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Receptor diffusion on cell surfaces modulated by locally bound concanavalin A

(surface modulating assembly/photobleaching recovery/concanavalin A-platelets/microtubules/3T3 mouse fibroblasts)

J. SCHLESSINGER*†, E. L. ELSON†, W. W. WEBB*, I. YAHARA‡, U. RUTISHAUSER‡, AND G. M. EDELMAN‡

* School of Applied and Engineering Physics and † Department of Chemistry, Cornell University, Ithaca, New York 14853; and ‡ The Rockefeller University, New York, N.Y. 10021

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ABSTRACT **In order to test the anchorage modulation hypothesis, the fluorescence photobleaching recovery method was used to measure the global inhibition of cell surface receptor mobility induced in 3T3 mouse fibroblasts by local binding of platelets labeled with concanavalin A (Con A). By measuring the diffusion of antibody-labeled cell surface receptors at various points on the cell surface, two states, immobile and mobile, were distinguished in the receptor population. Bound Con A-platelets, occupying between 4% and 30% of the cell surface, decreased the diffusion coefficient of the mobile population by a factor of 6. The magnitude of this effect was independent of distance from the sites of the bound Con A-platelets, demonstrating the propagated and nonlocal properties of the modulation effect. The immobile fraction of the population was not changed by Con A-platelet binding. Modulation of the diffusion constant of mobile receptors was partially reversed by treatment with microtubule-disrupting agents such as Colcemid and *Vinca* alkaloids. High doses of soluble Con A induced even higher levels of modulation than Con A-platelets, but reversal by microtubule-disrupting drugs was not observed. These experiments provide additional support for the anchorage modulation hypothesis and provide a measure of the nature and degree of mobility at the molecular level. They also put important constraints on the hypothesized interactions among submembranous components (microtubules and microfilaments) of surface modulating assemblies.**

The behavior of cell surface receptors has recently been studied intensively because of their strategic location in the cell and their role in controlling fundamental cellular functions. Several properties of surface receptors depend upon their lateral mobility in the membrane. Although the lipid bilayer in which many surface proteins move is fluid, great variation has been observed in the mobilities of various receptors, even within a specific receptor class (1–3). Moreover, it has been shown (4, 5) that the patching (and therefore the capping) of mobile receptors can be inhibited globally over the cell by the crosslinking of certain surface proteins with lectins. The inhibition has been ascribed to the prevention of lateral motion at the level of individual receptors. This effect, called anchorage modulation (6), is a propagated, nonlocalized event (4, 5) that appears to be mediated by a surface modulating assembly (SMA) containing microfilaments and microtubules (7). It has been shown that the state of the SMA is correlated with the expression of key cell functions such as growth control (6).

In the present experiments, we have used fluorescence photobleaching methods (8) to test the anchorage modulation hypothesis at the molecular level. The lateral diffusion of surface membrane components labeled with fluorescent antibodies was measured quantitatively in experiments detecting transport over distances of a few micrometers. Concanavalin A (Con A) coupled to platelets (5) was used to crosslink glycoproteins in small localized regions of the surface of 3T3 mouse fibroblasts and diffusion coefficients were measured at different distances from the bound Con A-platelets before and after addition of microtubule-disrupting agents. This procedure permitted us to determine the dependence of the diffusion coefficients and relative amounts of immobilized receptors upon the number and location of bound platelet groups as well as upon the state of cytoplasmic microtubules.

Abbreviations: Con A, concanavalin A; HBSS, Hanks' balanced salt solution; FPR, fluorescence photobleaching recovery; RaP388, rhodamine-labeled antibodies directed against mouse lymphoid P388 cell line; SMA, surface modulating assembly; CSP, the major extrinsic cell surface protein of fibroblasts.

MATERIALS AND METHODS

Reagents. Rabbit antibodies and Fab fragments directed against mouse P388 lymphoid cell lines were prepared as described elsewhere (9). These antibody preparations were crossreactive with 3T3 cells. Rhodamine-labeled Con A and antibodies were prepared using tetramethyl rhodamine isothiocyanate. Fresh human blood platelets were obtained from the American Red Cross (Syracuse, N.Y.) and were labeled with Con A as previously described (5).

