Incorporation of Fluorescent Gangliosides into Human Fibroblasts: Mobility, Fate, and Interaction with Fibronectin

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ABSTRACT Rhodamine- and fluorescein-labeled gangliosides were used as probes to investigate the distribution, dynamics, and fate of plasma membrane-bound gangliosides on cultured human fibroblasts. When sparse cultures of fibroblasts were incubated with the fluorescent ganglioside derivatives, their surfaces became highly fluorescent. The fluorescent gangliosides were taken up by the cells in a time- and temperature-dependent manner and were not removed from the cell surface by trypsin or serum. Thus, the gangliosides appeared to be stably incorporated into the lipid bilayer of the plasma membrane. Fluorescent photobleaching recovery measurements showed that the inserted gangliosides were free to diffuse in the plane of the membrane with a high diffusion coefficient of $\sim 10^{-8}$ cm²/s. When the ganglioside-treated cells were washed and incubated in fresh medium, the surface gangliosides became internalized with time and localized in the perinuclear region of the fibroblasts. In dense cultures of fibroblasts, a large fraction of the fluorescent gangliosides were organized in a fibrillar network and were immobile on the time scale of fluorescent photobleaching recovery measurements. Using antifibronectin antibodies and indirect immunofluorescence, these gangliosides were found to co-distribute with fibrillar fibronectin. Thus, exogenous gangliosides appear to be stably inserted into the lipid bilayer of the plasma membrane and to diffuse freely in its plane as well as form a less mobile state with the fibrillar networks of fibronectin associated with the cells.

The ability to insert exogenous gangliosides into the plasma membrane of various cells has proven to be a potent tool to study the biological function(s) of these membrane components. By using this technique, investigators have implicated gangliosides as membrane receptors for cholera toxin (1), Sendai virus (2), fibronectin (3), and migration inhibitory factor (4) and as receptor modulators for growth factors (5, 6), tumor promoters (7), and neurotransmitters (8). There still appears to be some controversy as to whether exogenous gangliosides become inserted and oriented in the lipid bilayer of the plasma membrane in the same manner as endogenous gangliosides. Wiegandt and co-workers (9–12) have reported that gangliosides taken up by cells can be removed by trypsin and serum and are sensitive to sialidase. In addition, it has been reported that exogenous gangliosides are neither internalized nor metabolized by the cells (10, 13). Other studies,

however, have indicated that exogenous gangliosides become functionally inserted into the outer leaflet of the membrane bilayer of cultured cells and are both internalized and metabolized by the cells (14–19). In the present study, we use fluorescent derivatives of gangliosides (20) and fluorescent photobleaching recovery (FPR)[1] measurements (21, 22) to explore the distribution, mobility, and fate of inserted gangliosides.

MATERIALS AND METHODS

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[1] *Abbreviations used in this paper:* FITC, fluorescein isothiocyanate; FPR, fluorescent photobleaching recovery.

tibodies were prepared as described elsewhere (23). Rabbit antifibronectin antibodies were kindly provided by Dr. B. Geiger (Weizmann Institute). 3,3'-dihexadecylcylindocarbocyanine iodide (diIC₁₆) and lissamine rhodamine B phosphatidylethanolamine were kindly provided by Dr. R. Blumenthal (National Cancer Institute). Fluorescein- and rhodamine-labeled gangliosides were prepared as described previously (20). Briefly, bovine brain gangliosides were oxidized with sodium periodate, reacted with hydrazide derivatives of fluorescein or rhodamine, reduced to stabilize the Schiff bond, and purified to remove any free fluorophore. The fluorophores were shown to be linked to the sialyl residues as follows. (a) Bovine brain gangliosides (1 mg) were heated at 80°C in 0.1 M HCl for 2 h to hydrolyze all of the sialic acids. After dialysis against water, the material was oxidized and reacted with the rhodaminyl hydrazide derivative and analyzed by thin-layer chromatography. No rhodaminyl glycolipid derivatives were detected. (b) The gangliosides (1 mg) were incubated with 0.5 U of *Vibrio cholera* neuraminidase for 2 h at 37°C, dialyzed, oxidized, and reacted with the rhodaminyl hydrazide derivative. Only rhodaminyl GM₁ was detected by thin-layer chromatography. This was expected as neuraminidase hydrolyzes complex gangliosides to GM₁, which is resistant to the enzyme.

