Synthesis and Characterization of *N*-Parinaroyl Analogs of Ganglioside G_{M3} and De-*N*-acetyl G_{M3}. Interactions with the EGF Receptor Kinase†

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Received September 10, 1992; Revised Manuscript Received February 12, 1993

ABSTRACT: A specific plasma membrane glycosphingolipid, known as ganglioside G_{M3}, can regulate the intrinsic tyrosyl kinase activity of the epidermal growth factor (EGF) receptor; this modulation is not associated with alterations in hormone binding to the receptor. G_{M3} inhibits EGF receptor tyrosyl kinase activity in detergent micelles, in plasma membrane vesicles, and in whole cells. In addition, immunoaffinity-purified EGF receptor preparations contain ganglioside G_{M3} (Hanai et al. (1988) *J. Biol. Chem. 263,* 10915–10921), implying that the glycosphingolipid is intimately associated with the receptor kinase in cell membranes. Both the nature of this association and the molecular mechanism of kinase inhibition remain to be elucidated. In this report, we describe the synthesis of a fluorescent analog of ganglioside G_{M3}, in which the native fatty acid was replaced with *trans*-parinaric acid. This glycosphingolipid inhibited the receptor kinase activity in a manner similar to that of the native ganglioside. A modified fluorescent glycosphingolipid, *N-trans*-parinaroyl de-*N*-acetyl ganglioside G_{M3}, was also prepared. This analog, like the nonfluorescent de-*N*-acetyl ganglioside G_{M3}, had no effect on receptor kinase activity. Results from tryptophan fluorescence quenching and steady-state anisotropy measurements in membranes containing these fluorescent probes and the human EGF receptor were consistent with the notion that G_{M3}, but not de-*N*-acetyl G_{M3}, interacts specifically with the receptor in intact membranes.

Many studies of glycosphingolipids (GSL[1]), based on analyses of the GSL composition of cell membranes and on observations of the effects of exogenously added GSL on cells, suggest that GSLs are involved in a wide variety of biological processes, including cell growth regulation, cell transformation, cell differentiation, cell–cell interaction and recognition, cell morphology changes, and interactions with bacteria, virus, and bioactive factors (Fenderson et al., 1990; Hakomori, 1984). However, a direct relationship between GSL composition and cell behavior was not established until recently, when Hakomori and co-workers discovered that specific gangliosides can modify the activity of growth-regulatory plasma membrane proteins.

Hakomori's group (Bremer et al., 1984, 1986) first discovered that G_{M3} and G_{M1} not only inhibited cell growth but also were capable of inhibiting ligand-stimulated phosphorylation of growth factor receptors in cell membrane preparations. Both G_{M3} and G_{M1} inhibited PDGF-stimulated PDGF receptor phosphorylation and cell proliferation; only G_{M3} inhibited EGF-stimulated EGF receptor phosphorylation, consistent with ganglioside effects on cell growth. Neither G_{M3} nor G_{M1} affected the binding of ^{125}I-EGF to its cell surface receptor. Phosphoamino acid analysis of the EGF receptor indicated that the reduction of phosphorylation induced by G_{M3} was entirely due to decreased phosphorylation of tyrosine residues. An inhibitory effect of G_{M3} on the EGF-stimulated phosphorylation of the EGF receptor was also observed in preparations of purified EGF receptor and in a membrane fraction derived from cells treated with G_{M3}. These results suggest that the inhibitory effect of G_{M3} on cell growth may be mediated by growth factor receptors and results from inhibition of the ligand-stimulated kinase activity of these receptors.

Later, Hakormori and co-workers found that the EGF receptor, purified on an anti-EGF receptor antibody–Sepharose column, contained G_{M3} (Hanai et al., 1988b). This observation suggested that G_{M3} interacts directly with the EGF receptor and further strengthened the argument that this ganglioside exerts regulatory effects on the EGF receptor. These workers also reported that some derivatives of G_{M3} affected cell growth and the autophosphorylation of the EGF receptor. De-*N*-acetyl G_{M3}, which has no acetyl group on its sialic acid residue, was reported to strongly enhance the kinase activity of the EGF receptor and to stimulate the growth of A431, Swiss 3T3, and B16 melanoma cells (Hanai et al., 1988a). Lyso G_{M3}, which has lost its acyl chain but retains an intact head group, inhibited the kinase activity of the EGF receptor and reduced the growth of those cells (Hanai et al., 1988b). Neither of these G_{M3} derivatives altered the affinity of EGF binding to its receptor. Moreover, it was found that de-*N*-acetyl G_{M3} and lyso G_{M3} were present in A431 and B16 cells. These data further defined the structural specificity of G_{M3} modulation of the EGF receptor and suggested that de-*N*-acetylation of the sialic acid moiety of G_{M3} may be an important physiological mechanism for modulation of EGF-dependent cell growth.

† The work was supported by grants from NASA (NAGW-2328) and the American Cancer Society (BC-507) to D.A.R. and a Wesley Foundation Fellowship to W.S. This is Contribution No. 93-63-J from the Kansas Agricultural Experiment Station.