Cells. The 3T3 cells were grown in 35 mm plastic petri dishes in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. Prior to labeling, the cells were washed twice with Hanks' balanced salt solution (HBSS), after which 100 μg of rhodamine-labeled anti-P388 antibodies (RaP388) or of their Fab fragments were added to cells covered with 1 ml of HBSS. After 15 min incubation at 37°, the cells were washed twice with HBSS and then incubated for 30 min at 37° with 2×10^7 Con A-platelets in 1 ml HBSS. Effects of microtubule-disrupting agents were tested by incubating unlabeled cells with the appropriate drug at 1 μM for 45 min at 37°, washing twice with HBSS, and then labeling with RaP388 and Con A-platelets. In most cases, incubation with these drugs resulted in a slight retraction of the cell periphery.

Photobleaching Measurements. Diffusion coefficients of RaP388 bound to cell surfaces were measured by the fluorescence photobleaching recovery (FPR) method (1–3, 8, 10). Fluorophores in a small (about 3 μm^2) area of the cell surface were irreversibly photobleached by a short pulse of intense focused laser light (λ = 520.8 nm). Rates of diffusion into the bleached region of unbleached fluorophores from the surrounding cell surface were determined from the recovery of the fluorescence measured in the bleached regions with attenuated laser excitation. Since the bleached area was small compared to that of the cells, measurements could be carried out at several spots at different distances from the Con A-platelets. The number of platelets on each cell was counted and the positions of each FPR measurement and of the bound platelets on each cell were recorded on Polaroid photomicrographs taken at the time of the experiment. The fraction of the cell surface area covered by the platelets was estimated from

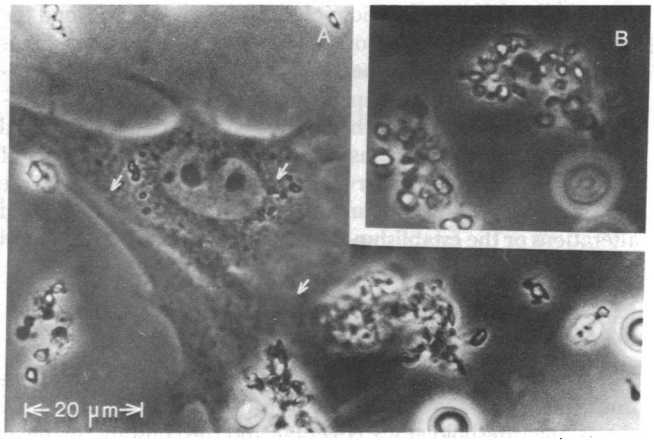

FIG. 1. Phase contrast photomicrograph of a 3T3 cell labeled with approximately 30 Con A-platelets. (*A*) Arrows indicate typical positions for FPR measurements. (*B*) An area containing platelets with plane of focus slightly altered to display the platelets more clearly.

these photographs. In most cases, the platelets were bound as aggregates of about 10 to 20 platelets each; this did not impede the measurement of areas or of fluorescence. The time interval from the beginning of the incubation with rhodamine antibody to the first measurement was 45–60 min. Two to three FPR measurements were performed on each cell over a period of 30–45 min.

RESULTS

Cell labeling

A 3T3 cell labeled with approximately 30 Con A-platelets is shown in Fig. 1. The possibility that Con A might have been dissociated from platelets and bound in a dispersed distribution over the surface was tested by the following experiment. 3T3 cells were labeled with Con A-platelets by the standard procedure and then with antibodies to Con A that were labeled with rhodamine. The fluorescence intensity from an illuminated spot of 5 μm radius was measured at different locations on the cell surface including the area covered by the platelets. The results of measurements on five cells are presented in Table 1. The fluorescence intensity measured on areas not covered by the Con A-platelets was not significantly greater than the background fluorescence intensity of an unlabeled cell. The sensitivity of the method was sufficient to exclude redistribution and rebinding of Con A at a level 1/5 of that produced by treatment with soluble Con A at 20 μg/ml, a concentration that did not modulate receptor mobility on these cells. Moreover, there was no visible transfer of rhodamine-labeled Con A from platelets labeled with rhodamine-Con A. Unlabeled platelets did not adhere to the cells. These and previous experiments (5) exclude the possibility that Con A released from platelets and rebound to the cell surface could have accounted for the modulation effects of Con A-platelets discussed below.

Table 1. Evidence for stability of Con A-platelet complexes on the cell surface

Cell treatment	Region of measurement	Fluorescence intensity (counts/sec)*
Con A-platelets	On platelets	29,000 ± 3,500
Con A-platelets	Membrane adjacent to platelets†	1,300 ± 320
No Con A	Membrane	1,250 ± 200
Soluble Con A (20 μg/ml)	Membrane	7,300 ± 1,300

* The cells were labeled with rhodamine-labeled anti-Con A and the fluorescence intensity was measured in an illuminated area of 5 μm radius. Each value represents the average of measurements on five different cells, ± standard deviation.

