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Restricted Mobility of Membrane Constituents in Cell-Substrate Focal Contacts of Chicken Fibroblasts

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ABSTRACT We studied the lateral mobility of membrane components in cell-substrate focal contacts using the fluorescence photobleaching recovery method. The measurements were performed on isolated substrate-attached membranes of chicken gizzard fibroblasts. The diffusion coefficients of a fluorescent lipid probe and rhodamine-conjugated surface proteins within contact regions (identified by interference-reflection microscopy) were significantly lower than those measured in nonattached areas along the ventral membrane. Complete recovery of fluorescence after photobleaching of the lipid probe was measured both in focal contacts and in nonattached areas with lateral diffusion coefficient (D) of $\sim 10^{-8} cm^2/s$. This indicated that the lipid probe is free to diffuse from and into the contact regions. Rhodamine-labeled surface components (mostly proteins) exhibited almost complete recovery after bleaching (~90%) in unattached regions of the ventral membrane with $D \simeq 10^{-9}$ cm^2/s. The rhodamine-labeled proteins in focal contacts showed only partial recovery (~50%), suggesting that large proportion of the membrane proteins in cell-substrate contacts are immobile (within the time scale of the experiments, $D \leq 5 \times 10^{-12}$ cm^2/s). The implications of these findings on the molecular dynamics of cell contacts are discussed.

It is now widely accepted that many of the processes mediated by the plasma membrane depend on the lateral movement of its various components. Ligand-induced redistribution of surface molecules was reported for a large number of systems over the last several years. The ligands used in such studies were of two major types. Initially, divalent or multivalent ligands were used, including antibodies or lectins, to induce the relevant membrane receptors to aggregate in the form of patches or polar caps (31, 32). The other type of ligands includes polypeptide hormones and growth factors such as insulin and epidermal growth factor which can induce clustering or capping of their respective receptors (23, 25, 30).

A quantitative method for the measurement of lateral mobilities of membrane components is fluorescence photobleaching recovery (FPR) (10, 20, 24, 26). Results obtained by FPR measurement indicated that both membrane proteins and lipids diffuse laterally in the plasma membrane, each with a specific diffusion coefficient (11, 24). Moreover, these measurements suggested that additional interactions beyond the "intrinsic viscosity" of the lipid matrix control the rate of receptor mobility (11, 24, 27, 28, 29). Thus, interaction with intracellular structures (i.e., cytoskeleton) or extracellular materials (i.e., extracellular matrix) could have a significant effect on the mobility of membrane components (17, 21, 27, 28). In this work we study by FPR measurements the dynamic properties of the cell membrane in a special membrane domain, namely the cell-to-substrate focal contact. In these sites the cell membrane forms a close association (~150 Å) with the substrate surface (1, 14, 16, 18, 19, 22). At their cytoplasmic faces, focal contacts are associated with the termini of actin filament bundles (1, 13, 16). It has been recently proposed that this linkage is mediated by the newly described intracellular protein, vinculin (12–15).

The dynamic properties of membrane lipids and proteins in focal contacts, as compared to their mobility in unattached areas of the membrane, are still not known. It is not clear in what way and to what extent the interactions of the cell membrane with the substrate or with the cytoskeleton may affect the lateral mobility of the various membrane components in these areas. Here we demonstrate that the movement of both lipids and proteins in focal contacts is impeded and that a significant proportion (~50%) of the labeled membrane proteins are essentially immobile in the time scale of FPR experiment.

MATERIALS AND METHODS

Reagents

The lipid probe 5-[3-γ-sodium sulfopropyl-6,7-benzo-2-(3H)-benzoxazolylidene-butinyldene]-1,3-dibutyl-2-thiobarbituric acid (WW591)[1] was a gift from A.

[1] G. Hillman and J. Schlessinger. 1982. The lateral diffusion of epidermal growth factor complexed to its surface receptor does not account for the thermal sensitivity of patch formation and endocytosis. *Biochemistry*. In press.

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Grinvald (The Weizmann Institute of Science). Rhodamine lissamine B was from Research Organics, Inc. (Cleveland, OH) and its sulfonylchloride derivative (RB200SC) was prepared according to Brandtzaeg (8) and stored at −70°C. Rhodamine-labeled wheat germ agglutinin was obtained from G. Hillman (The Weizmann Institute of Science).