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[1] Abbreviations: EGF, epidermal growth factor; CMC, critical micelle concentration; G_{M1}, Gal(β1–3)GalNAc(β1–4)[NeuAc(2–3)]Gal(β1–4)-GlcCer; G_{M3}, NeuAc(2–3)Gal(β1–4)GlcCer; GSL, glycosphingolipid; de-*N*-acetyl G_{M3}, G_{M3} containing unsubstituted neuraminic acid; HEPES, *N*-(hydroxyethyl)piperazine-*N'*-ethanesulfonic acid; PnA, parinaric acid; tPnA or *trans*-PnA, *all-trans*-9,11,13,15-octadecatetraenoic acid; PDGF, platelet-derived growth factor; *trans*-PnG_{M3}, G_{M3} containing *N*-acyl-*trans*-parinaric acid; *trans*-Pn de-*N*-acetyl G_{M3}, de-*N*-acetyl G_{M3} containing *N*-acyl-*trans*-parinaric acid.

0006-2960/93/0432-8602$04.00/0

Weis and Davis (1990) also investigated the regulatory functions of G_{M3} on cell growth and EGF receptor kinase activity *in vivo*. Their strategy was to use a mutant Chinese hamster ovary (CHO) cell line, which expresses a conditional defect in the biosynthesis of UDP-Gal and UDP-GalNAc. UDP-Gal and UDP-GalNAc are required for the biosynthesis of gangliosides. Under nonpermissive conditions, this mutant CHO cell line had a very low ganglioside content. Decreased levels of ganglioside expression were associated with increased EGF receptor autophosphorylation; this increased EGF receptor kinase activity stimulated cellular proliferation. The data of Weis and Davis provided additional evidence for a correlation between the cellular level of gangliosides and mitogenic signal transduction under physiological conditions. Unfortunately, the defect in the synthesis of UDP-Gal and UDP-GalNAc in the CHO variant used by Weis and Davis also caused the inhibition of the terminal glycosylation of the EGF receptor and other glycoproteins and the accumulation of precursors of GSLs. Even though there seemed to be no significant change in EGF binding affinity as a function of GSL content, problems due to potential defects in the structure of the EGF receptor (or other glycoproteins), as well as possible effects of GSL precursors, must be considered.

To date, it has not been unequivocally demonstrated that the cellular regulatory effects of GSLs are directly related to their effects on the growth factor receptor, since cell growth can be controlled by multiple signal transduction systems. In addition, many of the previous studies have included detergents in their standard EGF receptor kinase assays, which raises questions about the biological relevance of these experimental results. At low concentrations, detergents permeabilize biomembranes, and, at high concentrations, they cause the bilayer structure of biomembranes to dissociate, forming mixed micelles containing detergent and cellular lipids. Detergents also may change the secondary structure of proteins, resulting in denaturation. Moreover, since both GSLs and the EGF receptor are membrane components, a normal membrane environment might be required for physiologically relevant interactions of GSLs and the EGF receptor. In fact, Hakomori's group found that detergents did strongly affect the function of the EGF receptor (Igarashi et al., 1991; Hanai et al., 1988a,b). In the absence of GSLs, kinase activity of EGF receptor changed as a nonlinear function of detergent concentration, EGF receptor kinase activity in Triton X-100 was maximal when the detergent concentration was near the critical micelle concentration (CMC). G_{M3} exhibited only an inhibitory effect on the EGF receptor kinase at low detergent concentrations, while de-*N*-acetyl G_{M3} exhibited the highest stimulation at a detergent concentration close to the CMC (Hanai et al., 1988a). These workers also demonstrated that lyso PC, which has detergent-like properties, affected the EGF receptor kinase in a manner similar to Triton X-100 (Igarashi et al., 1991).

We examined the effect of the gangliosides in the absence of detergent in intact A431 cells and in membrane preparations from both A431 and 3T3 cells. The inhibitory effect of G_{M3} on receptor kinase autophosphorylation was confirmed in the presence and absence of detergent; the stimulatory effect of de-*N*-acetyl G_{M3} was observed only in the presence of detergent (Song et al., 1991).

For these and other reasons, it appears that alternative approaches will be required in order to gain definitive information about the specificity and nature of ganglioside/receptor interactions. In particular, spectroscopic methods, which have been valuable tools for dissection of lipid–protein interactions, should allow us to examine more closely the nature of these interactions and their physiological importance. In this report, we describe the synthesis and characterization of fluorescent probes which should be useful in such studies and show that these probes have different spectroscopic properties in membranes which are enriched for the human EGF receptor.

EXPERIMENTAL PROCEDURES

Materials. G_{M3} was purified as described previously (Song et al., 1991). *N-trans*-Parinaroyl sulfatide (tPn sulfatide) was synthesized as described by Hammarstrom (1971) and Rintoul et al. (1986). *N-trans*-Parinaroyl G_{M1} (PnG_{M1}) was synthesized as described previously (Song & Rintoul, 1989). *cis*- and *trans*-parinaric acids (PnA) were obtained from Molecular Probes, Inc. (Eugene, OR). TLC plates were obtained from Analtech (Dover, DE). All solvents and chemicals were reagent or HPLC grade from Allied Fisher Scientific Co. (Fairlawn, NJ).

