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Fluorescent Derivatives of Ganglioside G_{M1} Function as Receptors for Cholera Toxin

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ABSTRACT: A fluorescent derivative of ganglioside G_{M1} was prepared by oxidation of the sialic acid residue with sodium periodate and reaction of the resulting aldehyde with Lucifer yellow CH. The biological activity of the fluorescent derivative was compared with that of native G_{M1} using G_{M1}-deficient rat glioma C6 cells. When the cells were exposed to either native or fluorescent G_{M1}, their ability to bind ^{125}I-labeled cholera toxin was increased to a similar extent. This increase in binding was directly proportional to the amount of ganglioside added to the medium. The affinity of the toxin for cells treated with either native or fluorescent G_{M1} also was similar. More importantly, the fluorescent G_{M1} was as effective as native G_{M1} in enhancing the responsiveness of the cells to cholera toxin. Thus, the ganglioside-treated cells exhibited a 9-fold increase in toxin-stimulated cyclic AMP production over cells not exposed to G_{M1}. There was a similar increase in iodotoxin binding and toxin-stimulated cyclic AMP accumulation in cells treated with other G_{M1} derivatives containing rhodaminyl or dinitrophenyl groups. On the basis of these results, it is clear that these modified gangliosides retain the ability to function as receptors for cholera toxin. Consequently, fluorescent gangliosides are likely to be useful as probes for investigating the dynamics and function of these membrane components.

The function of many cell surface components may be related to their mobility in the plane of the membrane (Schlessinger & Elson, 1982). Various methods have been developed to determine the location and movement of plasma membrane constituents. One of the most successful approaches is the introduction of fluorescent probes into such molecules (Taylor et al., 1978; Wilchek et al., 1980; Kreis et al., 1982; Lipsky & Pagano, 1983; Spiegel et al., 1983; Sleight & Pagano, 1984). As a part of our strategy for studying the function of gangliosides, we have prepared fluorescent ganglioside derivatives with an unmodified lipid moiety (Wilchek et al., 1980; Spiegel et al., 1983). These derivatives were used to directly monitor the organization and dynamics of gangliosides in the plasma membrane (Spiegel et al., 1984a,b).

Whenever a biological molecule is chemically modified, a critical question arises as to whether the modification compromises its biological activity. Thus, it seemed important to investigate the effect that covalent attachment of fluorophores to the sialic acid residues of gangliosides has on their activity. In contrast to the vast knowledge available on the chemical structure of gangliosides [see Wiegandt (1982)], little is known about their function. The only well-established function for gangliosides is that of the ganglioside G_{M1}[1] as the receptor for choleragen (Fishman, 1982). When G_{M1}-deficient cells are cultured in medium containing exogenous G_{M1}, the cells take up the G_{M1} and exhibit increased choleragen binding and responsiveness (Moss et al., 1976; Fishman et al., 1980; Fishman, 1980, 1982). In the present study, this procedure was used to assess the biological activity of fluorescent G_{M1} derivatives.

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[1] Abbreviations: G_{M1}, II^{3}NeuAcGgOse$_4$Cer, Galβ1→3GalNAcβ1→4[NeuAcα2→3]Galβ1→4Glc-Cer; G_{M3}, II^{3}NeuAcLacCer, NeuAcα2→3Galβ1→4Glc-Cer; DNP, dinitrophenyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, Dulbecco's phosphate-buffered saline (pH 7.4) minus Ca^{2+} and Mg^{2+}; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Experimental Procedures

Preparation of Lucifer Yellow CH Labeled G_{M1}. G_{M1} (1 mg) was dissolved in 1 mL of 100 mM sodium acetate buffer (pH 5.5) containing 150 mM NaCl and 2 mM $NaIO_4$ and oxidized for 30 min at 0 °C (Veh et al., 1977; Fishman et al., 1980). The reaction was stopped by adding 0.1 mL of 50% glycerol, and the solution was dialyzed extensively against water and lyophilized. The oxidized G_{M1} was dissolved in 1 mL of PBS, and, after addition of Lucifer yellow CH to 5 mM, the solution was incubated overnight at 0 °C and dialyzed against 1 L of PBS. The material was reduced with 10 mM $NaCNBH_4$ (15 min at 23 °C), dialyzed against distilled H_2O, and lyophilized. The modified G_{M1} was analyzed by thin-layer chromatography on silica gel 60 coated glass plates, which were developed in chloroform/methanol/0.25% $CaCl_2$ (60:35:8 v/v), visualized with resorcinol reagent, and quantified by scanning densitometry (Fishman et al., 1979). The Lucifer yellow CH G_{M1} also was easily detected on the chromatograms under UV illumination.