† The center of the illuminated area was within 5 μm of the platelets.

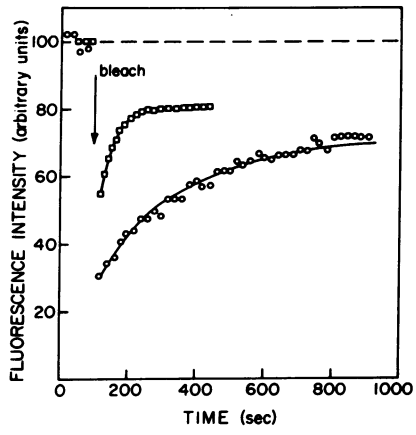

FIG. 2. Fluorescence photobleaching recovery curves of two cells labeled with RaP388 Fab. One cell had no Con A-platelets (□), the other had approximately 60 platelets covering 13% of its area (○). Both recoveries fit the theory for a single diffusion coefficient within experimental error (superimposed curves). $D = 1.25 \times 10^{-10}$ cm^2/sec, and $D = 1.8 \times 10^{-11}$ cm^2/sec, respectively.

The diffusion of surface antigens was modulated by Con A-platelets

Typical FPR recovery curves for RaP388 Fab on 3T3 cells with and without bound Con A-platelets are shown in Fig. 2. In this experiment, the recovery was about 7-fold slower on the cell with the Con A-platelets. Both recovery curves can be interpreted in terms of simple diffusion (10) with a single diffusion coefficient: D (with platelets) $= 1.8 \times 10^{-11}$ cm^2/sec; D (without platelets) $= 1.25 \times 10^{-10}$ cm^2/sec. Results obtained from measurements on 20 cells are presented in Fig. 3A. To rule out the possibility that the observed mobility might have resulted from dissociation of the RaP388 Fab from the cell surface with reassociation at a different location, cells were prefixed with 3% (wt/vol) glutaraldehyde for 45 min at 23° and then

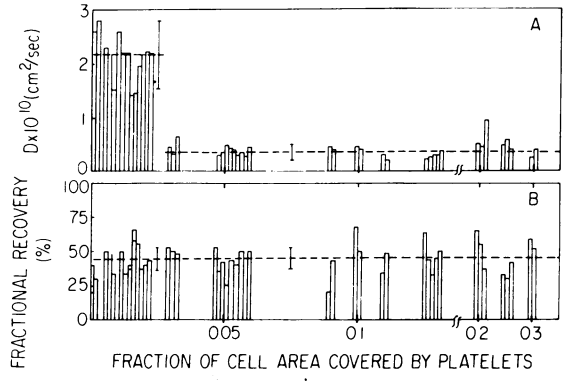

FIG. 3. Diffusion coefficients (*A*) and fractional fluorescence recoveries (*B*) versus the fraction of cell area covered by Con A-platelets. The data were obtained from experiments on 20 cells with two to three measurements at different locations on each cell. The broken lines represent the averages of measurements on cells with more and less than 4% surface coverage by platelets. Error bars indicate standard deviation.

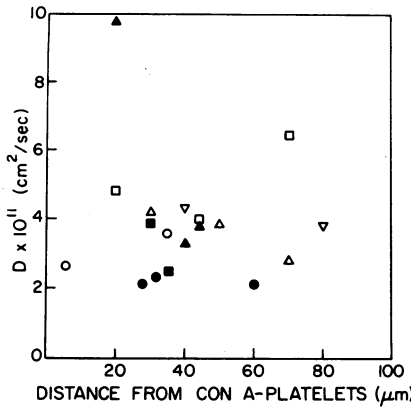

FIG. 4. Receptor diffusion coefficients at different distances from the center of the area covered by Con A-platelets. Each symbol represents a different cell.

treated as usual with RaP388 Fab and Con A-platelets. Fluorescence recovery after bleaching did not occur on such fixed cells.

When less than 4% of the upper surface of the cell was covered with Con A-platelets, no modulation of the diffusion coefficients of labeled cell surface receptors was seen (Fig. 3*A*). Above that value, the degree of modulation was constant for coverages up to 30%. The stated coverage refers only to the upper surface of the cell and is a maximum estimate assuming that every platelet associated with the cell was attached directly to it via Con A bridges.