Cell Culture: Human foreskin fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics under a humid atmosphere of 95% air/5% CO_2 at 37°C. Cells between the 25th and 30th doubling were used. The cells were plated on glass coverslips in 35-mm-diam dishes at 2,350 cells/cm² 2 d before an experiment for sparse cultures and at 47,000 cells/cm² 4 d before an experiment for confluent cultures.

Incorporation of Fluorescent Gangliosides by Fibroblasts: The cells were washed extensively with serum-free medium to remove any serum that interfered with ganglioside uptake by the cells. The cells were incubated in serum-free medium containing fluorescent gangliosides (20–50 μg/ml) for 30 min at 0° or 37°C in a humidified incubator. The coverslips were washed twice with PBS and examined for fluorescence or treated further as described below.

Treatment of Fibroblasts with Antibodies: Coverslips containing cultures of fibroblasts enriched with rhodaminyl gangliosides were incubated with antirhodamine (40 μg/ml) or antifibronectin (diluted 1 to 30) antibodies for 30 min at room temperature. After washing with PBS, the cells were incubated with FITC–protein A (20 μg/ml) for 30 min at room temperature. The cultures were rinsed three times with PBS and then fixed in PBS containing 3.7% formaldehyde and 5% sucrose for 60 min. After extensive washing, the cells were examined for fluorescence using a Zeiss microscope (Carl Zeiss, Inc., New York) modified for epifluorescence microscopy and equipped with a Neofluar G3X objective. Photographs were taken with a Pentax camera (Englewood, CO) equipped with specific interference filters for rhodamine and fluorescein; the filter systems were changed without altering the focus.

Fluorescent Photobleaching Recovery: Diffusion coefficients of the fluorescent gangliosides on the cell surface were determined by the FPR method (21, 22). All measurements were performed at room temperature.

RESULTS

Insertion of Fluorescent Gangliosides into Growing Fibroblasts

When sparse cultures of human fibroblasts were exposed to rhodamine- or fluorescein-tagged gangliosides, the surface of the cells became fluorescent (Fig. 1, *A* and *B*). The surface

location of rhodaminyl gangliosides also was demonstrated by indirect immunofluorescence using antirhodamine antibodies and FITC–protein A (Fig. 1, *C* and *D*). The distribution of rhodamine and fluorescein fluorescence was coincidental. When either unmodified gangliosides or normal rabbit IgG was substituted, no fluorescein fluorescence was detected (data not shown).

The intensity of the surface fluorescence was dependent on the ganglioside concentration and the time and temperature of the incubation. At 0°C, the incorporation was significantly reduced. The addition of 10% serum to the medium did not remove or reduce the surface fluorescence. When the ganglioside-treated cells were exposed to trypsin-EDTA, they retained their surface fluorescence even though they had become rounded upon removal from the substratum.

To follow the fate of the inserted gangliosides, we washed and incubated the cells in fresh medium. The fluorescence, which was initially confined to the outer surface of the plasma membrane, became internalized after 2 h (Fig. 2a); after 5 h, fluorescent endocytotic vesicles were observed in the perinuclear region of the cells (Fig. 2b). By this time, almost all of the fluorescent gangliosides on the cell surface had been internalized as determined by indirect immunofluorescence with antirhodamine antibodies and FITC–protein A.

