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Mobility and distribution of a cell surface glycoprotein and its interaction with other membrane components

(membrane-associated glycoprotein/cell adhesion/photobleaching recovery/protein mobility)

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ABSTRACT **Fluorescence photobleaching recovery and immunofluorescence methods have been used to study the lateral mobility and topographical distribution of a major cell surface glycoprotein (CSP). Both endogenous CSP and fluorescent-labeled exogenous CSP bind to the cell surface in a fibrillar pattern and are immobile on the experimental time scale. Azide, vinblastine, and cytochalasin B do not alter the immobility and cell surface distribution of the CSP molecules. Therefore, oxidative phosphorylation and the cytoskeleton do not seem to be responsible for the properties of the bound glycoprotein. The presence of immobile CSP fibrils does not, however, impede the diffusion of a lipid probe, a ganglioside analogue, or various surface antigens. Therefore, the fibrils apparently do not form a "barrier" across the lipid phase of the plasma membrane. In contrast, concanavalin A binds to CSP and is largely immobile in regions rich in CSP. The presence of immobile concanavalin A receptors in areas or on cells lacking CSP indicates that other types of immobile concanavalin A receptors also exist.**

CSP does not bind to lipid bilayers composed of phosphatidylcholine or oxidized cholesterol. It does bind to dextran-coated bilayers as a diffuse distribution of mobile molecules that can patch after addition of antibodies to CSP. The latter result suggests that CSP molecules do not interact strongly with other CSP molecules under these conditions. Exogenous CSP binds to regions on the cell surface that already bear CSP. In view of the apparent weakness of CSP–CSP interactions on the lipid bilayer, it seems possible that the assembly of CSP fibrils is nucleated by cell surface components in addition to CSP.

A large glycoprotein (molecular weight ~ 200,000) has recently been identified on the surface of many fibroblastic cells (1, 2). The amount of this protein decreases substantially after transformation (2). Designated cell surface protein (CSP) (3) or large, external, transformation-sensitive (LETS) protein (4), this protein has been isolated from chick embryo fibroblasts (CEF), in which it constitutes 3% of the total cell protein (1, 3). CSP agglutinates erythrocytes, suggesting that it may play a role in cell adhesion (5). This possibility is reinforced by the observation that addition of exogenous CSP to transformed cells partially restores the morphology, adhesiveness, and parallel alignment of cells that are typical of normal fibroblasts, although the added CSP has no effect on growth control (6). The effects of CSP on cell morphology have recently been interpreted as resulting from its enhancement of cell adhesion (1, 6).

This report describes an investigation of how CSP binds to the cell surface, focusing particularly on its lateral mobility, its interactions with the lipid phase of the membrane and with various other membrane components, and the factors that determine the characteristic fibrillar pattern of bound CSP. The fluorescence photobleach method (7–9) was used to measure rates of macroscopic lateral motion of fluorescently labeled CSP and antibodies to CSP and to assess the extent of interaction between CSP and other cell surface components, including unselected surface antigens, an exogenous fluorescent ganglioside analogue, a lipid probe, and concanavalin A (Con A) binding components.

Abbreviations: CSP, cell surface protein; CEF, chicken embryo fibroblasts; Con A, concanavalin A; P_i/NaCl, phosphate-buffered saline; F-CSP, fluorescein-labeled CSP; HBSS, Hanks' balanced salt solution; RaCSP, rhodamine-labeled antibodies against CSP; diI, 3,3'-dioctadecylindocarbocyanine iodide.

MATERIALS AND METHODS

Goat antibodies against isolated, electrophoretically purified CSP were prepared and affinity-purified (5). Rhodamine-labeled antibodies were prepared by using tetramethylrhodamine isothiocyanate (7). CSP was isolated and purified as described (6). The protein was stored at a concentration of 1.6 mg/ml in 10 mM cyclohexylaminopropanesulfonic acid, pH 11/0.15 M NaCl/1 mM $CaCl_2$ in liquid nitrogen. CSP was labeled with fluorescein isothiocyanate by dialyzing 1.1 mg of CSP (in 0.7 ml) for 24 hr against phosphate-buffered saline (P_i/NaCl) (Grand Island Biological) at 4°, followed by dialysis against 20 ml of fluorescein isothiocyanate (100 μg/ml) in 0.05 M bicarbonate-carbonate, pH 9.7/0.15 M NaCl overnight at 4°. To remove excess free dye, the CSP was dialyzed for 4 additional days against the bicarbonate-carbonate buffer at 4°. Fluorescein-labeled CSP (F-CSP) was kept at 1 mg/ml in aliquots of 0.2–0.3 ml in liquid nitrogen.