Detection of Gangliosides That Bind Choleragen. A modification (Critchley et al., 1981; Fishman et al., 1984) of the procedure of Magnani et al. (1980) was used to directly detect binding of ^{125}I-choleragen to G_{M1} and its derivatives on thin-layer chromatograms. Briefly, the gangliosides were separated on aluminum-backed silica gel in chloroform/methanol/0.2% $CaCl_2$ (5:4:1 v/v). The dried chromatogram was quickly soaked twice in 0.1% poly(isobutyl methacrylate) dissolved in hexane. After being air-dried, the chromatogram was sprayed with 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 1% bovine serum albumin. The following steps were performed rapidly. The chromatogram was soaked in the above buffer, drained, and immediately overlayed with ^{125}I-choleragen (10^6 cpm/mL) in the same buffer. After 30 min at 4 °C, the chromatogram was drained and dipped in four successive changes of the same ice-cold buffer without the bovine serum albumin. The chromatogram was air-dried, and the bound toxin was detected by autoradiography using Kodak X-Omat AR-2 film and quantified by densitometric scanning of the autoradiogram.

Cell Culture. Rat glioma C6 cells obtained from the American Type Culture Collection were used between passages 40 and 50 (Fishman et al., 1981; Mallorga et al., 1981) and were grown as described previously (Zaremba & Fishman, 1984). The cells were grown on multicluster plastic tissue culture dishes (24 × 13 mm wells from Costar, Cambridge, MA) for cAMP accumulation and in situ binding studies. For measurement of binding to cells in suspension, they were grown in 25 cm^2 flasks. The cells were seeded 6 days before an experiment and maintained in serum-free Dulbecco's modified Eagle's medium for 2 days prior to an experiment. Gangliosides were dissolved in sterile distilled water by warming at 37 °C for 1 h (Fishman et al., 1980). The concentrated ganglioside solutions then were diluted with serum-free medium and added to the cells. After the cells were incubated for 3 h at 37 °C, they were washed 3 times with ice-cold PBS and analyzed for toxin binding and responsiveness.

Binding of ^{125}I-Choleragen to Rat Glioma C6 Cells. Binding of iodotoxin to cells in situ was determined by using an established procedure (Fishman et al., 1980; Mallorga et al., 1981). Briefly, each 13-mm diameter well of cells was incubated with 1 mL of ^{125}I-choleragen (5–20 nM, 8400–13 350 cpm/pmol) in serum-free medium buffered with 25 mM Hepes and supplemented with 0.1% bovine serum albumin. After 1 h at 37 °C, the medium was removed, and the cells were rapidly washed 3 times with 1 mL of ice-cold PBS. The cells then were dissolved in 0.3 mL of 0.5 M NaOH and analyzed for protein and radioactivity. Binding to cells in suspension was determined by a filtration assay (Fishman & Atikhan, 1980) as modified by Miller-Podraza et al. (1982). The affinity of choleragen for its receptor was determined by a competition assay in which increasing amounts of unlabeled toxin were added to the binding assay (Critchley et al., 1981). Nonspecific binding of the iodotoxin was determined by adding 0.2 μM unlabeled choleragen to the assay. All values have been corrected for nonspecific binding and are the mean of quadruplicate determinations. Unless indicated, standard errors were less than 10% of the mean.