We examined the mobility of the fluorescent gangliosides inserted into the plasma membrane by FPR. Measurements were made at several different locations on the cell with at least two separate bleachings per location. The radius of the focused laser beam was ~1 μm and the data were analyzed as described previously (21). The FPR curves for fluorescein-labeled gangliosides indicated a high mobility for the inserted gangliosides with a diffusion coefficient of 10^{-8} cm²/s (Fig. 3). The gangliosides appeared to be completely mobile as the fluorescence in the plane of the membrane recovered almost to its original prebleached level. The diffusion coefficient of the gangliosides was similar to that of other lipids (24).

Incorporation of Fluorescent Gangliosides by Confluent Fibroblast Cultures

When nearly confluent cultures of human fibroblasts were incubated with fluorescent gangliosides, they exhibited in addition to a diffuse fluorescence on the cell surface, a fibrillar network around and above the cells that resembled the fibrillar matrix of fibronectin (Fig. 4a). When the cultures were exposed to trypsin, the fibrillar matrix was removed but the
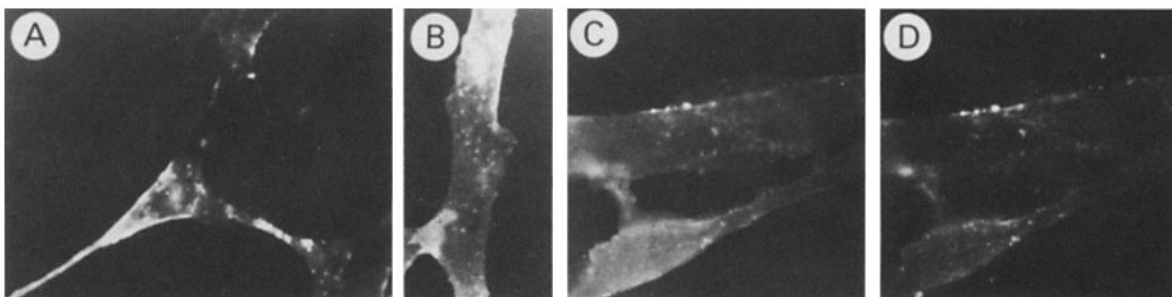