The fractional recovery of fluorescence after bleaching was not altered by Con A-platelets at any degree of coverage (Fig. 3*B*) and in no case was recovery complete. The fraction of unrecovered fluorescence in an FPR measurement was interpreted to be the fraction of immobile antibody-labeled receptors (1–3). Measurements on logarithmic phase, confluent, and serum-starved cells showed similar diffusion coefficients and fractional recoveries.

Modulation did not depend on distance from the Con A-platelets

Diffusion coefficients were measured as a function of distance from the center of the nearest region covered by Con A-platelets (Fig. 4). The differences among receptor diffusion coefficients

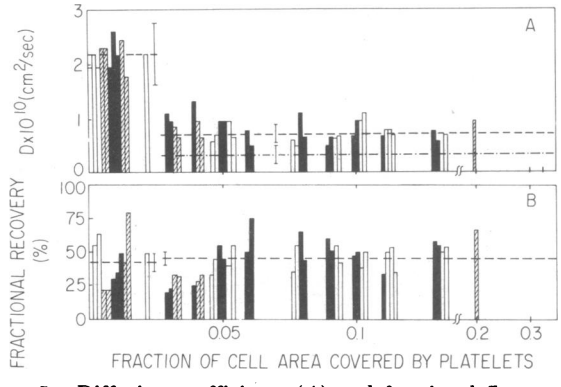

FIG. 5. Diffusion coefficients (*A*) and fractional fluorescence recoveries (*B*) versus fraction of cell area covered by Con A-platelets in the presence of microtubule-disrupting drugs. The labeled cells were pretreated as described with Colcemid (hatched bars), vinblastine (solid bars), and podophyllotoxin (open bars). The (- - -) lines indicate averages of measurements on cells with more and less than 4% area coverage. The line (·--·) indicates the average diffusion coefficient of untreated cells presented in Fig. 3.

measured at different locations on a single cell were beyond the range of experimental error. No systematic dependence was observed, however, and the amount of this intracellular variation appeared to be independent of the number of Con A-platelets on the cell. Nevertheless, it is possible that some spatial gradient of modulation was obscured by the heterogeneity of diffusion coefficients observed on a single cell. Moreover, the time required for the measurements was too long to detect rapid alterations or the establishment of spatial gradients over a short period.

Microtubule-disrupting agents partially reversed modulation of receptor diffusion

The effects on modulation of Colcemid, vinblastine, and podophyllotoxin are presented in Fig. 5*A*. In the presence of these drugs, the threshold at 4% coverage and the constant degree of modulation at higher coverages were retained, but the diffusion constant increased by a factor of 2, and thus the release of the modulation effect was not total. The drugs did not affect the diffusion coefficients on cells with coverages of less than 4% or on cells with no Con A-platelets. The drugs also did not affect fractional recoveries (Fig. 5*B*).

Soluble Con A induced higher levels of modulation

The diffusion rates of Con A bound to 3T3 cells and the effects of Con A binding on the diffusion of RaP388 Fab are summarized in Table 2. The mobility of rhodamine-labeled Con A was highly dose-dependent: at 20 μg/ml, $D = (1.5 \pm 0.3) \times 10^{-11}$ cm²/sec, but at 100 μg/ml, the Con A receptors were immobile ($D < 6 \times 10^{-12}$ cm²/sec). The mobility of bound RaP388 was not significantly affected by Con A at 20 μg/ml, but at 100 μg/ml it was reduced to $D = (1.6 \pm 0.5) \times 10^{-11}$ cm²/sec. It is noteworthy that at 100 μg/ml Con A, a dose at which rhodamine-labeled Con A was immobile, RaP388 retained some mobility. At the doses tested, drugs interfering with microtubule assembly had no effect on the modulation by soluble Con A, in contrast to the partial reversal of modulation induced by Con A-platelets. These data indicate that soluble Con A modulated the diffusion of surface antigens to a greater extent than did Con A-platelets. This observation and the lack of the drug effects

Table 2. Effect of Con A, Con A-platelets, and Colcemid on the mobility of cell surface components

Surface-binding molecule labeled with rhodamine	Con A (μg/ml)	Diffusion coefficient (10^{-11} cm²/sec)*
Succinyl-Con A (20 μg/ml)	—	3.9 ± 1.1
Con A (20 μg/ml)	—	1.5 ± 0.3
Con A (100 μg/ml)	—	<0.6†
aP388 Fab‡	0	26 ± 8
aP388 Fab‡	20	19.5 ± 5
aP388 Fab‡	100	1.56 ± 0.5
aP388 Fab‡	100 (+ Colcemid)§	1.31 ± 0.55
aP388 Fab‡	Platelets	3.6 ± 1.1
aP388 Fab‡	Platelets (+ Colcemid)§	6 ± 1

* Diffusion coefficient of rhodamine-labeled protein, ± standard deviation.