Primary and secondary CEF and 3T3 cells were grown in 35-mm plastic tissue culture dishes at 37° in a 95% air/5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium containing 5% (vol/vol) fetal calf serum. For labeling with F-CSP, the cells were incubated overnight with F-CSP added to the growth medium at 10 or 2 μg/ml. After the labeling, the cells were washed twice with Hanks' balanced salt solution (HBSS). Then 2 ml of HBSS was added for the fluorescence photobleaching recovery measurements. For antibody labeling the cells were washed with HBSS; then, 1 ml of HBSS containing 10 μg of rhodamine-labeled antibody to CSP (RaCSP) was added. After 15 min at 37°, the cells were washed twice with HBSS, and 2 ml of HBSS was added for the fluorescence photobleaching recovery measurements. The effects of drugs were tested by incubating unlabeled cells with sodium azide (10 mM, 45 min), vinblastine (1 μM, 45 min), or cytochalasin B (10 μg/ml, 45 min). For all drug treatments, the incubations were at 37°, and then the cells were washed three times with HBSS and labeled with RaCSP.

The planar lipid bilayers were made either from egg phosphatidylcholine or from oxidized cholesterol. Dextran derivatives containing trinitrophenyl groups, rhodamine, and stearate residues were a generous gift from P. Henkart (National Institutes of Health). The procedures for preparing and incor-

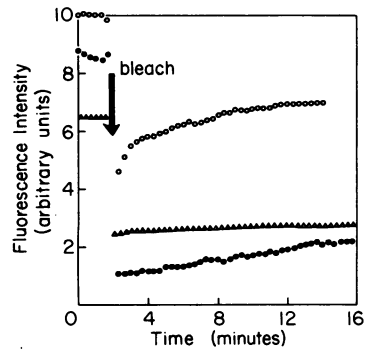

FIG. 1. Fluorescence photobleach recovery curves. △, RaCSP on CEF; $D \leq 5 \times 10^{-12}$ cm²/sec. ●, rhodamine–succinyl–Con A on CEF labeled with F-CSP in a CSP-rich area; $D \simeq 9 \times 10^{-12}$ cm²/sec; fractional recovery about 15%. ○, rhodamine–succinyl–Con A in a CSP-poor area; $D \simeq 4 \times 10^{-11}$ cm²/sec; fractional recovery ~ 50%.

porating the dextran derivatives into lipid bilayers have been described (10). About 10 μg of F-CSP in 1 ml of P_i/NaCl was added to the dextran-coated lipid bilayer for 30 min and then washed twice with the same buffer. Both sides of the bilayer were labeled with the dextran derivative.

In the experiments concerned with the interactions between CSP and other cell surface components, the cells were labeled with the following fluorescent probes under the specified conditions in addition to either RaCSP or F-CSP: (*i*) 25 μg of fluorescein-labeled ganglioside (GM_1) in 1 ml of HBSS for 15 minutes at 37° (the fluorescent-labeled ganglioside was a generous gift from H. Wiegandt); (*ii*) the lipid probe 3,3′-dioctadecylindocarbocyanine iodide (diI) (7); (*iii*) rhodamine–succinyl–Con A (7); (*iv*) fluorescein α-bungatotoxin (11); and (*v*) rhodamine-labeled antibodies against mouse lymphoid cell line P388 (12).

Diffusion coefficients of fluorescent-labeled cell surface components were measured by the fluorescence photobleaching recovery method (7–9). Fluorophores on a small area on the cell membrane (about 3 μm²) were irreversibly photobleached by an intense focused laser light. Rates of diffusion of unbleached fluorophores into the bleached region from the surrounding cell surface were determined from the recovery of the fluorescence measured in the bleached region with 1:1000 attenuated laser excitation. In some experiments the cell surface was labeled with two different markers that were labeled with fluorescein and rhodamine. In these experiments the mobility of the two fluorophores could be measured separately by using krypton laser lines at 482 and 568.2 nm, respectively.