FIGURE 1 Fluorescence patterns of growing human fibroblasts containing fluorescent gangliosides. Sparse cultures of human fibroblasts were incubated with (A) 20 μg/ml of rhodaminyl gangliosides or (B) 50 μg/ml fluoresceinyl gangliosides for 30 min at 37°C and washed and examined for surface fluorescence as described in Materials and Methods. (C and D) Same as in A except that cells were incubated with antirhodamine antibodies and stained with FITC-protein A. Note that indirect fluorescein immunofluorescence (D) corresponds with direct rhodamine fluorescence (C).
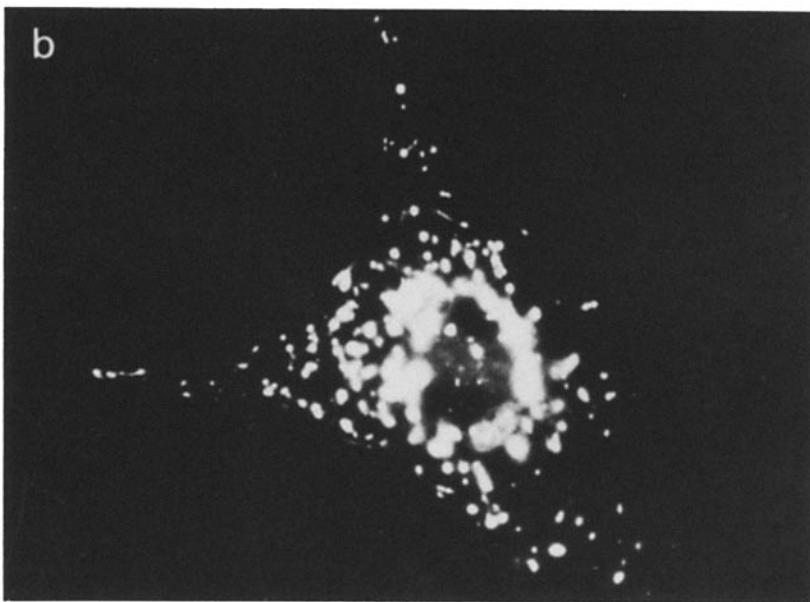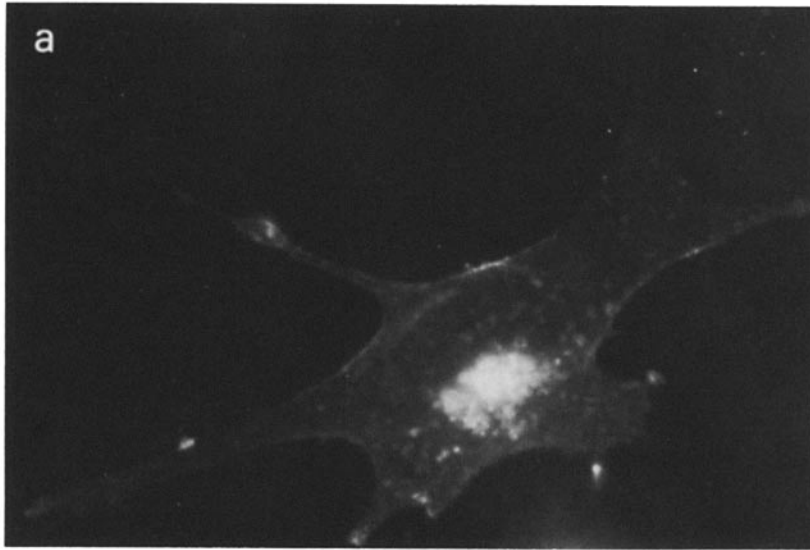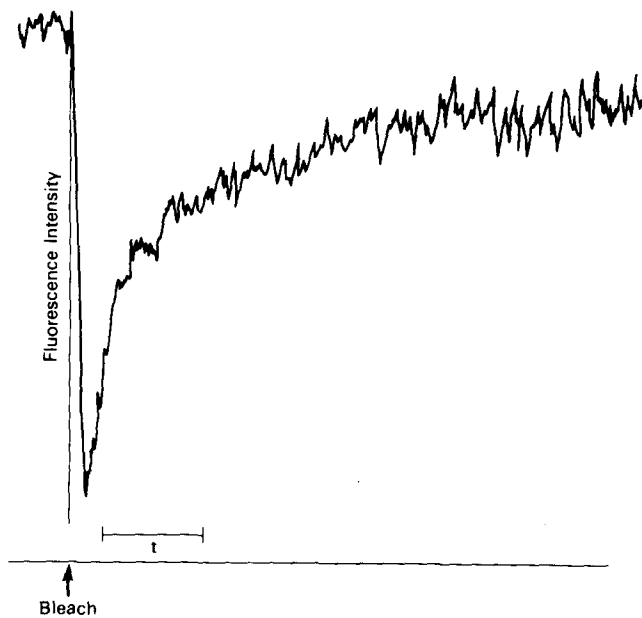
FIGURE 2 Fate of fluorescent gangliosides taken up by human fibroblasts. Sparse cultures were incubated with 50 μg/ml of rhodaminyl gangliosides for 30 min and washed extensively and incubated in complete medium at 37°C for 2 h (a) or 5 h (b).

surface fluorescence was retained (Fig. 4b). As gangliosides have been implicated as receptors for fibronectin (3, 25–27), we explored the possible connection between the fluorescent gangliosides and fibronectin. Very confluent cultures of fibroblasts were incubated with rhodaminyl gangliosides and then with antifibronectin antibodies followed by FITC–protein A. There was a striking correspondence between the two patterns; the distribution of the inserted gangliosides as visualized by the rhodamine fluorescence was similar to that of fibronectin as revealed by fluorescein immunofluorescence (Fig. 5, a and b).

