomyces*. Specificity is defined as resistance of I_{50} level inhibition to the addition of excess protein (ovalbumin) (I_{50} is the concentration in moles of the compound that produces 50 percent inhibition of control chitin synthetase activity). This test establishes that the specific compounds do not react nonselectively with polypeptide functional groups. Compounds active in the *Phycomyces* system include chlorinated hydrocarbons, triazines, nitrophenols, organophosphates, sulfenimides, and thiolanes. Many of these compounds were not pre-

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