Accumulation of cAMP by Rat Glioma C6 Cells. Accumulation of cAMP by the cells was determined by established procedures (Fishman, 1980; Fishman et al., 1980). Briefly, each 13-mm well of cells was incubated for 5 min at 37 °C in 1 mL of serum-free medium buffered with 25 mM Hepes and supplemented with 1 mM isobutylmethylxanthine and 0.01% bovine serum albumin. Then 10 μL of 1 μM choleragen was added and the incubation continued for an additional 1 h. The medium was rapidly removed, and 0.3 mL of 0.1 M HCl was added. After 30 min at room temperature, the acid extract was removed, lyophilized, and assayed for cAMP by using a radioimmune procedure (Zaremba & Fishman, 1984). The cell layer was digested in 0.3 mL of 0.5 M NaOH and assayed for protein (Lowry et al., 1951). Values are the mean of triplicate determinations, and standard errors were routinely less than 10% of the mean.

Materials. Choleragen was obtained from Calbiochem-Behring (La Jolla, CA) and iodinated by using Chloramine-T (Fishman & Atikkan, 1980). G_{M1} was purified as described previously (Pacuszka et al., 1978) from bovine brain gangliosides after exhaustive treatment with *Vibrio cholerae* neuraminidase which hydrolyzes all of the di-, tri- and polysialogangliosides to G_{M1}. Lucifer yellow CH was obtained from Aldrich Chemical Co. (Milwaukee, WI). Glass- and aluminum-backed silica gel 60 thin-layer chromatograms were obtained from E. Merck through EM Science (Gibbstown, NJ).

Results

Analysis of Lucifer Yellow CH Labeled Gangliosides. Oxidation of G_{M1} with $NaIO_4$ followed by reaction with Lucifer yellow CH resulted in the formation of two fluorescent derivatives which were readily separated from the free fluorophore by thin-layer chromatography (Figure 1A). The yield of Lucifer yellow CH labeled G_{M1} (30–75%) as well as the proportion of the two derivatives appeared to depend on the reaction time and concentration of fluorophore (compare lanes 1 and 2, Figure 1A). Both derivatives reacted with resorcinol reagent (not shown), and both had a lower mobility than G_{M1} on the chromatogram. As Lucifer yellow CH is both polar and negatively charged, the decrease in mobility is not unexpected.

The Lucifer yellow CH labeled G_{M1} derivatives were recognized by choleragen as revealed by direct binding of ^{125}I-choleragen to the chromatograms followed by autoradiography (Figure 1B). There was a direct correspondence between the fluorescence and autoradiogram (compare panels A and B of Figure 1). As expected, the toxin bound to unreacted G_{M1} but not to the free fluorophore (Figure 1B) or to other Lucifer yellow CH labeled gangliosides (not shown). The formation of two fluorescent derivatives of G_{M1} may be due to the presence of two oxidized gangliosides: one with aldehyde at carbon 8 of the sialic acid and the other at carbon 7 (Veh et al., 1977). There is an alternate possibility: in addition to the hydrazide, Lucifer yellow CH has a free amino group

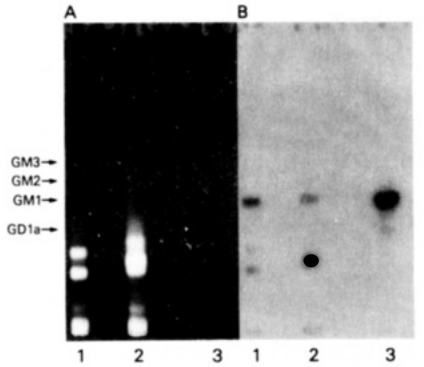

FIGURE 1: Separation of Lucifer yellow CH labeled G_{M1} by thin-layer chromatography and detection by UV light or ^{125}I-choleragen overlay and autoradiography. Lucifer yellow CH labeled G_{M1} was prepared and separated by thin-layer chromatography on silica gel as described under Experimental Procedures. The aluminum-backed chromatograms were either (A) photographed under UV light or (B) overlayed with ^{125}I-choleragen and the bound iodotoxin detected by autoradiography (16-h exposure). Lane 1, 0.4 nmol of oxidized G_{M1} reacted with 1 mM Lucifer yellow CH for 3 h; lane 2, 0.4 nmol of oxidized G_{M1} reacted with 5 mM Lucifer yellow CH for 12 h; lane 3, 1 nmol each of G_{M3}, G_{M2}, G_{M1}, and G_{D1a} (locations indicated by arrows). Note that Lucifer yellow CH itself is immobile in chloroform/methanol/0.2% $CaCl_2$ (5:4:1 v/v).