† Representing essentially immobile components.

‡ Cells were incubated with 100 μg/ml Fab. Similar results were obtained with aP388 IgG.

§ Cells were pretreated with 1 μM Colcemid. Similar data were obtained with vinblastine, podophyllotoxin, and colchicine.

suggest that mechanisms (5) in addition to modulation by the SMA may play a role in modulation by high doses of soluble Con A.

DISCUSSION

The major conclusions of this study can be summarized as follows:

(*i*) An untreated cell has receptors in two major states: immobile or anchored, A_1 (6) ($D < 5 \times 10^{-12}$ cm^2/sec) and mobile or free, F (6) (1.4×10^{-10} cm^2/sec $\leq D \leq 2.8 \times 10^{-10}$ cm^2/sec).

(*ii*) Both platelet-bound and soluble Con A induced a decrease in the diffusion coefficient of F state receptors, i.e., they converted them to the less mobile state A_2 (3×10^{-11} cm^2/sec $\leq D \leq 8 \times 10^{-11}$ cm^2/sec).

(*iii*) No changes in the amount of the immobile (A_1) fraction of the receptor population were induced by Con A-platelets or by lower doses (20 μg/ml) of soluble Con A.

(*iv*) The modulation effect showed a threshold and a plateau, i.e., occupancy of greater than 4% of the cell surface induced the effect but larger occupancies did not increase it. The effect was seen in all regions of the cell surface despite the fact that the platelets were localized on only a small region of the surface. The degree of modulation varied as much within a cell as among cells without systematic variations in position or time.

(*v*) The propagated nature (4, 5) of the modulation of receptor mobility is confirmed by the position independence of the effect. The propagation time was faster than the relatively long periods required for the present measurements.

(*vi*) Modulation by Con A-platelets was partially reversed by microtubule-disrupting agents; the extent of the reversal was independent of the area occupied by the Con A-platelets. At the levels tested, modulation by soluble Con A was not affected by these drugs.

These observations indicate that there are at least three classes of receptor mobility states, A_1, F, and A_2. In terms of diffusion constants, $D_F > D_{A2} \gg D_{A1}$. The molecular basis of the A_1 state is unknown but this state may involve a variety of mechanisms in addition to possible anchorage to cytoskeletal elements. For example, the major extrinsic cell surface protein on fibroblasts (CSP) has been found to be immobile (J. Schlessinger *et al.*, unpublished observations); inasmuch as CSP does not penetrate the bilayer, it must be immobilized by another mechanism.

At present, it is not known whether the same cell surface protein receptors are present in all of the receptor states. It has been shown, however, that complexes of IgE with Fc receptors on rat peritoneal mast cells (3), and of α-bungarotoxin with acetylcholine receptors on rat myotubes (11), exist in both mobile and immobile states. In contrast, as mentioned above, CSP seems to be exclusively immobile. Thus, it will be necessary to classify each receptor experimentally using the appropriate specific antiserum.

As shown previously (5), the mechanism for the modulation of mobility induced by platelet–Con A complexes cannot be simple external crosslinking of receptors by Con A. This possibility is further excluded by the present control experiments demonstrating the localization of Con A at the platelets. Although treatment with soluble Con A may modulate the receptor diffusion coefficient by simple lectin crosslinking as well as by propagated anchorage modulation, the retention of a finite diffusion coefficient suggests that *stable* crosslinking cannot be the origin of this effect. In any case, it is likely that the modulated state A_2 induced by *soluble* Con A involves mechanisms in addition to anchorage modulation. This is supported by previous observations (5) on cocapping of surface receptors induced by soluble Con A.