Preparation of Deacylated De-N-acetyl or De-N-glycolyl G_{M3}. G_{M3} (6 μmol) was hydrolyzed with 0.1 N KOH in aqueous 90% 1-butanol (6 mL) for 4 h at 80 °C; this procedure is adapted from that of Nores et al. (1989). Deacylated de-*N*-acetyl or de-*N*-glycolyl G_{M3} (so-called lyso ganglioside) was purified by column chromatography on Iatrobeads (Iatron Laboratories, Inc., Tokyo, Japan). A 1 × 120-cm column was eluted with a linear gradient from $CHCl_3/CH_3OH/NH_4OH$ (60/40/2) to $CHCl_3/CH_3OH/NH_4OH$ (30/70/4). The eluted fractions were analyzed by HPTLC. The purified deacylated de-*N*-acetyl or de-*N*-glycolyl G_{M3} showed a single band with an R_f lower than that of G_{M3} and greater than that of de-*N*-acetyl lyso G_{M3} and a positive reaction with both ninhydrin and resorcinol spray reagents (data not shown).

Preparation of De-N-acetyl Parinaroyl G_{M3}. De-*N*-acetyl parinaroyl G_{M3} (de-*N*-acetyl PnG_{M3}) was synthesized using a modification of the method which we developed for the synthesis of PnG_{M1} (Song & Rintoul, 1989). Lyso ganglioside (2 μmol) was mixed with *trans*-PnA (4 μmol), catalyst (4 μmol of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide HCl) and 0.4 mL of solvent (tetrahydrofuran/water, 19/1, v/v) in a screw-capped vial. The reaction mixture was flushed with N_2, sealed, and incubated, with stirring, for 1 h at 25 °C. The solvent was evaporated under N_2, the sample was dissolved in 100 μL of solvent $CHCl_3/CH_3OH$ (2/1, v/v), and the product was purified by latrobeads HPLC column chromatography (10 μm, 25 cm × 4.6 mm i.d.) using a programmed linear gradient of 2-propanol/hexane/3% ammonium hydroxide from 55/45/0 to 55/35/10 (v/v/v). The eluting fractions were monitored by absorbance at 320 nm, followed by HPTLC analysis. The purified de-*N*-acetyl *trans*-PnG_{M3} showed a single band and the same behavior as native de-*N*-acetyl G_{M3} on TLC plates (data not shown). In addition, the absorbance spectrum of de-*N*-acetyl *trans*-PnG_{M3} was the same as that of *trans*-PnA.

Preparation of N-Acetyl Parinaroyl G_{M3}. De-*N*-acetyl *trans*-PnG_{M3}, prepared as described above, was acetylated by the same method as described previously for the acetylation of de-*N*-acetyl parinaroyl G_{M1} (Song & Rintoul, 1989). *trans*-PnG_{M3} was purified by latrobeads HPLC column chromatography under the same conditions as described above. Approximately 10 nmol of PnGM3 was obtained from 1 μmol of bovine G_{M3}. The purified *trans*-PnG_{M3} showed a single band and was resorcinol-positive and ninhydrin-negative (data not shown). The absorbance spectrum of *trans*-PnG_{M3} was the same as that of *trans*-PnA.

Preparation of Plasma Membranes. Shed plasma membrane vesicles of A431 cells were prepared as described

previously (Song et al., 1991). Plasma membranes from 3T3 cell lines were prepared using nitrogen cavitation and discontinuous sucrose gradient centrifugation as previously described for LM cell membrane preparations (Rintoul et al., 1979).

EGF Receptor Kinase Assay. The inhibitory effect of gangliosides and fluorescent analogs on the kinase activity of the EGF receptor in A431 plasma membrane vesicles was determined as described previously (Song et al., 1991).

Preparation of Liposomes. Liposomes were prepared by ethanol injection in 3 mL of 20 mM HEPES (pH = 7.2) as described previously (Song & Rintoul, 1989).

CMC Measurements. G_{M3} in $CHCl_3/CH_3OH$ (2/1, v/v) and de-*N*-acetyl G_{M3} in $CHCl_3/CH_3OH$ (1/1, v/v) were dried under nitrogen and then dispersed in 20 mM HEPES buffer by vortexing, at final concentrations ranging from 10^{-9} to 10^{-5} M. *trans*- or *cis*-PnA at a final concentration of 3.3×10^{-7} M was added to each sample. The fluorescence of PnA is quenched when PnA is dissolved in water and in PnA micelles, while the emission is greatly increased when PnA is in a hydrophobic environment (Sklar, 1976). The CMC could be estimated as the concentration of gangliosides at which the fluorescent emission of PnA is dramatically increased. The emission data, obtained on a Spex Fluorolog spectrofluorometer, were normalized to the initial fluorescence emission.