RESULTS

RaCSP on CEF and 3T3 Cells Is Immobile. Fig. 1 presents a typical fluorescence recovery curve of RaCSP on a single CEF. The slight apparent recovery is at the limit of detection. These measurements cannot be extended to longer times because of interference from slow systematic motions of the cell surface such as might result from cellular locomotion. Therefore, only an upper bound to the rate of lateral transport on the membrane can be established. Although the mechanisms of possible motions slower than this limit are unknown, the limiting mobility is expressed as a diffusion coefficient, D, for consistency with other mobility measurements. For labeled CSP on these cells, D was less than 5×10^{-12} cm²/sec. (This corresponds to a flow velocity of less than 10^{-7} cm/sec.) The fractional recovery of fluorescence over the period of measurement was less than 10%. This protein can be considered essentially immobile.[§] The 3T3 cells showed lower levels of CSP on the cell membrane but with a fibrillar pattern similar to that seen on CEF. The CSP also was immobile on the 3T3 cells. On confluent CEF, the CSP was mainly concentrated at the edge of the cells in regions of cell-to-cell contact. The CSP was also immobile in this condition. Treating the cells with azide (an inhibitor of oxidative phosphorylation), vinblastine (a microtubule-disrupting agent), or cytochalasin B (which, among other effects, disrupts microfilaments and impairs gluocose transport) did not result in detectable lateral mobility of CSP. The last two agents do affect the mobility of some other cell surface components (12–14).

Mild trypsinization (5 μg/ml for 3 min at room temperature) removes the CSP from the cell surface. The CSP which appeared on trypsinized cells after 1 day in culture was distributed in a fibrillar pattern at the edges of the cells (15). This new CSP was also immobile.

Fluorescent-Labeled Exogenous CSP Binds to the Cell Surface. The F-CSP was fully active as revealed by the following assays: (*i*) F-CSP and unlabeled CSP agglutinated formalinized sheep erythrocytes to a similar extent, both having an activity of 200 units/mg (5); (*ii*) F-CSP and unlabeled CSP were equally active in altering the morphology of transformed fibroblasts, both causing flattening and elongation of simian virus 40-transformed 3T3 cells at concentrations as low as 1 μg/ml; and (*iii*) F-CSP bound to CEF, chicken embryo heart fibroblasts, and 3T3 cells with a typical fibrillar pattern identical to that observed after labeling with RaCSP.

The exogenous F-CSP began to appear on the cell membrane after about 3 hr of incubation at 37°. Incubation for longer times increased the amount of bound F-CSP without changing its pattern of binding. Binding of CSP at new sites at different locations on the cell surface was not detected, even after an overnight incubation which was sufficient for maximal CSP binding.

Mobility and Distribution of F-CSP on Cells and on Artificial Lipid Bilayer Membranes. Fig. 2 shows the pattern of binding of F-CSP and RaCSP on CEF labeled with both proteins. The high degree of coincidence of the two patterns is striking. Both the exogenously incorporated F-CSP and the RaCSP on the cell membrane were immobile (Table 1).

In contrast to its characteristic fibrillar pattern of binding to fibroblasts, F-CSP binds diffusely in some other systems—*e.g.*, fresh human erythrocytes after an overnight incubation. F-CSP did not bind in detectable amounts to planar black lipid membranes made of phosphatidylcholine or oxidized cholesterol. However, it did bind in substantial amounts to black lipid membranes made of the same lipids and coated with dextran that was coupled to fatty acids and labeled with 2,4-dinitrophenyl and rhodamine (10). The F-CSP on the dextran-coated black lipid membranes was in a diffuse distribution and was mobile [$D = (2.9 \pm 0.5) \times 10^{-9}$ cm²/sec]. The stearoyl dextran molecules in the presence of CSP had $D = (5.1 \pm 0.4) \times 10^{-9}$ cm²/sec. Increasing the concentration of the F-CSP 10-fold did not alter the pattern or diffusion coefficient of the bound protein. An additional layer of RaCSP induced patching of the F-CSP on the dextran-coated black lipid membrane within 5 min (Table 1). In contrast, 5-fold higher concentrations of RaCSP only gradually altered the distribution of the CSP on fibroblasts over a period of 2–3 hr.

[§] The diffusion coefficient, D, is equal to $w^2\gamma/4\tau_{1/2}$ in which w is the e^{-2} radius of the focus Gaussian laser beam, $\tau_{1/2}$ is the time required for half the recovery to occur, and γ is a factor that depends on the extent of bleaching and the beam profile (8). (In these experiments $\gamma \simeq 1.3$.) Those components having $\tau_{1/2} \geq 10$ min for $w \sim 1$ μm are defined as immobile.