Cells and Membrane Preparations

Chicken gizzard cells were cultured at 10–25% confluence on 18-mm square cover slips in Dulbecco's modified Eagle medium (DME) supplemented with 10% fetal calf serum (FCS) (Biolab Inc., Jerusalem, Israel). Substrate-attached membranes of these cells were prepared by the $ZnCl_2$ method as described elsewhere (5). In brief, the cells were incubated for 2 min in buffer A (50 mM 2,(*N*-morpholino)ethane sulfonic acid [MES] buffer, 5 mM $MgCl_2$, 3 mM EGTA 1 mM $ZnCl_2$, pH 6.0), then washed and sheared with a stream of phosphate-buffered saline (PBS). Analysis of these preparations with interference-reflection and scanning electron microscopes indicated that most of the cells were opened up, leaving behind their ventral membranes and the associated cytoskeleton (for details, see reference 5).

Fluorescence-labeling of Cells and Membranes

Cells were reacted with the lipid probe WW591 or with the amino group specific rhodamine derivative RB200SC according to the following procedure: WW591 was dissolved in ethanol yielding 1.6 optical density (OD) at 500 nm, and aliquots of 100 μl were dried under nitrogen. The dye was redissolved in 1 ml of PBS or buffer A and added to cells at a 1:10 final dilution for 5 min at room temperature.[1]

For conjugation of RB200SC, cultured chicken gizzard cells were suspended by a short treatment with Trypsin-EDTA (Biolab Inc.) or by EDTA alone. They were washed once with DME and twice with PBS. The cells ($\sim 10^6$) were finally suspended in 1 ml of PBS and mixed with 1–1.5 μg of RB200SC (in ~1 μl of acetone) for 2 min. Then, the cells were diluted 10-fold in PBS and rinsed twice. After centrifugation, the cell pellet was suspended in DME containing 10% FCS and plated on 18-mm cover slips in 35-mm tissue culture dishes ($\sim 10^5$ cells/plate). Ventral membranes were prepared after stable contacts were formed ~5 h after the cells were plated.

FPR and Image Intensification Microscopy

The lateral diffusion coefficients of the fluorescently labeled membrane components were measured by the FPR method as previously described (2, 10, 20, 24, 26). We have added to the FPR apparatus a Silicon Intensified Target camera. Using this camera, we could localize even faintly staining fluorescent reagents on the cell. We also used this camera to align and focus the laser beam (Argon, 514 nm) on the cell surfaces. We have used an oil-immersion objective (× 63, NA 1.25), and the radius of the focused laser beam was 1.5 μm (9). After the laser beam was focused, we applied a brief (10 ms) intense pulse which irreversibly bleached the fluorescence in a small region on the cell surface. The time course of recovery of fluorescence in the bleached region by fresh fluorophores from adjacent regions of the cell membrane was recorded. Diffusion coefficients (D, cm^2/s) were calculated from the FPR curves as previously described (2). Incomplete fluorescence recovery was interpreted as an indication that a fraction of the fluorophores is immobile on the time scale of the experiments. $D \leq 5 \times 10^{-12}$ cm^2/s is considered to indicate immobility. The microscope of the FPR apparatus is also equipped with the proper filter and aperture settings for interference reflection microscopy. This method was used to specifically localize cell-to-substrate focal contacts (5, 14, 18, 19). The fluorescence and the interference reflection images were visualized with the Silicone Intensified Camera which was connected to time-lapse-videotape (Panasonic VTR-NV-8030) and a 9-inch TV monitor. The intensified cellular images were videotaped and micrographs were taken, with a Polaroid camera, from a television screen.

RESULTS

Labeled Cells and Ventral Membranes

Cells and ventral membranes labeled with either WW591 or RB200SC were morphologically indistinguishable from unlabeled cells. Scanning electron microscopy of such specimens indicated that the cell membrane was generally smooth with few filopodia and ruffles near or at the leading edge (Fig. 1 *A*). Ventral membranes were similar to those described earlier (4), with some filamentous networks attached to their exposed cytoplasmic faces (Fig. 1 *B*).

Interference reflection analysis of whole cells indicated the locations of focal contacts, close contacts and loosely attached areas (Fig. 1*C*). The isolated ventral membranes used here were prepared by relatively mild treatment and thus contained both focal contacts and loosely attached membrane fragments. The interference reflection analysis was performed at pH 7.0–7.2. When observed at pH <6.5 an extensive close contact (not focal) was reversely formed with the substrates as described in detail elsewhere (5).