When the rhodaminyl ganglioside-treated cells were stained with antirhodamine antibodies and FITC–protein A, a similar co-distribution of the two fluorescent patterns was observed

FIGURE 3 FPR measurements on human fibroblasts containing rhodaminyl gangliosides. Sparse cultures were incubated with rhodaminyl gangliosides (20 μg/ml) and washed and analyzed by FPR as described under Materials and Methods. From the curve, a diffusion coefficient of $\sim 10^{-8}$ cm^2/s and a fractional recovery of 83% were calculated.
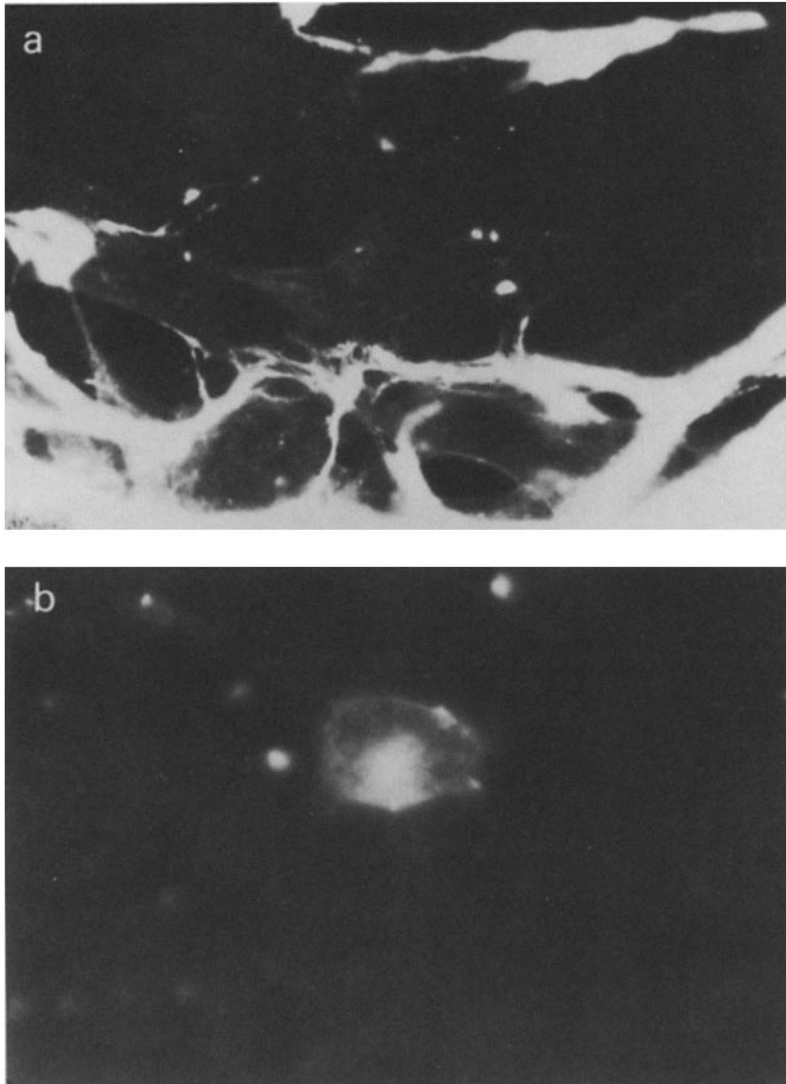
FIGURE 4 Fluorescence patterns of nearly confluent human fibroblasts containing fluorescent gangliosides. (a) Cultures incubated with rhodaminyl gangliosides (20 μg/ml). (b) Same as in a except that cells were treated with trypsin.