Fluorescence Anisotropy Measurements. A431 cell plasma membrane vesicles, containing 60 μg of protein and 120 nmol of phospholipid, were frozen and thawed with 1 nmol of fluorescent probe in 3 mL of 20 mM HEPES buffer. Fluorescence anisotropy was determined and calculated as described previously (Song & Rintoul, 1989). Corrections for scattering depolarization (Lentz et al., 1979) were made when necessary.

Quenching of Tryptophan Fluorescence of A431 Cell Plasma Membranes by Fluorescent Probes. The fluorescence emission of tryptophan was measured with a Spex Fluorlog spectrofluorometer at 25 °C. Excitation and emission wavelengths were 286 (bandpass 5 nm) and 338 nm (bandpass 10 nm), respectively. The emission readings were taken before and after incubation of A431 plasma cell membrane vesicles (20 μg of protein and 40 nmol of phopsholipid in 400 μL of the EGF receptor kinase buffer) with fluorescent probes (1 nmol). Quenching of tryptophan fluorescence was calculated as the difference between the tryptophan emission measurements before and after incubation with parinaroyl probes. The data were normalized relative to the initial tryptophan fluorescence emission.

Analysis of the Fatty Acid Composition of G_{M3}. G_{M3} (400 nmol) was hydrolyzed in 1.5 mL of methanolic HCl at 70 °C for 5 h. Fatty acid methyl esters were extracted with pentane, dried under nitrogen, and acetylated with 0.5 mL of pyridine/acetic anhydride (1/1, v/v) at room temperature overnight. The methyl esters were analyzed by gas–liquid chromatography utilizing a Hewlett-Packard 5790 GLC with flame ionization detector, a 6-ft × $^1/_4$-in. column of 10% SP-2330 on 100/120 Chromosorb, and a Spectra Physics 4270 integrator.

Other Methods. Protein was assayed as described by Smith et al. (1985). Gangliosides were assayed as described by Svennerholm (1957) or by using a modification of the method of Naoi et al. (1974). Phospholipid was assayed according to the method of Ames (1966). The concentration of PnA and parinaroyl isomers was determined by absorbance, using known extinction coefficients (Sklar, 1976).

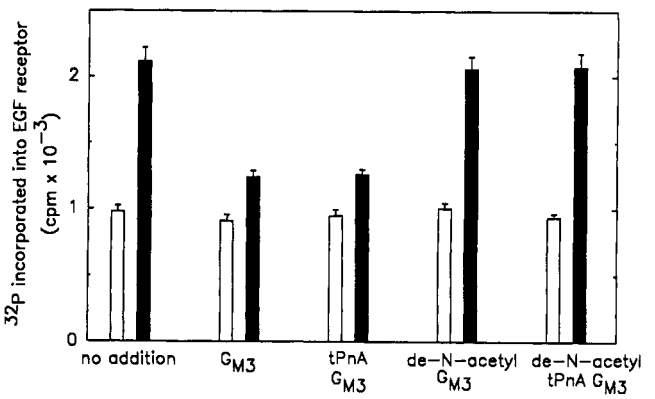

FIGURE 1: Effect of *N*-parinaroyl gangliosides on the kinase activity of the EGF receptor. Shed plasma membrane vesicles (20 μg per assay replicate), prepared from A431 cells as described by Cohen et al. (1982), were permeabilized by eight freeze–thaw cycles. Gangliosides (20 nmol) were added before the last freeze–thaw cycle, and autophosphorylation of the EGF receptor was assayed as described in Experimental Procedures. Open bars, no EGF; filled bars, 100 ng of EGF.

RESULTS

Biological Function of N-Parinaroyl G_{M3} and De-N-acetyl Parinaroyl G_{M3}. *N-trans*-Parinaroyl analogs of G_{M3} and de-*N*-acetyl G_{M3} were synthesized and purified. Previous studies showed that G_{M3} inhibited EGF receptor kinase activity in A431 cell plasma membrane vesicles, while de-*N*-acetyl G_{M3} had no effect on kinase activity in this preparation (Song et al., 1991). In order to examine the ability of the parinaroyl analogs to mimic the functions of native gangliosides, the effect of PnG_{M3} and de-*N*-acetyl PnG_{M3} on EGF-dependent receptor autophosphorylation in A431 cell plasma membrane vesicles permeabilized by freezing–thawing cycles was examined (Figure 1).

In this experimental system, addition of EGF to the frozen and thawed membranes resulted in a two-fold stimulation of receptor autophosphorylation activity. *trans*-PnG_{M3} inhibited the receptor autophosphorylation stimulated by EGF. The magnitude of this inhibition was similar to the inhibition produced by native G_{M3}. Like nonfluorescent de-*N*-acetyl G_{M3} (made from native G_{M3}), de-*N*-acetyl-*trans*-PnG_{M3} did not affect EGF-stimulated receptor autophosphorylation. These results indicate that substituting *trans*-PnA for the acyl chains of G_{M3} and de-*N*-acetyl G_{M3} had no effect on their ability (or inability) to modulate EGF receptor autophosphorylation.