FPR Measurements with Lipid Probe WW591

Upon incubation with WW591, the viable cultured fibroblasts exhibited uniform fluorescence over their entire surface. After photobleaching, a complete recovery of fluorescence was obtained with an apparent diffusion coefficient of $\sim 10^{-8}$ cm^2/s (see Table I). The extent of fluorescence recovery was 90–100% when the cells were either maintained at pH 6.0 or pretreated with 1 mM $ZnCl_2$. The lateral diffusion coefficient was somewhat larger when the FPR measurements were performed with cells in buffer A (pH 6.0). Nevertheless, pretreatment of the cells at pH 6.0 (with or without $ZnCl_2$) did not significantly affect the diffusion coefficients as long as the FPR measurements were performed in PBS. The variability of the values obtained for the lateral diffusion coefficients was with standard deviations in the order of 25–40% of the mean values (see Discussion).

The FPR measurements in intact cells were performed at random areas over the upper (dorsal) membrane. These measurements, with isolated ventral membranes, were selectively performed on focal contact and on unattached areas (the results obtained with close contacts were indistinguishable from those of unattached areas). The photobleached area was approximately half of the focal contact area. Hence, we have chosen relatively large focal contact areas for the FPR experiments. In all cases tested, we have found complete recovery of fluorescence following bleaching (see Table I and Fig. 2). The mean values obtained for the diffusion coefficient of WW591 in unattached membrane areas was the same as those found for intact cells, while the lateral mobility of the probe in focal contacts was approximately two-fold smaller. It should be pointed out that (*a*) the extent of labeling with WW591 was identical over the entire membrane, including the focal contact areas (Fig. 2 *C*), (*b*) the microscope observations were performed under low illumination using the light intensification system to minimize possible bleaching and photo damage, and (*c*) the variabilities of the values obtained for the diffusion coefficients of WW591 in the defined membrane domains were significantly lower (10–15% of the mean value) than those obtained by measuring at random sites.

FPR Measurements with Rhodamine-labeled Cells and Ventral Membranes

The short labeling of suspended cells with RB200SC resulted in an extensive and uniform labeling of their surface without affecting their viability or their adhesive properties. However, after 5 h of incubation (the time required for complete attachment and spreading), parts of the labeled membranes were endocytosed and exhibited an intracellular granular fluorescence. However, the isolated ventral membranes exhibited a diffuse uniform label (see Fig. 4 *C*). Selective extraction of the labeled cells with chloroform-methanol (2:1) solubilized only a small amount of label (~10%). The majority of the fluorescence labeling was found to be associated with surface proteins