A number of factors are important in considering models and mechanisms of anchorage modulation. First, the microtubule-disrupting drugs and cytochalasin B do not affect the diffusion of a lipid probe incorporated in the lipid bilayer of the plasma membrane (2). Second, microtubule-disrupting agents reverse anchorage modulation (4–6, 12) and in particular partially reverse modulation of receptor diffusion in the present experiments. Disruption of microtubules by *src* gene products of Rous sarcoma virus in transformed chick embryo fibroblasts also is accompanied by a large decrease in anchorage modulation (13). Third, cytochalasin B reduces the mobility of many receptors by a factor of 5 to 10 (1–3). Fourth, as shown previously (4, 5) and in these experiments, the local crosslinking of glycoproteins by lectins is an effective stimulus for a propagated global response. Fifth, there appears to be a threshold below which propagation does not occur and above which it is fully activated. Finally, the modulation of mobility reported here neither immobilizes F state receptors completely in forming A_2 nor increases the immobilized population A_1.

The model for the SMA proposed previously (6) is generally consistent with these observations, particularly those implicating cytoskeletal interactions. This model suggests that the receptors penetrate the fluid bilayer and interact indirectly with microtubules via microfilamentous structures and associated proteins. The assembly of tubulin to form microtubules is assumed to be critical in the anchorage process, but is not necessarily the only factor in anchorage of state A_1. It should be added that this model may have to be extended to account for the retention of mobility in the modulated state A_2 and the absence of change of the immobile fraction in A_1. Because of the lack of detailed information, this model does not specify the precise chemical nature of the interactions among the components of the SMA. Global modulation could be propagated from a nucleation site of localized Con A-platelet binding by several different mechanisms. For example, changes in the intracellular concentration of Ca^{2+} ions or phosphorylation of tubulin could alter polymerization equilibria that control the spatial extent and interactions of cytoskeletal structures. The

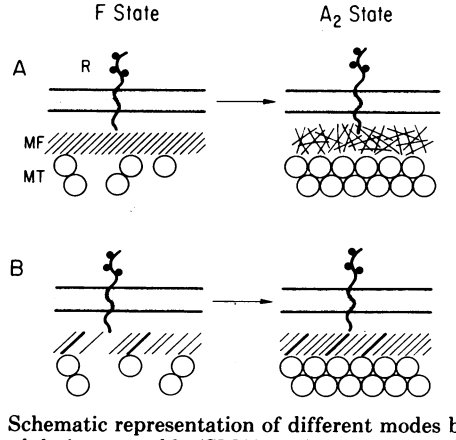

FIG. 6. Schematic representation of different modes by which a surface modulating assembly (SMA) might act to cause a change in receptor state from F to A_2. The various elements of the SMA have not been drawn to scale. (*A*) Modulation event induced by local crosslinkage of glycoprotein receptors (R) results in alteration of the submembranous components of the SMA with gelation of fibrils (MF) and restricted diffusion of the receptor. (*B*) Modulation results in enhanced binding of the cytoplasmic base of the receptor to submembranous structures. In either case, it is assumed that some intact microtubules (MT) are essential for modulation to occur.

sharp threshold at 4% coverage with the constant degree of modulation at higher coverage suggests a highly cooperative step—either in triggering or in propagation—that is in accord with earlier suggestions (4, 5).

As discussed previously (6), the disorderly gelation of subunits of actin or tubulin in a submembranous location (Fig. 6*A*) must be considered as an alternative to specific interactions of receptors with cytoskeletal components (Fig. 6*B*) as a mechanism for restriction of receptor diffusion. This mechanism is reconcilable with the observation that microtubule-disrupting agents partially reverse modulation, particularly if the postulated gel is also dissociated by these agents or if intact microtubules are necessary for formation of the gel. The fact that cytochalasin B retards receptor diffusion but not lipid diffusion (1–3) raises the possibility that it promotes disorderly gelation. These observations are consistent with a gelation model for modulation in which disassembled constituents of the SMA form a submembranous layer that increases the viscous drag on the mobile receptor. Recent observations of patch and cap formation that are in accord with receptor–microfilament interactions (14) are also consistent with this suggestion. As yet, however, a clear-cut choice between the two models in Fig. 6 cannot be made.

The present experiments confirm the phenomenon of modulation of receptor mobility at the molecular level and raise an interesting unanswered question about the nature of the anchorage of receptors in the A_1 state which are not detectably involved in modulation. The relation of the A_1 state to the A_2 state, in which there is anchorage modulation, will remain obscure until it is determined whether F and A_1 state receptors are interchangeable. In addition, determination of the nature and mechanism of propagation of the signal for modulation induced by local crosslinkage may require kinetic measurements within a time domain shorter than that accessible by the current techniques of mobility measurement. Such measurements, coupled with structural and reconstitution experiments, should allow formation of a more precise model of surface modulation and also help to specify the role (6, 13) of components of surface modulating assemblies in growth control.

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