(Fig. 5, c and d). The antifibronectin antibodies were shown not to cross-react with rhodamine derivatives inasmuch as only antirhodamine but not antifibronectin antibodies reacted with rhodamine-labeled bovine serum albumin (data not shown). Treatment of the rhodaminyl ganglioside-labeled fibroblasts with either antirhodamine or antifibronectin antibodies did not induce redistribution of the gangliosides on the cell surface (Fig. 5, b and d). This is consistent with the observation that the extensive fibronectin fibrils found in dense cultures are immobile and stable to antibody induced redistribution (28).

FPR measurements on confluent fibroblasts indicated that on the fibers of fibronectin, all of the fluorescent gangliosides were immobile in the time scale of the FPR measurement ($D > 5 \times 10^{-12}$ cm^2/s). We attempted to evaluate this very slow recovery by bleaching a continuous line across a parallel array of fibers using a focused laser beam. We then recorded the recovery of fluorescence in the bleached area by video intensified time-lapse cinematography (29). The dark line was clearly visible even 30 min after bleaching (data not shown).

In contrast to the behavior of the fluorescent gangliosides taken up by confluent human fibroblasts, other fluorescent lipid probes behaved as expected. When the cells were incubated with either 3,3′-dihexadecylcylindrocarbocyanine io-

dide or rhodaminyl phosphatidylethanolamine, their surfaces became uniformly and highly fluorescent. There was no indication of any organization of these lipophilic probes into a fibrillar network. When the cells were stained for fibronectin, the fibrillar network as detected by immunofluorescence did not correspond to the direct fluorescence of the lipid fluorophores (data not shown). These results support the specificity of the ganglioside–fibronectin interaction as both the ganglioside and the phospholipid derivatives have the same rhodaminyl fluorophore attached to them.

DISCUSSION

It is very important to assess whether exogenous gangliosides are in fact inserted into the lipid bilayer of the plasma membrane, if such an approach is to be useful to investigate ganglioside function. From their structure, one would expect on energetic grounds that both hydrocarbon chains of the gangliosides would be inserted into the bilayer. Using fluorescent gangliosides, we found that they became stably incorporated into the plasma membranes of cultured human fibroblasts. The gangliosides were not removed from the cell surface by serum or by trypsin, although the latter detached the cells from their substratum and removed their fibrillar
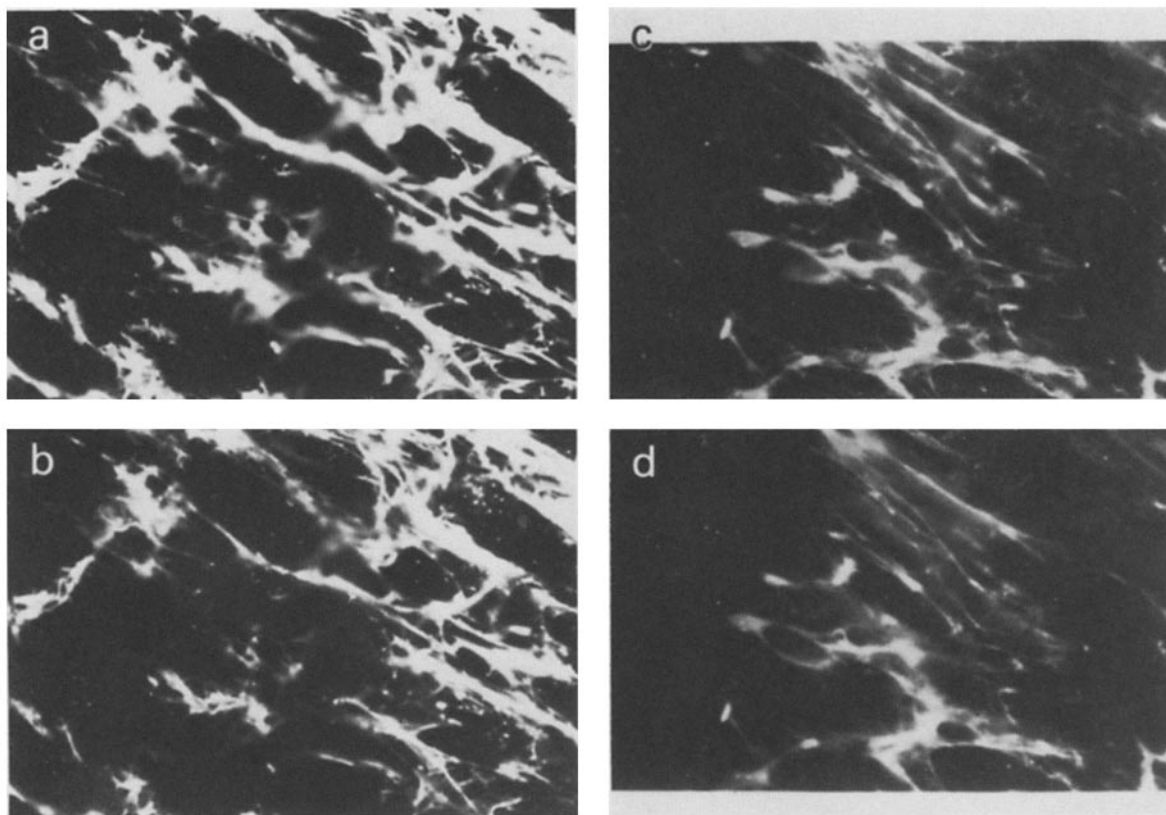
FIGURE 5   Fluorescence patterns of heavily confluent human fibroblasts containing fluorescent gangliosides. (a and b) Cultures labeled with rhodaminyl gangliosides were incubated with antifibronectin antibodies followed by FITC-protein A and examined for rhodamine (a) and fluorescein (b) fluorescence. (c and d) Same as in a and b except that cells were incubated with antirhodamine antibodies.