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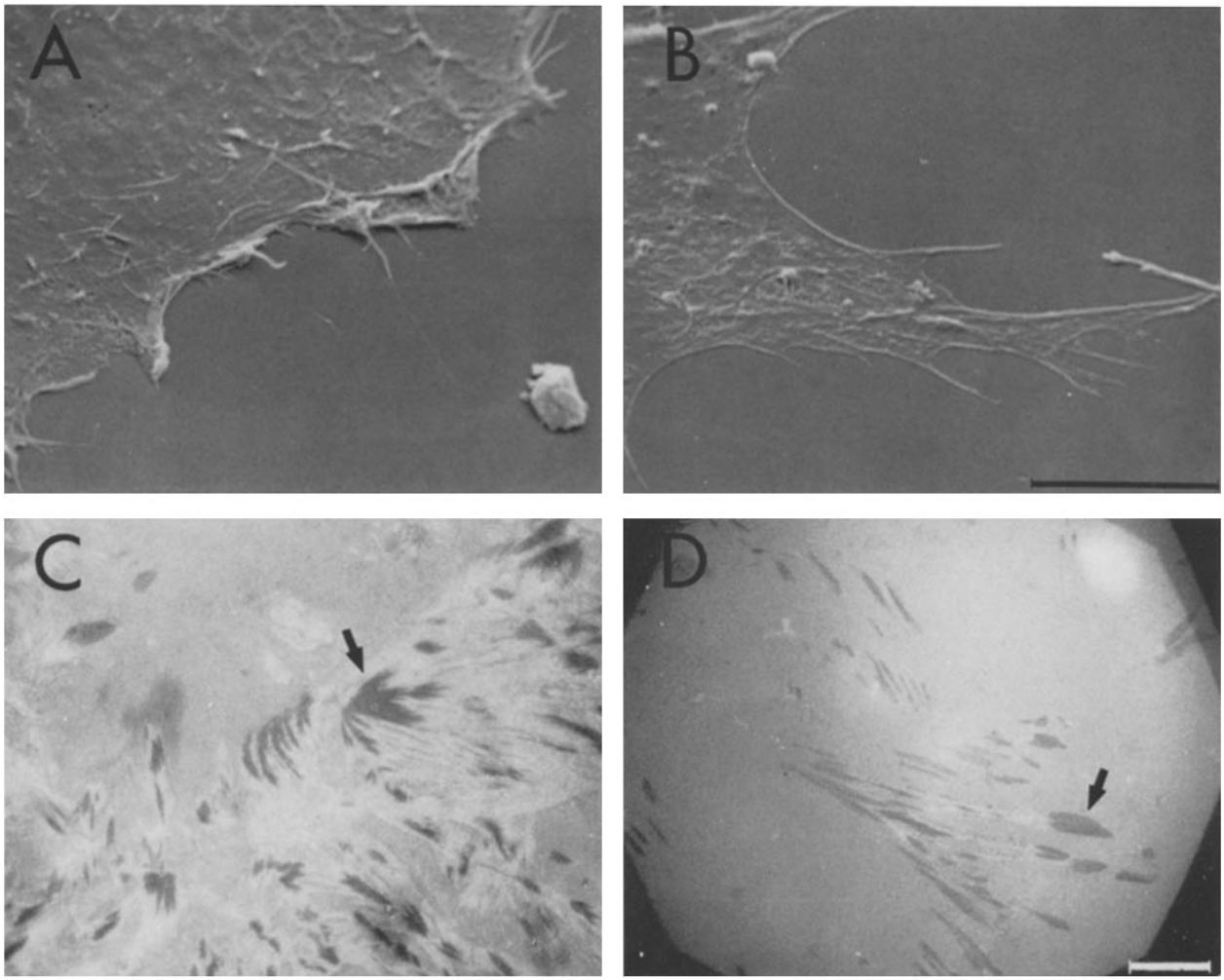

FIGURE 1 Scanning electron micrographs (*A* and *B*) and interference reflection images (*C* and *D*) of intact chicken gizzard cells (*A* and *C*) or isolated substrate-attached membranes prepared by the $ZnCl_2$ method (*B* and *D*). Typical focal contacts in *C* and *D* are indicated by arrows. Bar, 10 μm.

TABLE I

Fluorescence Photobleaching Recovery Measurements with Cells and Isolated Ventral Membranes

Fluorescent probe	Sample	D (cm^2/s) × 10^{-9}	% Recovery
WW591	Whole cells, in PBS	10.6 ± 4	90–100
WW591	Whole cells in buffer A	17.0 ± 6	90–100
WW591	Whole cells in PBS, pretreated with $ZnCl_2$ in buffer A	12.6 ± 3	90–100
WW591	Ventral membranes in focal contact areas	7.7 ± 0.8	90–100
WW591	Ventral membranes in nonattached areas	12.8 ± 2.0	90–100
RB200SC	Ventral membranes in focal contact areas	0.8 ± 0.18	47 ± 9
RB200SC	Ventral membranes in nonattached areas	1.4 ± 0.21	90 ± 4
Rhodamine WGA	Whole cells in PBS	2.2 ± 0.7	49 ± 9
Rhodamine WGA	Whole cells in PBS, pretreated with $ZnCl_2$ in buffer A	1.3 ± 0.3	45 ± 8
Rhodamine WGA	Whole cells in buffer A in the presence of $ZnCl_2$	1.2 ± 0.3	48 ± 7

of the cultured cells. The electrophoretic separation of cellular proteins, shown in Fig. 3, indicates that only a limited number of proteins was labeled by the lissamine-rhodamine sulfonyl chloride. The Coomassie-Blue-stained gel (Fig. 3 *a*) shows that the major peptides are actin, myosin (marked with arrows), and several additional proteins comigrating with filamin, vimentin, desmin, and tropomyosin α chain. The corresponding areas were, however, devoid of fluorescently-labeled peptides (Fig. 3 *b*). The fluorescence was associated with two major bands with apparent molecular weights of 220,000 (probably fibronectin) and ~70,000 and with several minor bands, as indicated in the figure.