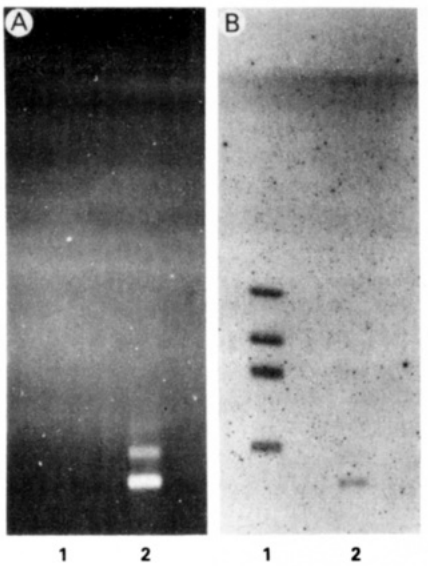

FIGURE 2: Thin-layer chromatography of purified Lucifer yellow CH labeled G_{M1}. Lucifer yellow CH labeled G_{M1} was purified and separated by thin-layer chromatography on silica gel coated glass plates which were developed in chloroform/methanol/0.25% $CaCl_2$ (60:35:8 v/v) as described under Experimental Procedures. (A) Visualization under UV light. (B) Visualization with resorcinol spray. Lane 1, Ganglioside standards (1 nmol of each) from top to bottom, G_{M3}, G_{M2}, G_{M1}, and G_{D1a}; lane 2, purified Lucifer yellow CH labeled G_{M1} (0.4 nmol).

which could react with the aldehyde on the oxidized G_{M1}.

The major Lucifer yellow CH labeled G_{M1} derivative was purified from the minor derivative, free fluorophore, and unreacted G_{M1} by scraping the band from the chromatograms and eluting with silica gel with 10 mL of chloroform/methanol/H_2O (10:10:3 v/v). Upon further chromatography, the fluorescent G_{M1} appeared as a single resorcinol-positive band (Figure 2B, lane 2) which corresponded to the major fluorescent band (Figure 2A). To further assess its purity, the even more sensitive overlay technique with iodotoxin was used (Figure 3). Again, the fluorescence corresponded to the bound iodotoxin. There appeared to be only a trace of unreacted G_{M1} (Figure 3B) on the basis of densitometric scanning of the autoradiogram (Figure 3C).

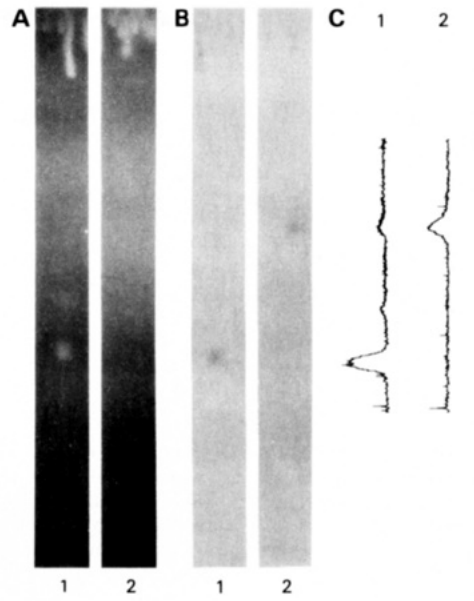

FIGURE 3: Analysis of Lucifer yellow CH labeled G_{M1} by thin-layer chromatography. Purified Lucifer yellow CH labeled G_{M1} was separated by thin-layer chromatography ass described in the legend to Figure 1. The chromatograms were either (A) photographed under UV light or (B) overlayed with ^{125}I-choleragen and the bound iodotoxin detected by autoradiography (5-h exposure). (C) Densitometric scans of autoradiogram shown in (B). Lane 1, Purified Lucifer yellow CH labeled G_{M1} (0.6 nmol); lane 2, ganglioside standards (0.25 nmol of each).

