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LATERAL DIFFUSION OF CONCANAVALIN A RECEPTORS IN THE PLASMA MEMBRANE OF MOUSE FIBROBLASTS

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Summary

Lateral diffusion of receptors binding fluorescein labeled concanavalin A and its succinylated derivative has been measured by bleaching portions of the labeled surface and following return of fluorescence to the bleached spot. Binding of either concanavalin A or its succinylated derivative causes restriction of mobility of the surface receptors for this lectin. The degree of restriction is a function of time after binding the lectin.

Cell membranes may be considered as continuous fluids in which membrane proteins are free to move by diffusion [1]. However, many experiments indicate that cells place constraints on membrane protein movements, either as part of cell differentiation (for example, intestinal epithelial cells or muscle cells), or in response to external manipulations, such as addition of antigens or lectins to cultured cells [2,3]. The latter, in particular the lectin concanavalin A, may immobilize surface receptors by stimulating changes in the polymerization of submembranous microtubule systems [3]. Here we use a method developed for measuring protein diffusion in the membranes of single cultured cells to measure the diffusion of concanavalin A receptors.

Our method measures return of fluorescence to a spot bleached in an otherwise uniformly labeled cell surface. Similar experiments have indicated that erythrocyte membrane proteins are immobile [4], but that the integral proteins of a transformed cultured fibroblast cell, C1 1d, move with a diffusion constant $D \approx 2 \cdot 10^{-10}$ cm$^2 \cdot$s^{-1} [5]. In our present experiments we find slower rates of diffusion than this for concanavalin A receptors on non-transformed cells, as well as a dependence of mobility on time after addition of concanavalin A. Our results suggest that concanavalin A sites may be clusters of molecules or otherwise anchored and that addition of concanavalin A or its succinylated divalent derivative alter clustering or anchoring of receptor molecules so as to greatly inhibit their diffusion in the plane of the plasma

A similar experimental system has been used to evaluate concanavalin A receptor movement in other cultured cells [6]. Our data are in general agreement with results obtained in that system. Discrepancies in rates of diffusion and rates at which it is inhibited may well depend on differences in the cells used, the levels of concanavalin A applied, and the choice of geometries for bleaching and measurement.

Concanavalin A (Sigma) was conjugated with fluorescein isothiocyanate by permitting 100 mg in 25 ml of isotonic saline (buffered to pH 7.5) to react with 5 mg of fluorescein isothiocyanate (Baltimore Biological Laboratories), with stirring in the cold room for 5 h. The conjugate was freed of excess dye by passage over Bio Gel P-6. It was then bound to Sephadex G-50 and eluted with 0.1 M glucose. Glucose was removed by two passages over Bio Gel P-6. The conjugate's molar ratio of fluorescein to protein obtained was as determined by corrected $A_{280\ nm}$ and $A_{496\ nm}$. A portion of this material was succinylated as described [7]. The succinylated material showed a single peak on analytical centrifugation, which migrated more slowly than native concanavalin A. It failed to agglutinate C1 1d cells at a concentration of 100 μg per ml; the same cells were readily agglutinated by intact, fluorescent concanavalin A at a concentration of 3 μg/ml.

C1 1d, a clone of L-M cells [8] has been maintained in tissue cultures in our laboratory for 8 years. Embryo fibroblasts were prepared from eviscerated 13—15 day C57BL/10 mouse fetuses [9]. They were used either as primary cultures or in passages 1—5 after preparation. Cells grown on coverslips were washed free of medium by rinsing with phosphate-buffered saline. They were then bathed with fluorescein-concanavalin A or succinyl-fluorescein concanavalin A at concentrations of 0.2—6.0 mg/ml in Hepes-buffered Hanks solution at 22—24°C. At the highest concentrations of lectin, minimal time of exposure for adequate labeling was 15 s. Maximum labeling time used was 2 min. After labeling, cells were washed free of excess label and observed in a Leitz fluorescence microscope. All labeled cells ring-stained, though the intensity of the stain varied from cell to cell. Binding of labeled lectin was specific; it was blocked by 0.1 M α-methyl mannoside.

To measure diffusion, a 441 nm laser beam was focused on a portion of the periphery of a labeled cell. The fluorescence in the spot bleached was followed with a photomultiplier operated in the photon counting mode. Intensity in the spot was read from an analogue signal applied to a chart recorder. Further details of the instrument are given in [5]. The spot bleached was always at the edge of the cell chosen. In C1 1d this resulted in a spherical spot on a sphere. Embryo fibroblasts were bleached at the edges of flattened cylindrical processes.