matrix. Uptake of fluorescent gangliosides by the fibroblasts was highly temperature dependent; this would be consistent with their insertion into the fluid lipid bilayer. The gangliosides, when incorporated into sparsely cultured fibroblasts, were completely mobile in the plane of the membrane and had a diffusion coefficient of 10^{-8} cm^2/s.[2] This value is in the range of other fluorescent lipid probes and is 20 times higher than those usually observed for membrane proteins (24). Thus, we believe that the fluorescent gangliosides have become stably inserted into the membrane and not just adsorbed to less mobile, trypsin-sensitive complexes on the cell surface.

In this regard, our results are in contrast to those of Wiegandt and co-workers (9–12), who found that exogenous gangliosides taken up by chick and mouse fibroblasts and

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[2] In a previous study, the mobility and diffusion coefficient of a fluorescent ganglioside derivative taken up by chick embryo fibroblasts was measured by FPR (28). The fluorescein-labeled analog of GM$_1$ contained an N-fluoresceinyl-octadecylamino group instead of the normal ceramide moiety. The derivative had a diffusion coefficient of 5×10^{-10} cm^2, was completely mobile, and did not appear to interact with immobile fibronectin of chick embryo fibroblasts. This derivative, however, has only one hydrocarbon chain and bulky fluorescein group close to, if not partially imbedded in, the lipid bilayer. Thus, it may behave differently than native gangliosides or fluorescent derivatives with a normal ceramide group. Other studies have shown that GM$_1$ does not interact as strongly with fibronectin as more complex gangliosides (3, 24–27). We used fluorescent derivatives of bovine brain gangliosides, which are mainly the complex gangliosides that interact with fibronectin.

chick and canine erythrocytes were easily removed by serum or trypsin treatment. From their studies, they proposed that the bulk of the exogenous gangliosides are attached to trypsin-sensitive membrane components and are not inserted into the membrane in the same manner as endogenous gangliosides. Other studies, however, are consistent with our present findings. Fluorescent gangliosides taken up by lymphocytes were not removed by trypsin (30). Exogenous GM$_1$ incorporated into GM$_1$-deficient cells was trypsin resistant (17) and behaved as a functional receptor for cholera toxin (14–18).