Table I: Binding of Choleragen to Rat Glioma C6 Cells Treated with Gangliosides[a]

ganglioside added	^{125}I-choleragen bound (fmol/well) expt 1	expt 2
none	4.1	4.6
Lucifer yellow CH labeled G_{M3}	31	ND[b]
Lucifer yellow CH labeled G_{M1}	1065	1050
G_{M1}	1060	1230

[a] Cells were incubated with 2 μM aliquots of the indicated gangliosides for 3 h at 37 °C, washed, and incubated with 5 nM ^{125}I-choleragen for 1 h at 37 °C. The cells then were assayed for bound iodotoxin as described under Experimental Procedures. Values have been corrected for nonspecific binding as measured in the presence of 200 nM unlabeled toxin. Each 13-mm diameter well contained 120 ± 32 μg of protein. [b] ND, not determined.

Binding of Choleragen to Rat Glioma C6 Cells Treated with G_{M1} and Its Fluorescent Derivatives. The ability of Lucifer yellow CH labeled G_{M1} to serve as a cell surface receptor for choleragen was tested with G_{M1}-deficient rat glioma C6 cells. These cells only bind trace amounts of the toxin (Fishman, 1980; Fishman & Atikkan, 1980; Fishman et al., 1980; Mallorga et al., 1981) and have no detectable G_{M1} when analyzed by chemical or cell surface labeling techniques (Miller-Podraza et al., 1982). The C6 cells readily take up G_{M1} from the medium with a corresponding increase in choleragen binding (Fishman, 1980; Fishman et al., 1980). The cells were incubated at 37 °C for 3 h with G_{M1} or purified Lucifer yellow CH labeled G_{M1}, extensively washed to remove any free ganglioside, and then incubated in situ with ^{125}I-choleragen. The treated cells bound substantially more iodotoxin than did the untreated cells (Table I). Both G_{M1} and its fluorescent derivative were able to create new binding sites whereas Lucifer yellow CH labeled G_{M3} was ineffective.

The increase in ^{125}I-choleragen binding was proportional to the concentration of G_{M1} added to the medium, and at equivalent concentrations, the fluorescent G_{M1} was as effective

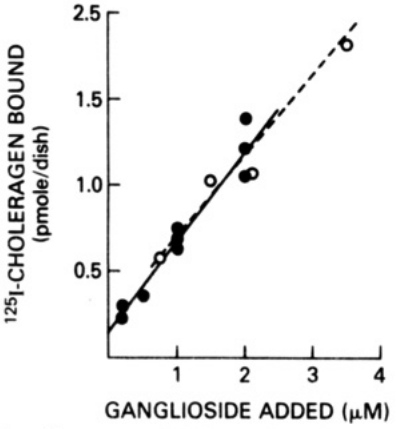

FIGURE 4: Effect of treatment with G_{M1} and Lucifer yellow CH labeled G_{M1} on binding of choleragen to rat glioma C6 cells. Cells were incubated with increasing concentrations of G_{M1} (●) or Lucifer yellow CH labeled G_{M1} (○) for 3 h at 37 °C, washed, and assayed for specific binding of ^{125}I-choleragen as described under Experimental Procedures. Untreated cells bound only 0.004–0.008 pmol/well.

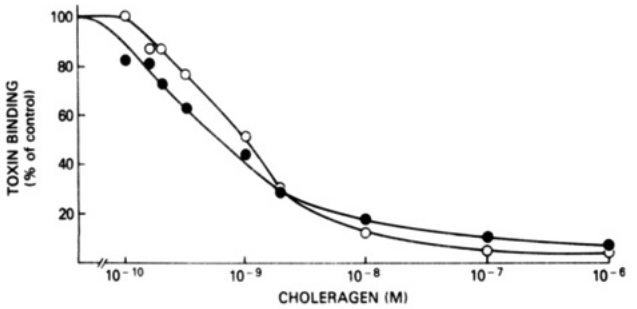

FIGURE 5: Affinity of choleragen for G_{M1} and Lucifer yellow CH labeled G_{M1} inserted into rat glioma C6 cells. Cells were incubated with 0.2 μM G_{M1} (○) or Lucifer yellow CH labeled G_{M1} (●) for 2 h, washed, detached from the flasks by mechanical scraping, and collected by centrifugation. The cells were suspended in buffer (Miller-Podraza et al., 1982) and assayed for binding of ^{125}I-choleragen (0.4 nM) in the presence of increasing concentrations of unlabeled toxin as described under Experimental Procedures.