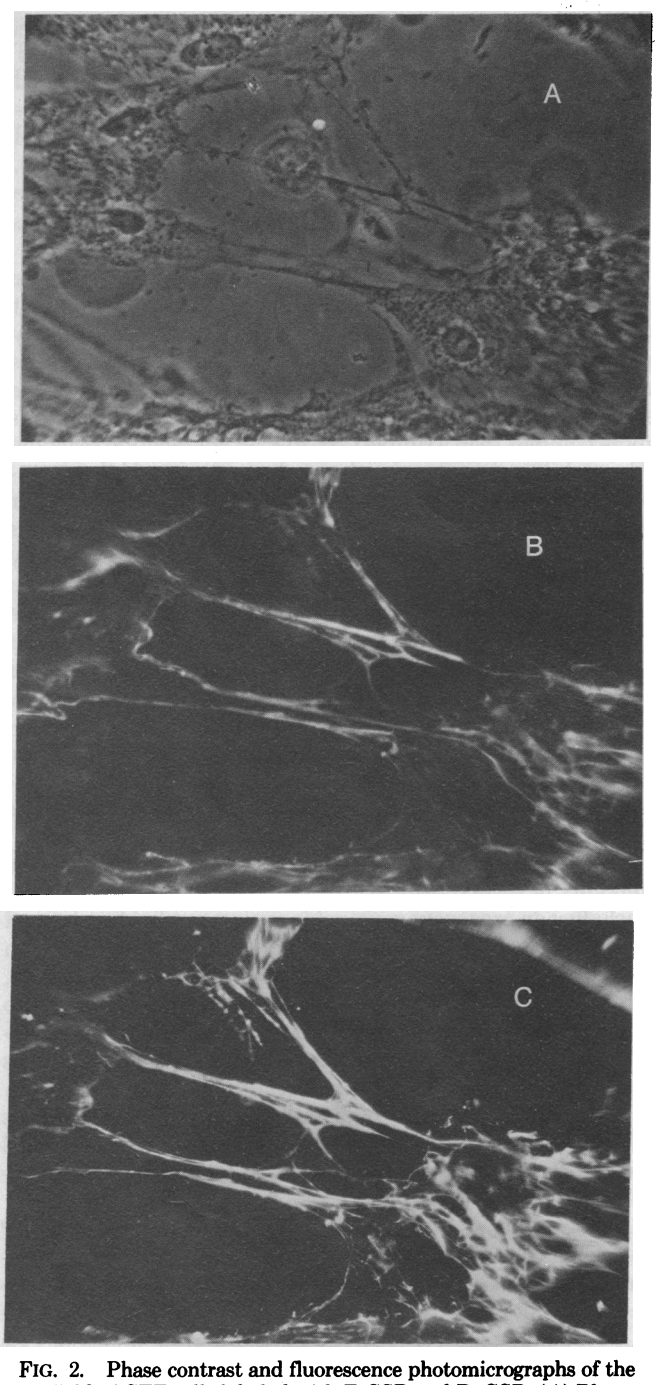

FIG. 2. Phase contrast and fluorescence photomicrographs of the same field of CEF cells labeled with F-CSP and RaCSP. (*A*) Phase contrast; (*B*) F-CSP pattern; (*C*) RaCSP pattern. (×300.) Note typical distribution of CSP on cell surfaces with dense concentrations in fibrils and large areas deficient in CSP.

Interactions between CSP and Other Cell Surface Components. The interactions between CSP and other cell surface components were studied by labeling the cells with exogenous F-CSP or RaCSP and with other specific cell surface probes. The diffusion coefficients could then be measured and the distribution of the external probe observed in areas on the membrane that were either rich or poor in CSP. The small area illuminated by laser lines at 482 or 568.2 nm permitted measurement of the diffusion coefficients of specific components in spots of diffraction-limiting size (radius of about 1 μm).

(*a*) *CSP versus Con A and succinyl-Con A.* Fig. 1 shows recovery curves of rhodamine–succinyl–Con A on CEF that were labeled first with F-CSP and then with rhodamine–succinyl–Con A. The mobility and fractional recovery were much lower in areas rich in CSP than in areas deficient in this protein. The CSP was confined to a fibrillar pattern while the rhodamine–succinyl–Con A appeared in both diffuse and fibrillar distributions. The fibrillar pattern of the rhodamine–succinyl–Con A largely coincided with that of F-CSP. These results are presumably due to the fact that Con A binds to CSP (16).