No recovery of fluorescence was observed when C1 1d cells were labeled with fluorescein-concanavalin A, even when time between labeling and bleaching of a single cell was as short as 4 min (19 cells). Cells labeled with divalent succinyl-fluorescein concanavalin A also showed no recovery of fluorescence in bleached spots on their peripheries (10 cells). However, large scale movement of bound lectin did occur; C1 1d formed fluorescent caps after labeling with tetrameric concanavalin A and formed patches after labeling with succinyl-concanavalin A. In contrast, confluent primary mouse

embryo fibroblasts labeled with concanavalin A showed slow, but definite recovery of fluorescence in bleached spots for about 1 h after labeling. At times greater than 1 h after labeling, no recovery of fluorescence was detectable in bleached spots (46 cells).

Much more rapid recovery of fluorescence was seen if succinyl-fluorescein concanavalin A was applied to confluent primary fibroblasts (20 cells). The recorder trace for one such cell is shown in Fig. 1. In Fig. 2, data derived from recordings of the type shown in Fig. 1 are plotted as $\hat{I} = (I_t - I_0)/(I_i - I_0)$ where I_i is intensity before bleaching, I_t is intensity measured at some time after bleaching and I_0 is an estimate of intensity immediately after bleaching. The curves are from cells at times up to 2 h after labeling; it can be seen that the rate of recovery of fluorescence decreases with time after labeling. No recovery was seen if sparse isolated cells were bleached.

Recovery of fluorescence in the bleached spot might be due to: (a) Reversion of bleached fluorescein molecules to fluorescent forms. (b) Release of bleached succinyl-fluorescein concanavalin A molecules from their receptors and diffusion into the bleached spot of free, unbleached, succinyl-fluorescein concanavalin A molecules dissociated from their receptors. (c) Bulk flow of labeled receptors into the bleached area; that is the process thought to be underlying "cap" formation. (d) Diffusion of labeled receptors in the plane of the membrane.

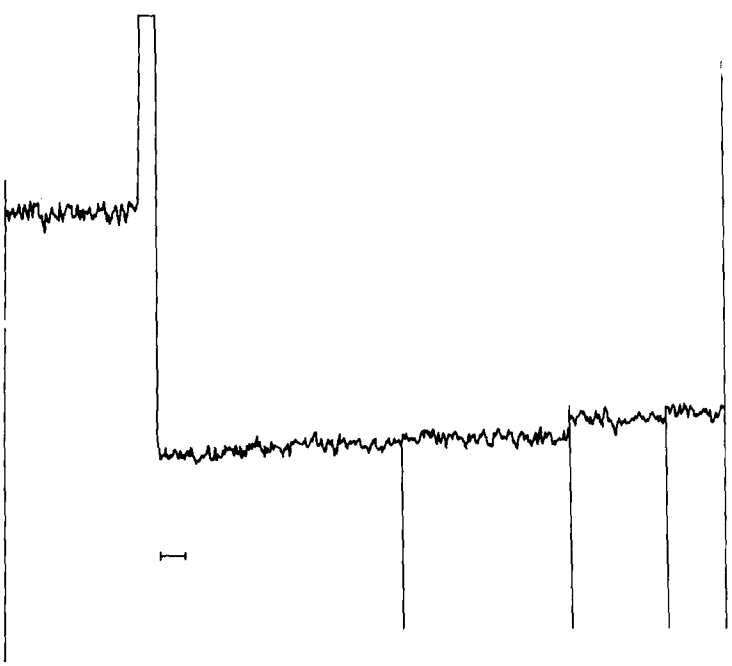

Fig. 1. Recorder trace of the fluorescence intensity in a spot bleached at the periphery of a succinyl-fluorescein concanavalin A-labeled embryo fibroblast. The record to the left is for intensity in the spot before bleaching. The upward spike indicates application of the laser beam. Measurements were taken continuously for 60 s and then from 105 to 150 s, from 400 to 420 s and from 540 to 555 s (9 min after bleaching). The downward vertical lines indicate breaks in the record between each of the periods listed. Scale: 1.2 cm vertical = 0.1 V; 1 cm horizontal = 6 s. Bar indicates 1 cm.

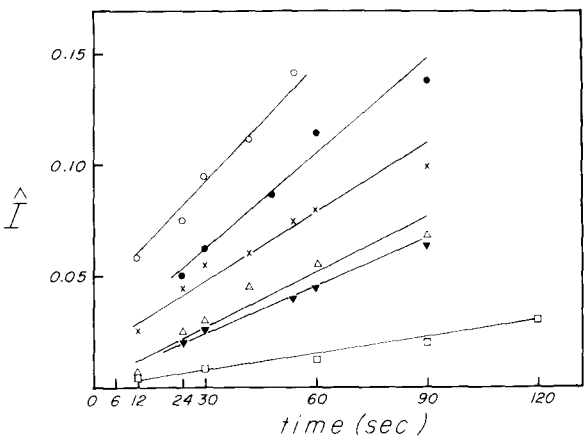

Fig. 2. $\hat{I}$, the ratio of corrected intensity after bleaching to corrected intensity before bleaching for primary mouse embryo fibroblasts. The ordinate is expanded; recovery is probably not linear. 5 min after adding intact fluorescein concanavalin A. All other plots are for times after adding succinyl-fluorescein concanavalin A. ○, 10 min; △, 40; X, 60; □, 70; ▽, 110 min after adding succinyl-fluorescein concanavalin A.