as unmodified G_{M1} (Figure 4). The apparent affinity of ^{125}I-choleragen for cells treated with G_{M1} or Lucifer yellow CH labeled G_{M1} was similar (Figure 5). The concentration of unlabeled choleragen required to inhibit by 50% the binding of tracer amounts of iodotoxin was 0.65 nM for G_{M1}-treated cells and 1.1 nM for cells treated with fluorescent G_{M1}. These values are similar to the concentration of toxin (0.5 nM) required to half-saturate the receptors on untreated C6 cells (Fishman & Atikkan, 1980; Mallorga et al., 1981). Thus, the apparent affinity of choleragen for endogenous or exogenous G_{M1} on the surface of viable cells is similar, and addition of a fluorophore to the sialyl residue of the ganglioside has little effect on binding or affinity of the toxin.

Biological Activity of Lucifer Yellow CH Labeled G_{M1}. In addition to creation of new choleragen binding sites on rat glioma C6 cells, treatment of the cells with G_{M1} also enhanced their responsiveness to choleragen (Fishman, 1980; Fishman et al., 1980, 1981; Mallorga et al., 1981). The ability of Lucifer yellow CH labeled G_{M1} to sensitive C6 cells to choleragen was determined, and the results of two separate experiments are shown in Table II. Although rat glioma C6 cells of low passage number do accumulate some cAMP when exposed to choleragen (Fishman et al., 1981; Mallorga et al., 1981), the response is greatly enhanced in G_{M1}-treated cells (8.8-fold greater than in cells not treated with G_{M1}). The purified Lucifer yellow CH labeled G_{M1} also was very effective in increasing toxin responsiveness (7.5-fold) whereas Lucifer yellow CH labeled G_{M3} was, if anything, slightly inhibitory.

Table II: Effect of Ganglioside Treatment on Responsiveness of Rat Glioma C6 Cells to Choleragen[a]

ganglioside added	cAMP accumulation (pmol/well) expt 1	expt 2
none	18.7 ± 1.9	21.7 ± 2.2
Lucifer yellow CH labeled G_{M3}	5.3 ± 0.4	ND
Lucifer yellow CH labeled G_{M1}	141 ± 2.7	151 ± 12.5
G_{M1}	166 ± 37.4	190 ± 24.6

[a] Cells were incubated with 1 μM aliquots of the indicated gangliosides for 3 h, washed, and incubated with 10 nM choleragen for 1 h at 37 °C. The cells then were assayed for cAMP content as described under Experimental Procedures. Values have been corrected for basal cAMP content (0.13–0.3 pmol/well) and are the mean ± SD of triplicate wells.

Table III: Treatment of Rat Glioma C6 Cells with Rhodaminyl-Labeled G_{M1}: Effects on Choleragen Binding and Action[a]

ganglioside added	^{125}I-choleragen bound (fmol/well)	cAMP accumulation (pmol/well)
none	10	42.2
rhodaminyl-labeled G_{M1}	413	342
G_{M1}	382	368

[a] Cells were incubated with a 0.5 μM aliquot of the indicated ganglioside for 3 h, washed, and incubated with 5 nM ^{125}I-choleragen for 45 min or 10 mM unlabeled choleragen for 1 h at 37 °C. The cells then were assayed for bound iodotoxin or cAMP accumulation as described under Experimental Procedures. Values have been corrected for nonspecific binding or basal cAMP content (5.3–6.5 pmol/well).

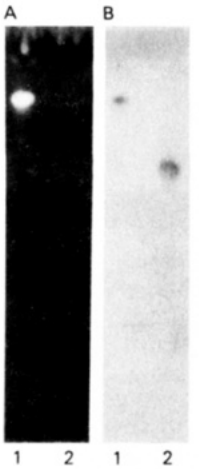

FIGURE 6: Analysis of purified rhodaminyl-labeled G_{M1} by thin-layer chromatography. Rhodaminyl-labeled G_{M1} was prepared as described previously (Wilchek et al., 1980), purified by preparative thin-layer chromatography, and separated on aluminum-backed silica gel as described in the legend to Figure 1. The chromatograms were either (A) photographed under UV light or (B) overlayed with ^{125}I-choleragen and bound iodotoxin detected by autoradiography (5-h exposure). Lane 1, Purified rhodaminyl-labeled G_{M1} (0.25 nmol); lane 2, native G_{M1} (0.5 nmol).