The results obtained from FPR measurements of rhodamine-labeled membranes in focal contacts and in unattached areas are shown in Fig. 4. The diffusion coefficient of the rhodamine-labeled surface components in nonattached areas was 1.4 × 10^{-9} cm^2/s, while in focal contacts significantly lower values were obtained (0.8 × 10^{-9} cm^2/s). A remarkable difference between the two areas was found in the extent of fluorescence recovery. Thus, while the recovery in unattached membrane

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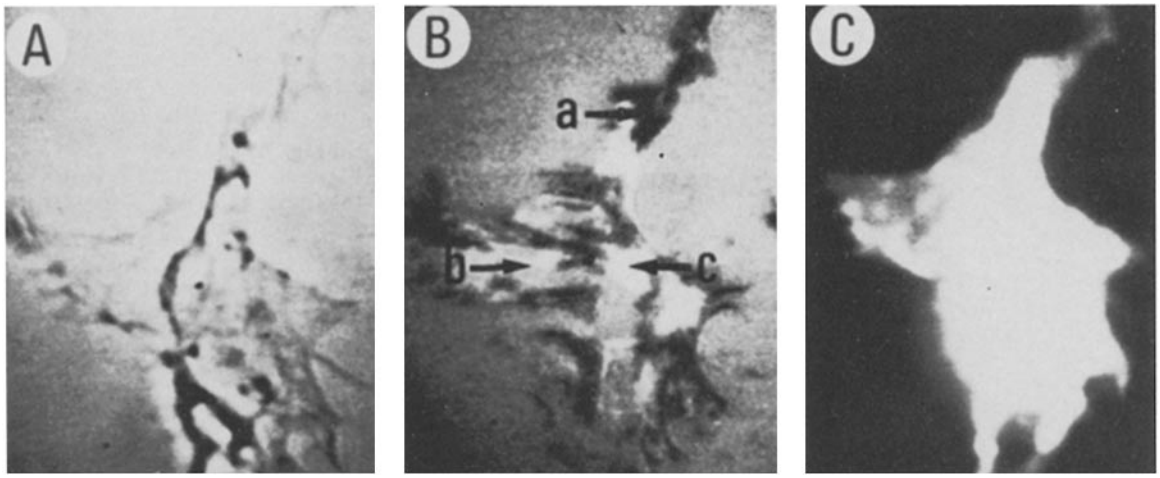

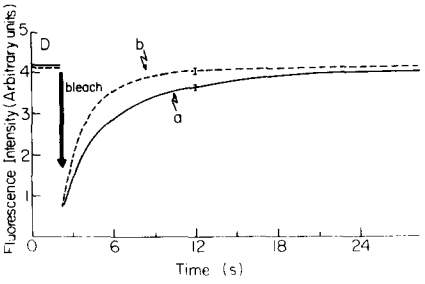

FIGURE 2. FPR measurements with the lipid probe WW591 on isolated ventral membrane. (*A*) Phase-contrast photomicrograph of an isolated ventral membrane. (*B*) Interference-reflection photomicrograph of the same membrane. *a* indicates area of focal contact; *b* and *c* are loosely attached regions. (*C*) Fluorescence image of the same membrane as visualized by the Silicon Intensified Camera. (*D*) The recovery of fluorescence of WW591 in the membrane shown in *A–C* after bleaching the nonattached area (*b*, broken line) or the focal contact (*a*, solid line). The horizontal lines on the left indicate the initial fluorescence intensity before bleaching.

areas was ~90%, only ~50% recovery was obtained in focal contacts (Fig. 4 and Table I).

Since the intact rhodamine-labeled cells exhibited a considerable intracellular fluorescence, we have estimated the rate of lateral diffusion of membrane proteins in the intact cells, using rhodamine-conjugated WGA as a probe. We have found (Table I) that pretreatment with $ZnCl_2$ reduced the diffusion coefficient of the lectin-bound receptors,[2] but the values obtained for the pretreated cells were identical to those obtained for the rhodamine-conjugated components in unattached areas of the isolated ventral membrane. However, while the components directly labeled with rhodamine exhibited almost complete recovery after photobleaching (~90%), only part (~50%) of the lectin-bound receptors were mobile at the time scale of the experiment.