In another experiment, Con A was added to CEF prelabeled with RaCSP. The Con A did not cause patching of the antibodies and did not change the value of the antibody mobility. The Con A itself, however, patched but did not cap. Conversely, addition of RaCSP at 20 μg/ml to cells prelabeled with rhodamine–Con A did not affect the mobility or distribution of the lectin. These results also are most simply interpreted in terms of the binding of Con A to immobile CSP as well as to other mobile receptors.

(*b*) *CSP versus cell surface antigens.* 3T3 cells were labeled with F-CSP and with rhodamine-labeled Fab fragments of antibodies directed against cell surface antigens of a mouse lymphoid (P388) cell line. Neither diffusion coefficients nor fractional recoveries of the anti-P388 antibodies differed significantly in areas rich or poor in CSP. The anti-P388 antibodies were uniformly distributed whereas the CSP appeared in a typical fibrillar pattern (Table 2). Hence, CSP fibrils do not impede the motion of the unselected surface antigens that bind anti-P388 antibodies.

(*c*) *CSP versus a ganglioside analogue.* The possibility of interactions between CSP and gangliosides was examined by labeling CEF with RaCSP and an exogenous fluorescein-labeled ganglioside analogue. The diffusion coefficient of the fluorescent ganglioside was not significantly different in areas rich and poor in CSP (Table 2). Moreover, it appeared to be uniformly distributed on the cell membrane whereas the CSP assumed a typical fibrillar pattern.

(*d*) *Diffusion of a lipid probe on CEF.* Extensive overlap of the excitation spectra of the lipid probe, diI, with those of fluorescein and rhodamine prevented direct correlation of diI mobility with the local content of CSP. Therefore, diI diffusion was measured in areas that typically have high levels of CSP (e.g., the edges of confluent cells) or low levels (e.g., the area above the cell nucleus). The measured diffusion coefficients were independent of position; the range of values did not exceed that obtained in repeated measurements on the same spot

Table 1. Diffusion coefficient and distribution of CSP on lipid bilayers and cells

Cell or membrane	Fluorescent marker	Diffusion coefficient (cm^2/sec) and distribution
Lipid bilayer	F-CSP	No labeling
Dextran-coated lipid bilayer	F-CSP	$(2.9 \pm 0.5) \times 10^{-9}$ diffuse
Dextran-coated lipid bilayer	F-CSP + RaCSP	D not measured "patches"
CEF	F-CSP or RaCSP	$<5 \times 10^{-12}$ "fibrillar"
CEF	F-CSP + RaCSP	$<5 \times 10^{-12}$ "fibrillar"
3T3	F-CSP or RaCSP	$<5 \times 10^{-12}$ "fibrillar"
Human erythrocytes	F-CSP	Weak labeling diffuse

Table 2. Diffusion of cell surface components in the presence or absence of CSP

Cell membrane marker*	Diffusion coefficient, cm^2/sec	
	In CSP-rich areas	In CSP-poor areas
R-succinyl-Con A†	$\sim 9 \times 10^{-12}$	$\sim 4 \times 10^{-11}$
R-anti-P388	$(2.1 \pm 0.7) \times 10^{-10}$	$(2.0 \pm 0.5) \times 10^{-10}$
Fluorescein ganglioside analogue	$(4.2 \pm 0.3) \times 10^{-10}$	$(4.6 \pm 0.7) \times 10^{-10}$
diI	$(9 \pm 3) \times 10^{-9}$	$(7 \pm 3) \times 10^{-9}$

* R = rhodamine. All the experiments were done on CEF except those with R-anti-P388. They were done with 3T3 cells.

† The fractional recovery of rhodamine-succinyl-Con A was decreased from about 50% in areas deficient in CSP to about 15% in areas rich in CSP. The fractional recovery of all other components was similar in CSP-rich and CSP-poor regions.

(Table 2). Therefore, the diffusion coefficient of a lipid probe is not affected by the existence of an extracellular immobile matrix of CSP. This is consistent with the previous finding that complete immobilization of cell surface proteins by Con A and anti-Con A did not affect the diffusion of diI (13).

(*e*) *CSP versus acetylcholine receptors on myotubes.* When chicken embryo myotubes were labeled with a fluorescein derivative of α-bungarotoxin, a snake toxin that binds specifically to the acetylcholine receptor, the receptor was found to be in either of two conditions: a mobile, diffusely distributed state and an immobile state in which the receptor was confined to large patches on the cell membrane (11). On myotubes labeled with both fluorescein α-bungarotoxin and RaCSP, the CSP appeared mainly on the cell edges and did not show any spatial correlation with the patches of the acetylcholine receptor. Lower levels of CSP were also detected on L6 myoblasts and on L6 myotubes (17).