Assay of Other G_{M1} Derivatives. Rhodamine-labeled G_{M1} was prepared as described previously (Wilchek et al., 1980), separated by thin-layer chromatography, and also observed to directly bind ^{125}I-choleragen by the overlay technique (Figure 6). In contrast to the Lucifer yellow CH labeled G_{M1}, the rhodaminyl derivative had a faster mobility than G_{M1} which is consistent with the more hydrophobic nature of rhodamine. When taken up by rat glioma C6 cells, rhodaminyl-labeled G_{M1} also enhanced choleragen binding and responsiveness (Table III). To test the effects of adding a

Table IV: Treatment of Rat Glioma C6 Cells with DNP-Labeled G_{M1}: Effects on Choleragen Binding and Action[a]

ganglioside added	concn (μM)	^{125}I-choleragen bound (fmol/well)	cAMP accumulation (pmol/well)
none		1.0	11.7
DNP-labeled G_{M1}	0.15	60	124
	0.30	139	157
G_{M1}	0.30	182	156
	1.0	637	214

[a] Cells were incubated with the indicated gangliosides for 3 h, washed, and incubated with 10 nM ^{125}I-choleragen for 45 min or 10 nM unlabeled choleragen for 1 h at 37 °C. The cells then were assayed for bound iodotoxin or cAMP accumulation as described under Experimental Procedures. Values have been corrected for nonspecific binding or basal cAMP content (5.3–8.9 pmol/well).

nonfluorescent group on G_{M1}, DNP-labeled G_{M1} was prepared (Spiegel et al., 1979). The DNP-labeled derivative was slightly more mobile than native G_{M1} by thin-layer chromatography and also directly bound ^{125}I-choleragen (data not shown). DNP-labeled G_{M1} was taken up by the C6 cells and increased their ability to bind ^{125}I-choleragen and to accumulate cAMP in response to the toxin (Table IV).

Discussion

In the present study, derivatives of G_{M1} containing either fluorophores (Lucifer yellow CH or rhodamine) or dinitrophenol were prepared and tested for their retention of biological activity. The modification procedure does not appear to impair the ability of a ganglioside-binding protein, i.e., choleragen, to recognize the ganglioside. Using a variation of the elegant and sensitive technique developed by Magnani et al. (1980), the toxin was shown to directly bind to the G_{M1} derivatives separated on thin-layer chromatograms even though the modification had altered the mobility of the ganglioside. The change in mobility had the added advantage of allowing separation of the derivative from unmodified G_{M1} and free reagents. The various derivatives of G_{M1} also were inserted into biological membranes, namely, viable rat glioma C6 cells, and shown to retain their ability to function as receptors for choleragen. Lucifer yellow CH labeled G_{M1} was as effective as G_{M1} in enhancing the binding of the toxin to the cells and had an affinity for choleragen similar to that of the native ganglioside. More importantly, the fluorescent G_{M1} behaved similarly to G_{M1} in increasing toxin-stimulated cAMP accumulation in the cells. Rhodamine-labeled G_{M1} and DNP-labeled G_{M1} also were able to increase toxin binding and responsiveness in rat glioma C6 cells.

Introduction of a bulky fluorscent or dinitrophenol group into the side chain of the sialic acid residue of G_{M1} did not interfere with its ability to be recognized by choleragen. Previous studies showed that periodate oxidation and borohydride reduction of G_{M1} did not destroy its function as a choleragen receptor (Fishman et al., 1980). The latter investigators also found that G_{M1} containing *N*-glycolylneuraminic acid instead of *N*-acetylneuraminic acid was a functional receptor for the toxin. Reductive decarboxylation of the sialic acid appears, however, to destroy the ability of G_{M1} to be recognized by choleragen (Sattler et al., 1977).