Measurements of CMC. The critical micelle concentration (CMC) is an intrinsic property of any amphiphilic molecule. It is the concentration above which the amphiphile forms multimolecular aggregates in water. CMCs of G_{M3} and de-*N*-acetyl G_{M3} were measured by following the emission of a constant concentration of PnA as the ganglioside concentration was increased (Figure 2). The fluorescence emission drastically increased at about 10^{-7} M G_{M3} and 10^{-6} M de-*N*-acetyl G_{M3}. These concentrations were considered to be the approximate CMCs of G_{M3} and de-*N*-acetyl G_{M3}.

Steady-State Anisotropy of Fluorescent Probes in A431 Cell Plasma Membrane Vesicles. Specific lipid–protein interactions in cell membranes often result in a reduction of the rotational motion of the lipids (Silvius, 1981). Fluorescence anisotropy measurements can be a useful indicator of fluorescent lipid motion and have been widely used with both parinaroyl and more traditional fluorescent probes (Hudson & Cavalier, 1988). Figure 3 shows the steady-state fluorescence anisotropy of free *trans*-PnA, as well as that of *trans*-

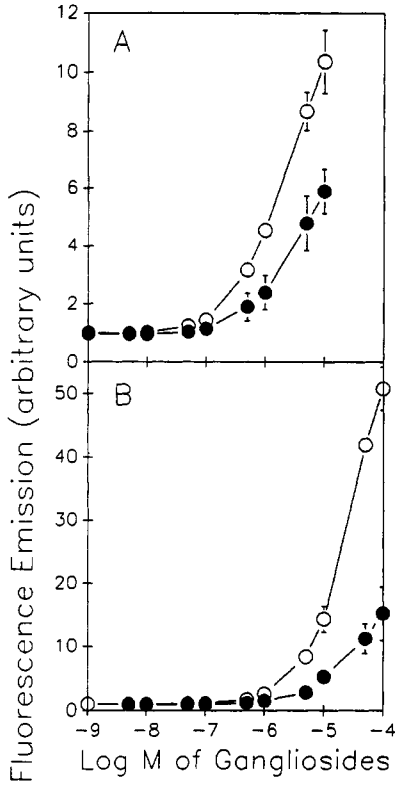

FIGURE 2: Fluorescence emission of PnA at 420 nm, normalized to initial fluorescence emission, as a function of the concentration of G_{M3}. Emission of PnA in G_{M3} is shown in A. Emission of PnA in de-*N*-acetyl G_{M3} is shown in B. Open circles (○) represent the data obtained from *trans*-PnA. Filled circles (●) represent the data obtained from *cis*-PnA.

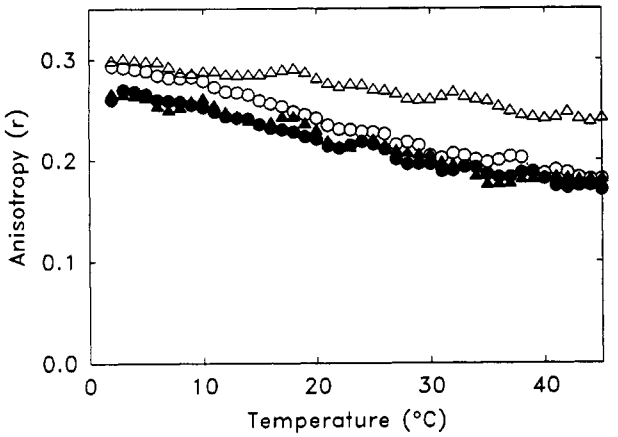

FIGURE 3: Steady-state fluorescence anisotropy of parinaroyl probes in A431 cell plasma membrane vesicles. A431 cell plasma membrane vesicles, prepared as described in Experimental Procedures and containing 60 μg of protein and 120 nmol of phospholipid, were resuspended in 3 mL of HEPES (20 mM, pH 7.2). Fluorescent probe (1 nmol) was incorporated into the membrane by a freeze–thaw cycle. The samples were cooled from 45 to 2 °C. Steady-state fluorescence anisotropy was determined and calculated as described previously [2127]. ○, *trans*-PnA; ●, *trans*-Pn sulfatide; △, *trans*-PnG_{M3}; ▲, *trans*-Pn de-*N*-acetyl G_{M3}.

parinaroyl isomers of G_{M3}, de-*N*-acetyl G_{M3}, and sulfatide in A431 cell plasma membrane vesicles. Unlike the kinase activity determinations shown in Figure 1, these measurements were made in buffer that lacked divalent cations, since the free fatty acid probe interacts with these ions to form insoluble, nonfluorescent soaps. Similar experiments with the sphingolipid probes were performed in the presence of divalent cations (at a concentration identical to that used in the receptor kinase assays shown in Figure 1), and in these cases the