The ultimate fate of the inserted fluorescent gangliosides is not yet clear. We observed that they became internalized with time and appeared in endocytic vesicles in the perinuclear space of the fibroblasts. Cholera toxin, which binds specifically to GM$_1$, is internalized by various cells (31–33) including human fibroblasts (34). By using peroxidase-conjugated toxin and [³H]GM$_1$, Gonatas et al. (33) showed both markers underwent endocytosis and had a similar distribution in the perinuclear region of the cell including lysosomes and vesicles at the trans aspect of the Golgi apparatus. More recently, it was shown that GM$_1$-deficient cells incorporated exogenous GM$_1$, internalized it, and metabolized it to both higher and lower homologs (19). O'Keefe and Cuatrecasas (13), however, reported that GM$_1$ taken up by GM$_1$-deficient, transformed Balb c/3T3 cells was not internalized. And, Keenan et al. (10) reported that [³H]GM$_1$ taken up by Swiss 3T3 cells was not degraded.

There are several possibilities to explain these differences in the way exogenous gangliosides are taken up by cells,

internalized, and metabolized. Different cell types may vary in their ability to incorporate and metabolize exogenous gangliosides. The presence of serum is known to interfere with ganglioside incorporation (35). The metabolic state of the cells and cell density may also be important. As discussed below, we found that the distribution of fluorescent gangliosides was different on sparse and dense cultures. In the latter, part of the gangliosides was associated with the trypsin-sensitive fibrillar matrix surrounding the fibroblasts. Finally, the physical state of the gangliosides may be very important. Gangliosides form micelles in solution and self-associate at concentrations as low as 10^{-9} M (36, 37). It is reasonable to assume that the monomeric form is inserted into the lipid bilayer and that the micellar form in turn may only be absorbed to the cell surface. More recently, Facci et al. (38) reported that the stable incorporation of gangliosides by neuroblastoma cells depended on the ganglioside concentration, the incubation time, and the cell density. Using spin-labeled analogues of gangliosides, Schwarzmann et al. (39) found that the portion of gangliosides taken up by mouse fibroblasts that was trypsin-resistant appeared to be anchored in the hydrophobic region of the plasma membrane.

By using fluorescent gangliosides, we were able to directly demonstrate that gangliosides bind to the organized fibronectin network of the fibroblasts. Other studies have implicated an interaction between gangliosides and fibronectin (2, 25–27). Although the direct binding of soluble fibronectin to gangliosides appears to be weak (27), we observed a tight interaction between the fluorescent gangliosides and fibrillar fibronectin. The gangliosides were not removed by extensive washing or by serum and were completely immobile by FPR measurements. It is possible that gangliosides have a much higher affinity for fibrillar fibronectin than for soluble fibronectin due to some form of cooperative interaction. Although our results clearly demonstrate that gangliosides bind to the fibronectin matrix, they do not prove that gangliosides are cell surface receptors for fibronectin. Preliminary results indicate that fluorescent gangliosides, when taken up by ganglioside-deficient cells, promote the retention of fibronectin and co-distribute with the fibronectin matrix (40). Thus, fluorescent gangliosides may be useful probes for elucidating the function of gangliosides.[3]

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[3] In this regard, we have been able to show that fluorescent GM₁ derivatives function as receptors for cholera toxin. When GM₁-deficient rat glioma C6 cells (16) were exposed to the derivatives, the binding of iodotoxin to the cells increased substantially. The affinity of the toxin for cells treated with native or fluorescent GM₁ also was the same. More important, the cells exhibited a ninefold increase in cyclic AMP production compared with control cells treated with the toxin (manuscript in preparation).