In a previous study, fluorescent gangliosides were found to be taken up by human fibroblasts, to initially be restricted to the external face of the plasma membrane, and to be freely mobile in the plane of the membrane with a diffusion coefficient of $\sim 10^{-8}$ cm^2/s (Spiegel et al., 1984a). In other studies (Wilchek et al., 1980; Spiegel et al., 1983, 1984b), it was found that fluorescent gangliosides were taken up by lymphocytes and stably inserted into the external half of the plasma membrane. The inserted gangliosides also appeared to be mobile in the plane of the lymphocyte membrane as they underwent patching and capping when the cells were exposed to anti-rhodamine antibodies (using rhodaminyl-labeled gangliosides) or choleragen (using Lucifer yellow CH labeled G_{M1}) (Spiegel et al., 1984b). Thus, fluorescent gangliosides appear to be very useful for studying the dynamics of these membrane components.

Gangliosides have been implicated as recognition molecules for toxins (Fishman, 1982), fibronectin (Kleinman et al., 1979; Yamada et al., 1983), and viruses (Markwell et al., 1981) and as modulators of receptors for growth factors (Bremer et al., 1984). The availability of fluorescent gangliosides that retain their biological function should facilitate research in this area. Gangliosides containing DNP and biotinyl groups have also been prepared (Spiegel et al., 1979; Spiegel & Wilchek, 1981); when they were inserted into lymphocytes, the modified gangliosides promoted stimulation of DNA synthesis in cells exposed to anti-DNP antibodies and avidin, respectively. Biotinylated gangliosides as well as neutral glycosphingolipids also have been used to determine their localization and distribution by electron microscopy (Spiegel et al., 1982; Tillack et al., 1983). The ready availability of dinitrophenylhydrazine, biotin hydrazide, and Lucifer yellow CH and the simplicity of the modification procedure make the preparation of these ganglioside derivatives relatively easy. The present demonstrated that such modified gangliosides retain their biological activity should promote their use as probes for investigating the distribution, dynamics, and function of these membrane components.

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Characterization of the Oligosaccharides of Prolyl Hydroxylase, a Microsomal Glycoprotein†

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ABSTRACT: Prolyl hydroxylase is a tetrameric glycoprotein that catalyzes a vital posttranslational modification in the biosynthesis of collagen. The enzyme purified from whole chick embryos (WCE) possesses two nonidentical subunits, α and β, and has been shown by several techniques to reside in the endoplasmic reticulum of chick embryo fibroblasts. The studies described here demonstrate that the larger of the two subunits (α) exists in two forms in chick embryo fibroblasts (CEF); these two forms differ in carbohydrate content. The larger α subunit, α', contains two N-linked high mannose oligosaccharides, each containing eight mannose units; the smaller subunit, α, contains a single seven-mannose N-linked oligosaccharide. Both oligosaccharides could be cleaved by endo-β-*N*-acetylglucosaminidase H and completely digested with α-mannosidase to yield mannosyl-*N*-acetylglucosamine.

Prolyl hydroxylase [prolyl-glycyl-peptide, 2-oxoglutarate: dioxygenase (4-hydroxylating), EC 1.14.11.2] catalyzes a critical posttranslational modification during the biosynthesis of collagen (Kivirikko & Myllyla, 1980; Davidson & Berg, 1981). Electron microscopy with ferritin-labeled antibodies to prolyl hydroxylase and subcellular fractionation have demonstrated that the enzyme is located in the endoplasmic reticulum of fibroblasts (Olsen et al., 1973; Peterkofsky & Assad, 1976). It has been purified from a number of sources (Kivirikko & Myllyla, 1980) and shown to be a tetramer composed of two pairs of nonidentical subunits ($\alpha_2\beta_2$) (Berg et al., 1979). Two forms of the α subunit have been observed in immunoprecipitates of embryonic chick tendon cell homogenates (Berg et al., 1980); both forms were found to be present in tetrameric enzyme. Only one of these forms, α, is found in enzyme purified from 13-day-old whole chick embryos (WCE).[1] The α subunit contains at least two residues of *N*-acetylglucosamine and a larger amount of mannose (Berg

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[1] Abbreviations: Endo H, endo-β-*N*-acetylglucosaminidase H; Con A, concanavalin A; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CEF, chick embryo fibroblast; WCE, whole chick embryo; DTT, dithiothreitol; PPO, 2,5-diphenyloxazole, Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; RNase, ribonuclease.

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