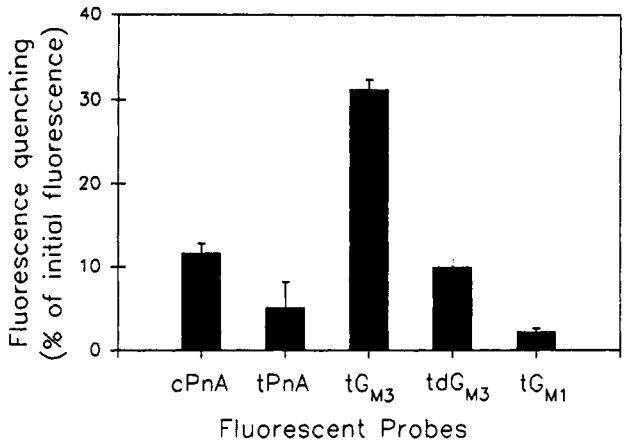

FIGURE 4: Quenching of tryptophan fluorescence of A431 cell plasma membrane vesicles by parinaroyl probes. A431 cell plasma membrane vesicles, prepared as described in Experimental Procedures and containing 20 μg of protein and 40 nmol of phospholipid, were suspended in 400 μL of EGF receptor kinase buffer as described in Experimental Procedures. Tryptophan fluorescence of A431 plasma membrane vesicles at 25 °C was measured at 338 nm (bandpass 5 nm), and excitation was at 286 nm (bandpass 10 nm). Tryptophan fluorescence from the vesicles alone was measured first; after incubation of the vesicles with the indicated probes (1 nmol), fluorescence was measured again. Quenching was calculated as the difference between the tryptophan emission before and after addition of probe and normalized to the initial fluorescence emission value.

anisotropy values were indistinguishable from the values depicted in Figure 3 (data not shown). All probes except for *trans*-PnG_{M3} exhibited a notable decrease in anisotropy as the temperature was increased. A phase transition can be recognized around 10–20 °C but was not highly cooperative, probably because of the heterogeneous lipid composition of the membrane. The anisotropy values of these probes indicated that the membrane lipids had mobilities similar to those of lipid crystalline lipids at the higher temperatures examined and similar to those of gel-phase lipids at the lower temperatures. The anisotropy of *trans*-PnG_{M3} decreased much more slowly than that of other probes with increasing temperature and was substantially higher than that of other probes at or near the physiological temperature of 37 °C. These data indicate that the rotational motion of *trans*-PnG_{M3} in A431 cell plasma membrane is restricted at physiological temperatures and that *trans*-PnG_{M3} is more restricted even when the majority of the lipids in the membrane are fluid, as indicated by results with other fluorescent probes.

Quenching of Tryptophan Fluorescence of A431 Cell Plasma Membrane Vesicles by Fluorescent Probes. Because the absorbance spectra of *trans*- and *cis*-PnA and the emission spectrum of tryptophan overlap, the interaction of lipid and protein can be analyzed with our synthetic probes (Kimelman et al., 1979; Hudson & Cavalier, 1988). Using fluorescent analogs of different GSLs, we were able to determine if GSLs interacted specifically with membrane proteins. Quenching of tryptophan fluorescence of A431 cell plasma membrane vesicles by *cis*- and *trans*-PnA, *trans*-PnG_{M1}, *trans*-PnG_{M3}, and *trans*-Pn de-*N*-acetyl G_{M3} at 25 °C is compared in Figure 4. Similar experiments were performed with the sphingolipid probes in buffers containing divalent cations; the data obtained were not distinguishable from those depicted in Figure 4 (data not shown). Among these probes, *trans*-PnG_{M3} showed the greatest quenching of tryptophan fluorescence. This indicates that the quenching of tryptophan fluorescence by *trans*-PnG_{M3} is probably not due to nonspecific interactions but may result from a close contact of that G_{M3} analog with one or more membrane proteins. In addition, the quenching (and hence