DISCUSSION

We focused this study on the characterization of the dynamic properties of different components in a defined structural and functional domain in cell membranes, namely the focal contact. Most of the studies on the lateral mobility of membrane receptors which were described in the last few years were performed on random sites over the cell surface. In that fashion the lateral mobilities of a variety of membrane antigens, lectin receptors, hormone-receptor complexes, and chemically modified surface molecules were measured (11). Only in few cases were the lateral diffusion coefficients of specific receptors in a defined membrane structure determined. Such areas were the acetylcholine receptor clusters in differentiating myotubes in which the receptor molecules were essentially immobile, while they were free to move in other areas of the cell membrane (3).

[2] This observation may be related to the effective action of Zn^{+2} in the isolation of plasma membranes (6, 33), though its exact mode of action is largely unclear.

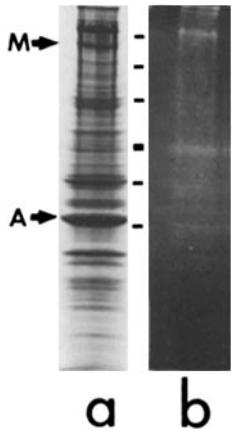

FIGURE 3 SDS PAGE of rhodamine-labeled chicken gizzard cells. The labeled cells were lysed in electrophoresis sample buffer and applied to a slab 10% polyacrylamide gel. After electrophoresis the gel was photographed with a Polaroid camera under ultraviolet illumination (*b*) and then stained with Coomassie Blue (*a*). The lines on *a* mark the location of the detectable, fluorescently labeled peptides. The arrows point to actin (*A*) and myosin (*M*).

Another aspect of measuring lateral mobilities using FPR is that in most measurements on cultured cells the measured fluorescence is emitted from two membranes through which the light passes. In measurements with lymphoid cells the relative contribution of light by the membrane which is not in focus is not significant (9, 26). In flat cells such as cultured fibroblasts, however, fluorescence of both membranes may contribute to the measured signal. However, we performed the measurements on defined sites (determined by interference reflection optics) in isolated single membranes.

To determine the significance of our results on the dynamic properties of the intrinsic membrane elements, we should consider the properties of the fluorescent probes used: the amphipatic compound WW591[1] and the amino group specific dye RB200SC. We have no direct evidence whether the lipid probe became indeed intercalated into the membrane bilayer. Nevertheless, it seems to represent well the rate of diffusion of the lipid matrix of the membrane as measured previously with fluorescently derivatized gangliosides (28), or with carbocyanine dyes (29). The rhodamine-lissamine sulfonylchloride

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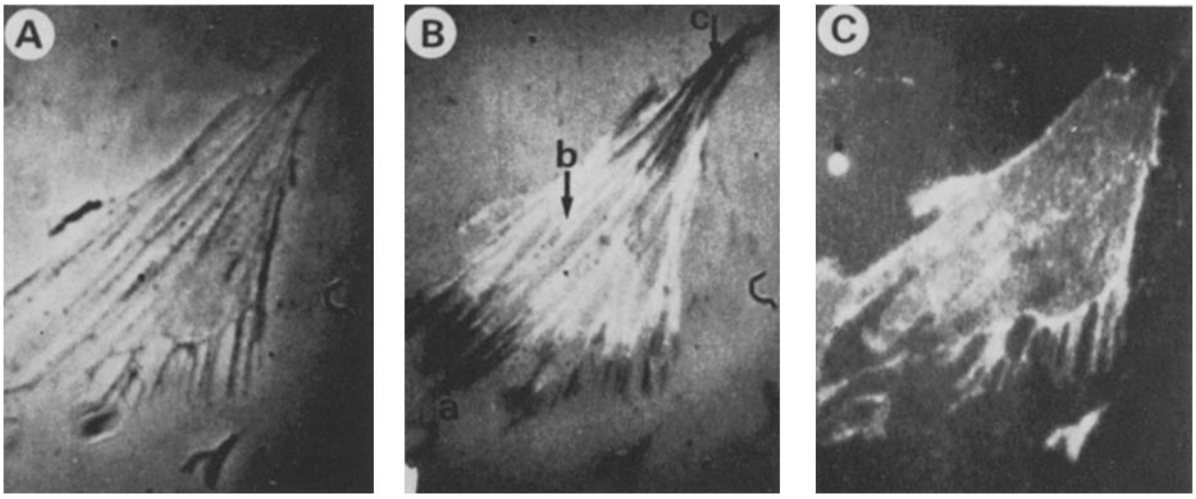