DISCUSSION

The difference in behavior of CSP on bilayer model membranes and on cell surfaces indicates which types of interactions may be important for the binding of the glycoprotein to cells. The failure of CSP to bind to phosphatidylcholine or oxidized cholesterol bilayers suggests a minor role for direct interaction between it and the lipid phase of the plasma membrane. The substantial binding of CSP to dextran-coated black lipid membranes, however, indicates that important interactions can occur between the glycoprotein and a membrane-bound polysaccharide. (Interactions with the trinitrophenyl groups attached to the dextran may also be significant.) This type of interaction may be relevant to the binding of CSP to cell surfaces. Nevertheless, cell-bound CSP differs in important respects from that bound to dextran-coated bilayers. The cell-bound protein is assembled into fibrillar structures. It is immobile on the time scale of our experiment and is not redistributed into patches by antibodies to CSP. In contrast, the CSP binds uniformly over the dextran-coated bilayers. Moreover, it is freely mobile and is patched by antibodies to CSP on these model membranes.

It seems reasonable that the immobility of CSP on the cell surface might be due to its assembly into fibrillar structures. If so, the mobility of CSP on dextran-coated bilayers is consistent with its lack of discernable organized structure. The immobility of a CSP fibril might result from relatively nonspecific "frictional" resistance generated by interactions between CSP and charged groups imbedded in the membrane (possibly glycoproteins and glycolipids) mediated by Ca^{2+} and Mg^{2+}. Alternatively, the CSP might bind specifically to immobile membrane receptors. The failure of drugs to induce the mobility of cell-bound CSP argues against the involvement of a cytoskeletal anchor. Other mechanisms may also contribute to the immobilization of the cell-bound CSP. It appears that CSP is involved in adhesion of cells to other cells and to the substratum (5, 6). Therefore, CSP molecules may be immobilized by adherence to the substratum or by the simultaneous binding of CSP fibrils between two cells.

The coincidence of the independently measured cell surface patterns formed by exogenous F-CSP and RaCSP indicates that the exogenous CSP binds either to CSP molecules already present on the cell surface or to other CSP binding components associated with the preexisting CSP molecules. Some evidence suggests, however, that the mechanism of fibril assembly is more complex than a simple CSP-to-CSP aggregation reaction. The mobility of CSP molecules on dextran-coated bilayers indicates that they do not interact strongly enough with each other to form large, immobile, supramolecular aggregates in this environment. [The formation of immobile aggregates by crosslinking of mobile molecules has been demonstrated in both natural and model (10) membrane systems.] Furthermore, the diffuse distribution of CSP bound to erythrocytes and the failure of the CSP bound to dextran-coated bilayers to form fibrils suggest that some other, possibly carbohydrate-containing, cell surface component is necessary for the formation of the characteristic fibrillar pattern seen on primary fibroblasts. Thus, fibril formation might be guided or initiated by strong interaction with a "CSP nucleation" component that remains to be discovered.

The presence of CSP on the cell surface has no effect on the mobility of several different kinds of membrane components including an exogenous ganglioside analogue, a lipid probe, and cell surface antigens. Moreover, no correlation of CSP with acetylcholine receptors on myotubes was observed. Therefore, it appears that CSP fibrils do not provide a general barrier to the mobility of membrane components, although the movement of gold particles attached to the dorsal surface of 3T3 cells is reportedly suppressed where CSP fibrils are abundant (18). The absence of effects on lipid probe diffusion further suggests that the lipid phase of the membrane underlying the CSP fibril is not densely filled with portions of stationary CSP molecules.

The situation is different for Con A, however, because CSP is itself a Con A binding site. Therefore, Con A is largely immobile in areas that have high levels of this protein. The presence of CSP explains in part earlier observations of immobile Con A binding sites (7). Nevertheless the fact that immobile Con A receptors are seen in areas low in CSP or on cells that lack CSP (14) indicates that other kinds of immobile Con A receptors exist.

This study provides mechanistic insight into the current view that CSP is involved in cell adhesion but not directly in the control of growth or metabolism. The immobile fibrils of CSP on a cell's surface are suitable for binding it to the substratum and to other cells. These fibrils do not, however, impede the lateral motion of various kinds of membrane components. If the mobility of some of these components is necessary for the control of normal cell functions, this result is consistent with the lack of correlation between the presence or absence of CSP and control of cell growth (1).

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