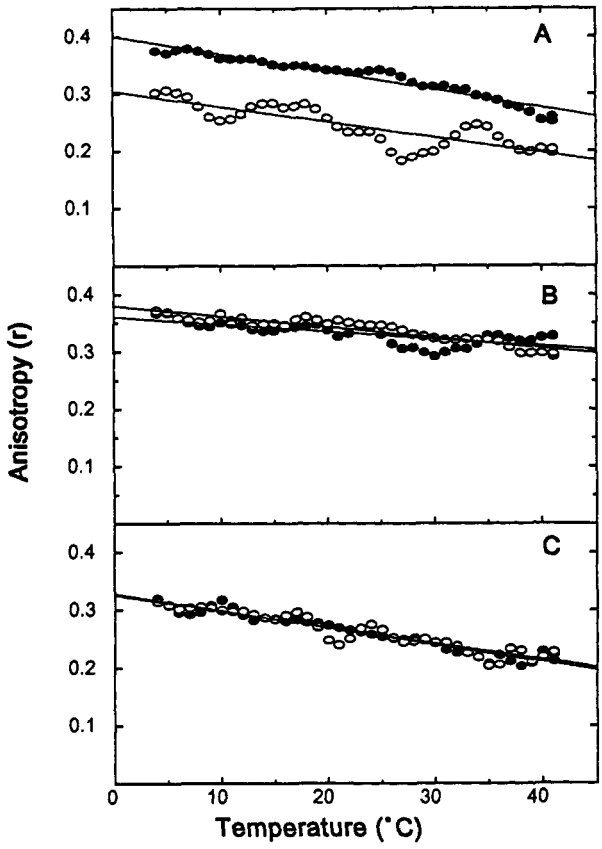

FIGURE 5: Steady-state fluorescence anisotropy of parinaroyl probes in 3T3 cell plasma membrane vesicles. 3T3 cell plasma membrane vesicles, prepared as described in Experimental Procedures and containing 60 μg of protein and 120 nmol of phospholipid, were resuspended in 3 mL of HEPES (20 mM, pH 7.2). Fluorescent probe (1 nmol) was incorporated into the membrane by a freeze–thaw cycle. The samples were cooled from 45 to 2 °C. Steady-state fluorescence anisotropy was determined and calculated as described in Figure 2. Panel A shows the fluorescence anisotropy of *trans*-PnAG$_{M3}$ in cell membranes from parental NR6 cells (○) and transfected EGFR-NR6 cells (●). Panel B shows the fluorescence anisotropy of *trans*-PnA in similar membrane preparations. Panel C shows the fluorescence anisotropy of *trans*-Pn de-*N*-acetyl G$_{M3}$ in similar membrane preparations.

the lipid–protein interaction) requires the intact G$_{M3}$ head group, since the de-*N*-acetyl G$_{M3}$ probe does not exhibit increased quenching of tryptophan fluorescence. This correlates with the restriction of the rotational motion observed for *trans*-PnG$_{M3}$, but not *trans*-Pn de-*N*-acetyl G$_{M3}$, in A431 cell plasma membrane (Figure 3). The addition of EGF (33 μM) had no effect on the anisotropy or quenching values under these conditions (data not shown). These data suggest that there is a direct and specific interaction of *trans*-PnG$_{M3}$ with a protein or proteins in A431 plasma membrane. The identity of the protein(s) remains to be determined.

Steady-State Fluorescence Anisotropy of Parinaroyl Probes in 3T3 Membranes. Figure 5 shows the steady-state fluorescence anisotropy of *trans*-PnA, *trans*-Pn G$_{M3}$, and *trans*-Pn de-*N*-acetyl G$_{M3}$ in crude membranes from two 3T3 cell lines, one of which (NR6) lacks an endogenous EGF receptor and one of which (EGFR-NR6) is transfected with the gene for the human EGF receptor (Velu et al., 1989). The latter cell line expresses the human EGF receptor gene and contains approximately 400 000 cell surface EGF receptors per cell (Velu et al., 1989). Lipid analyses and SDS-PAGE analyses of membrane proteins from these 3T3 clones did not reveal any reproducible differences between the parental and transfected strains, with the exception of the presence of the 170 000-Da EGF receptor in the transfected line (data not shown). The increased anisotropy value seen for *trans*-PnAG$_{M3}$ in the EGFR-NR6 membranes, when compared to the anisotropy observed in the membranes from the untransfected cells (panel A), indicates that this lipid is significantly more motionally restricted in the transfected cell membranes. This restriction is specific for the ganglioside probe, since the anisotropy values for *trans*-PnA (panel B) and *trans*-Pn de-*N*-acetyl G$_{M3}$ (panel C) are similar in the two membrane preparations. The addition of EGF had no effect on these anisotropy values (data not shown). These data demonstrate that the restriction of *trans*-PnG$_{M3}$ motion observed in the A431 cell plasma membranes (Figure 3) is not an inherent property of this ganglioside and are consistent with the notion that *trans*-PnG$_{M3}$ is interacting specifically and avidly with the EGF receptor protein in a way that restricts the ganglioside rotational motion.

DISCUSSION

Fluorescence spectroscopy has been a very powerful tool for studying the lipid organization of membranes. Lipids containing PnA are among the best probes for membrane studies. These molecules closely mimic natural lipids (Sklar, 1976) and are very sensitive to thermotropic phase transitions and phase separations. The free fatty acid probes partition differentially into fluid and solid lipid domains, with *trans*-PnA preferentially partitioning into solid phase and *cis*-PnA partitioning equally into solid and fluid phases (Rintoul et al., 1982; Sklar et al., 1979). Additionally, their absorbance spectra overlap with the emission spectrum of tryptophan, making them very useful for studies of lipid–protein interactions (Kimelman et al., 1979; Hudson & Cavalier, 1988).

Modifying a natural lipid to produce a fluorescent analog often changes its physical and biological properties, and thus many probes do not correctly reflect the molecular behavior of native lipids in biomembranes. We have demonstrated, however, that our synthetic *trans*-PnGM3 retained the inhibitory effect of native G$_{M3}$ on EGF receptor autophosphorylation. Similarly, neither *trans*-Pn de-*N*-acetyl G$_{M3}$ nor native de-*N*-acetyl G$_{M3}$ had any effect on the kinase activity of EGF receptor.