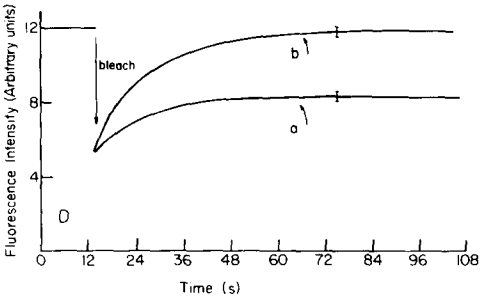

FIGURE 4. FPR measurements with rhodamine-labeled membrane components. The photomicrographs *A*–*C* show the same substrate-attached membrane observed with phase-contrast (*A*), interference-reflection (*B*), and fluorescence (*C*) microscopies. The area marked in *a* and *b* represent focal contacts and unattached membrane regions, respectively. (*D*) FPR measurement of the membrane shown in *A*–*C* in the focal contact (*a*) and in the nonattached region (*b*). The horizontal line on the left represents the level of fluorescence intensity before the bleaching.

reacts specifically with the amino groups (8), exposed on the surface of the cells. This dye apparently does not penetrate into the cells. This was expected from its chemical properties and was directly verified by electrophoresis (Fig. 3). It was found that RB200SC, when added to living cells, does not modify intracellular cytoskeletal proteins in the cells. The cells were labeled with RB200SC in suspension to assure that the reagent has a free access to the entire cell surface, and to avoid the staining of substrate-associated matrix components. Indeed, at short times after plating, the cell-associated fluorescence was fairly uniform, though upon incubations for 1 h or more many intensely labeled intracellular vesicles were noted. For that reason, we were able to measure the lateral mobility of the labeled surface components only with the isolated ventral membranes. The lateral diffusion of the membrane components labeled with RB200SC was smaller by a factor of ~10 than the values obtained for the lipid probe. This diffusion coefficient is somewhat larger than those commonly obtained for the diffusion of membrane proteins (11). It should be pointed out that similar values were also obtained for the diffusion of membrane-bound WGA for similarly treated intact cells (see Table I).

Quantitative analysis of the dynamic properties of the lipid and protein probes (both the extent and the rate of fluorescence recovery) suggests the following: (*a*) the lateral mobility of both lipid and protein probes in focal contacts is slower almost by a factor of 2, compared to loosely attached sites on the ventral membrane or to the dorsal membrane of the cells; (*b*) the treatment of pH 6.0 (with or without $ZnCl_2$) has a reversible effect on the rate of movement of the lipid probe (the decrease in the lateral mobility at pH 6.0 may be related to the extensive formation of close cell-to-substrate contacts at this pH, as previously described); (*c*) the recovery of the fluorescence of the lipid probe after bleaching was always close to 100% regardless of whether the bleached area was related to site of focal contact or not; (*d*) the rate of movement of the rhodamine-labeled proteins was reduced by the $ZnCl_2$ treatment at pH 6.0, but it did not cause their immobilization (thus, in nonattached areas the recovery of fluorescence was almost complete [~90%]); and (*e*) the extent of recovery of protein fluorescence was significantly lower (~50%) in focal contacts (but not in the interference-reflection-grey-close contacts) compared to the rest of the membrane.

It should be emphasized that although ~50% of the labeled focal contact proteins were essentially immobile, this does not imply that a similar proportion of the membrane protein is directly involved in the contact with the substrate. Thus, it is possible that membrane components which do not interact directly with the substrate become trapped in the focal contacts and thus appear immobile. It has been recently shown that the immobile acetylcholine receptors of rat myotubes are concentrated in areas of abroad close contact, although they seemed not to be directly associated with individual contact points but rather to be localized between them (7). This type of apparently indirect interactions with immobile constituents in the membrane or the cytoskeleton could contribute not only to the low recovery but also to the decreased mobility.

It should be pointed out that in spite of the relatively limited mobility of proteins in the focal contacts these sites are still largely mobile. This dynamic feature of focal contacts may relate to a recent observation that after the formation of focal contacts fibroblastic cells may actively remove underlying matrix proteins (such as fibronectin). This apparently requires that some specific component(s) may bind to the fibronectin and remove it from the focal contact (4). It is conceivable that the capability of membrane proteins to move into or away from mature focal contacts is involved in the regulation of cell attachment, spreading, and motility.

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