Possibility (a) is apparently ruled out since: (i) recovery of fluorescence is a function of the time elapsing between labeling and observing, and (ii) when an entire cell is bleached, no change in fluorescence can be observed for at least 3 min after bleaching. We attempted to control for possibility (b) by fixing labeled cells in 0.5% paraformaldehyde in phosphate-buffered saline, pH 7.5. Cells fixed in this solution for 1 h at 0°C still showed recovery of fluorescence in bleached spots. The fixation would be expected to cross-link concanavalin A to its receptors. Also, divalent labeled concanavalin A could not be displaced from cells by an excess of unlabeled tetravalent concanavalin A. This would not be true if lectin and receptor were only loosely associated, and lectins could exchange places with one another.

Possibility (c) is controlled for in 2 ways. First, the fixation experiment described above ought to have arrested cell metabolism. (However, it should be noted that C1 1d so fixed still appeared to patch their concanavalin A receptors.) Second, no caps, visible evidence of membrane bulk flow, could be observed in labeled primary fibroblasts, even after 1 hour at room temperature, though local patches of succinyl concanavalin A did form.

The controls strongly suggest that fluorescence in a bleached spot recovers because of lateral diffusion of lectin receptors. The fixation procedure described above is relatively mild and would not be expected to block lateral diffusion. Furthermore, this fixation does not interfere with the intermixing of heterokaryon antigens (Edidin and Wei, unpublished), a process that has been shown to occur independently of cell metabolism [10]. Fixation in 5% paraformaldehyde in phosphate-buffered saline for 3 h at 0°C does block recovery of fluorescence in the bleached spot, consistent with the much more extensive denaturation of membrane protein caused by this concentration of fixative.

We were able to monitor the lateral mobility of concanavalin A receptors after binding fluorescent concanavalin A or its succinylated, divalent, derivative

to cultured cells. Our observations are, to some extent, paradoxical.

First, C1 1d cells, which have highly mobile membrane proteins [10], did not show any motion of concanavalin A receptors. Despite this, receptors did collect into caps.

Second, primary fibroblasts, whose membrane proteins diffuse at least 10-fold more slowly than those of transformed cells [11] and which do not cap showed some degree of concanavalin A receptor mobility, though this mobility decreased with time. Perhaps the immobilization of receptors involves a diffusion-dependent step which occurs more rapidly in C1 1d than in primary fibroblasts. Cross-linking of receptors due to random collisions is one such step, and aggregates formed in this way would diffuse slowly, mobility being linearly proportional to radius. However, aggregates would have to be very large in order to inhibit diffusion, approximately 1000-fold larger than a single molecule of membrane protein. Such aggregates could be detected as large fluorescent patches or spots on the surface of a 12—15 μm diameter cell. We failed to see such patches.

A second process might be clustering of a few concanavalin A receptors leading to immobilization of these receptors, via cytoplasmic anchoring systems (cf. 3). Here initial diffusional mobility of receptors could be a factor determining the rate of microcluster formation. If this mobility is indeed higher in C1 1d than in primary fibroblasts, we expect faster initiation of the steps leading to complete anchoring of receptors.

Quantitative observations with heterokaryons indicate differences between the mean mobility of H-2 antigens in primary fibroblasts and transformed cells of 6—10 fold [11]. Such magnitudes might be accounted for if the receptors of non-transformed cells were clustered into oligomers approximately 6 times larger than those on transformed cells, or if individual receptors were anchored to small structures in the cell cytoplasm. We propose that the following sequence of events occur when concanavalin A or s-concanavalin A is bound to cells:

(a) Transformed cells, bearing highly mobile receptors, rapidly re-form these receptors into aggregates. Aggregation of receptors triggers their immobilization by anchoring into a submembrane cytoplasmic system, perhaps similar to that described in lymphocytes by Edelman and coworkers [3,12]. (b) Primary fibroblasts and other contact-inhibited cells bear receptors in aggregates roughly an order of magnitude larger than those in transformed cells. These aggregates may be receptor to receptor or receptor to cytoplasmic anchor. Addition of concanavalin A or succinyl concanavalin A results in bringing these pre-existing aggregates together, again anchoring them. As noted in larger scale experiments, tetrameric concanavalin A is more efficient in causing immobilization than is succinylated concanavalin A, either because of more efficient cross linking or because a second type of membrane binding site is modified in the succinylated derivative [12].

We suggest then that concanavalin A receptors exist in various degrees of cross linking in different cell types.

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