The value of 10^{-7} M for the CMC of G$_{M3}$, as estimated by measurements of PnA emission, is higher than that reported by Sonnino et al. (1990). These workers, using static and dynamic light-scattering measurements, measured the CMC of G$_{M3}$ to be 1.25×10^{-8} M. Our fluorescence method may not be very sensitive, since a high molar ratio of PnA in lipid aggregates could cause self-quenching of PnA; this could happen at the time when lipids just start to aggregate. On the other hand, the bovine adrenal G$_{M3}$ used in the present study contained both *N*-acetyl and *N*-glycolyl G$_{M3}$ and was relatively unsaturated (29.1% unsaturated fatty acids; Table I). The bovine brain G$_{M3}$, used by Sonnino et al., contained only *N*-acetyl G$_{M3}$ and contained only 6.8% unsaturated fatty acids (Sonnino et al., 1990). These compositional differences may account for the different CMCs obtained. In any case, the de-*N*-acetyl G$_{M3}$ used in our experiments was prepared from G$_{M3}$; thus the G$_{M3}$ and de-*N*-acetyl G$_{M3}$ used in these studies should have the same fatty acid composition. Thus, our results clearly establish that the higher CMC of de-*N*-acetyl G$_{M3}$, compared to the acetylated molecule, is due to the difference in the head group structure.

In addition to the structural differences between G$_{M3}$ and de-*N*-acetyl G$_{M3}$, there are also functional differences. G$_{M3}$ inhibits both EGF receptor kinase activity and EGF-stimulated

Table I. Fatty Acid Composition of Bovine Adrenal G_{M3}[a]

fatty acid	mol %	fatty acid	mol %
16:0	19.9	22:1	17.5
16:1	1	24:0	5.2
18:0	15.4	24:1	10.5
18:1	0.1	other	1.9
22:0	28.5		

[a] Each fraction included both non-hydroxyl and hydroxyl fatty acids. Gangliosides were prepared from bovine adrenal medullae as described in Experimental Procedures. Fatty acid methyl esters were prepared and analyzed by GLC as described; the numbers represent mole percentages of the individual fatty acids.

cell proliferation, but de-*N*-acetyl G_{M3} affects neither, as described previously (Song et al., 1991). *trans*-PnG_{M3}, which is both fluorescent and biologically active, should be very useful in studies designed to investigate the relationship of structure and function of this GSL. *trans*-PnG_{M3} showed a substantially higher anisotropy in A431 cell plasma membranes (Figure 3) and in transfected 3T3 cell membranes (Figure 5) at physiological temperatures, compared to *trans*-Pn de-*N*-acetyl G_{M3}, *trans*-Pn sulfatide, and *trans*-PnA. This indicates that the *trans*-PnG_{M3} is more ordered in these biomembranes, while the other probes were more disordered. Additionally, we observed that tryptophan fluorescence of A431 plasma membrane vesicles was quenched to a greater degree by *trans*-PnG_{M3} than by other products (Figure 4). This indicates that *trans*-PnG_{M3} had closer contact with tryptophan-containing membrane proteins when compared to other *trans*-PnA-containing probes. All of these observations strongly suggest that one or more membrane proteins interact with *trans*-PnG_{M3}, specifically restricting the rotational motion of *trans*-PnG_{M3} in a fluid membrane. The EGF receptor is an obvious candidate for interaction with this GSL; our data, particularly those shown in Figure 5, are consistent with this hypothesis.

The different physical and functional attributes of *trans*-PnG_{M3}, compared to *trans*-Pn de-*N*-acetyl G_{M3}, suggest that head group structure is an important determinant of physical properties as well as biological functions of G_{M3}. It should be mentioned that our data do not rule out an interaction of the de-*N*-acetyl ganglioside with one or more membrane proteins, as proposed by Hanai et al. (1988a). It is possible that this glycosphingolipid interacts with membrane proteins, perhaps even the EGF receptor, but that this interaction either is of lower affinity or does not result in the motional changes that occur in the case of G_{M3}. The observed interaction between G_{M3} and proteins is clearly determined by the carbohydrate head group structure; further experiments will have to be performed in order to evaluate the influence of the acyl chain structure on these interactions.

More quantitative analyses of the interaction between G_{M3} and the EGF receptor will require a purified and reconstituted preparation of the receptor. Theoretically fluorescence quenching data, using various concentrations of EGF receptor and fluorescent gangliosides, should allow us to determine stoichiometric and affinity parameters for this interaction. Such quantitative analyses are not possible in the present system, due to the heterogeneity of the membrane proteins and lipids in the preparations used. However, the qualitative results indicate that these fluorescent analogs will be quite valuable in future experiments using purified and reconstituted EGF receptor preparations.

In summary, carbohydrates head group structure can affect the physical properties and biological function of ganglioside G_{M3}. De-*N*-acetylation of the sialic acid residue of G_{M3} raised the CMC of the molecule. *trans*-PnG_{M3}, but not other fluorescent probes, was found to directly interact with proteins in A431 cell plasma membrane and in membranes from mouse fibroblasts expressing the human EGF receptor. Fluorescent analogs of gangliosides are not only physically sensitive but are also biologically active and have great potential for elucidation of the molecular bases of biological functions of gangliosides.

ACKNOWLEDGMENT

The authors thank Drs. Emin Ulug and Jim Guikema for helpful discussions and criticism of the manuscript.

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