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MODIFIED GANGLIOSIDE AS A POSSIBLE MODULATOR OF TRANSMEMBRANE SIGNALING MECHANISM THROUGH GROWTH FACTOR RECEPTORS: A PRELIMINARY NOTE*

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SUMMARY: Two types of modified GM_3 strongly alter EGF-dependent phosphorylation of the EGF receptor in opposite directions, i.e., de-N-acetyl-GM_3 (amino-GM_3; $NeuNH_2\alpha2\rightarrow3Gal\beta1\rightarrow4Glc\beta1\rightarrow1Ceramide$) strongly promotes tyrosine phosphorylation of the EGF receptor of A431 cells, while lyso-GM_3 ($NeuNAc\alpha2\rightarrow3Gal\beta1\rightarrow4Glc\beta1\rightarrow1Sphingosine$) as well as GM_3 inhibit tyrosine phosphorylation of the EGF receptor in the same cells under the same conditions. A hypothesis is proposed that de-N-acylation of gangliosides, in either the sialic acid or ceramide moiety, is a crucial event in triggering a positive or negative transmembrane signal.

Cell surface glycolipids (or gangliosides) may have two basic cellular functions, i.e., (i) to mediate cell social functions, either cell-cell, cell-microbial, or cell-molecule interactions; and (ii) to modulate membrane-associated cellular functions through interaction with functional membrane proteins, such as receptors and transporters (1). As evidence for the second function, modulation of membrane proteins, gangliosides have been reported to affect internalization of FGF (2) and tyrosine phosphorylation of PDGF receptor (3) and EGF receptor (4).

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Abbreviations: EGF, epidermal growth factor; EGTA, ethylene glycol-bis-(β-aminoethyl) N,N,N',N'-tetraacetic acid; HEPES, (N-3-hydroxyethylpiperazine-N'-2-ethane sulfonic acid); PBS, 0.14M NaCl 2mM phosphate buffer. Gangliosides are assigned as defined by Svennerholm (19). Lyso-glycolipids are defined as in Ref. 10,12.

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Exogenous addition of GM_3 or GM_1 but not other gangliosides or neutral glycolipids influences FGF-, EGF-, and/or PDGF-dependent cell growth stimulation (2-4). More recently, data have been presented that indicate that polysialogangliosides inhibit C-kinase activity (5) and modulate phosphorylation of myelin membrane proteins (6). Interestingly, sphingosine and various types of lysoglycosphingolipids were shown to have a non-specific, common inhibitory effect on C-kinase activity by Bell and associates (7,8). As an extension of our studies on the effect of GM_3 on EGF receptor kinase activity (4), we studied, in the presence of various concentrations of detergent, the effect of modified GM_3, which produced quite different results on receptor kinase activity than native GM_3.

MATERIALS & METHODS

Preparation of GM_3 and its Derivatives: GM_3 was prepared from dog erythrocytes and extensively purified by DEAE-Sephadex followed by HPLC on an Iatrobeads column (6RS 8010) (Iatron Laboratories, Toyko, Japan). Lyso-GM_3 (de-fatty acylated GM_3) was prepared by treatment of GM_3 with 1 N KOH in n-butanol (9-11), followed by isolation of de-N-acetyl and de-N-fatty acyl GM_3 (de-N-acetyl lyso-GM_3; CII compound, Fig. 1), and N-acetylation of sialic acid by carbodiimide and acetic acid while protecting the amino group of sphingosine by hydrophobic interaction with phosphatidylcholine in the form of a liposome. The yield of lyso-GM_3 by this method is greater than that of the previously published method (12) (Nores, G., Hanai, N., and Hakomori, S., unpublished data). A part of the CII compound preparation used in these experiments was kindly donated by Professor T. Taketomi, Shinshu University, Japan. De-N-acetyl-GM_3 (CI compound, Fig. 1) was found as one of the major early degradation products of treat-

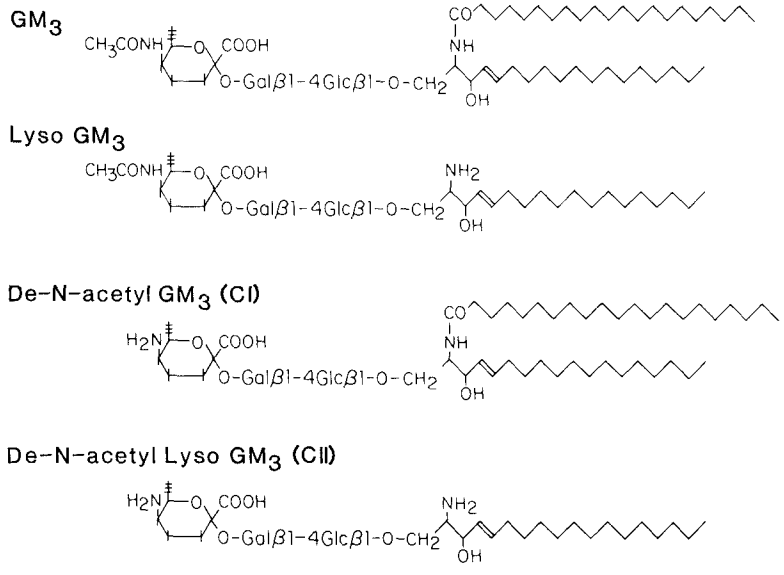

Figure 1. Structures of GM_3 and its derivatives used in this study.

ment of GM_3 with 0.1 N KOH in n-butanol. These compounds were characterized by NMR and mass spectrometry. Preparation and characterization of these compounds will be described elsewhere (Nores, G., Hanai, N., Eaton, H., Levery, S., and Hakomori, S., manuscript in preparation).

Cells and Receptor Kinase Assay: Human ovarial epidermoid carcinoma A431 cells (13) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. In order to maintain reproducibility, the plasma membrane fraction of A431 cells was prepared, aliquotted, and stored in liquid nitrogen until use. Confluent cell culture in 150 mm plastic dishes was scraped, pelleted in PBS (800 x g), and resuspended in 7 ml of 20 mM HEPES buffer (pH 7.4), 1 mM EGTA, 0.5 mM phenylmethylsulfonylfluoride in 250 mM sucrose, and homogenized in Dounce homogenizer with a tight-fitting pestle (Wheaton Scientific, Millerville, NJ) and centrifuged (3000 x g, 10 min). The supernatant fraction was centrifuged at 100,000 x g 1 hour, and the pellet was resuspended in 300 μl of 20 mM HEPES buffer pH 7.4, aliquotted, and frozen. Phosphorylation of membrane proteins, particularly EGF receptors, was modified from the method previously described (4). The concentration of ATP added to the membrane assay system in the previous study was 10 nM, which was employed by Pike et al. to assay the PDGF receptor (14). Since the physiological concentration of intracellular ATP is greater than 1.0 μM, the assay in the present study was performed with this concentration. In the previous paper, 0.2% Triton X-100 (oxidant- and nitrilo-free, Pierce Chemical Co., Rockford, IL) was used in the membrane assay system. However, it was found that the detergent concentration greatly affects the receptor kinase activity (Torres-Mendez, C.-R., Cooper, J., and Hakomori, S., unpublished observation) (15), and therefore, we studied the effect of GM_3 and its derivatives at various concentrations of Triton X-100. The assay system is detailed in the legend of Fig. 2. Aliquots of the incubation mixture were subjected to SDS-polyacrylamide gel electrophoresis, and the radioactivity associated with the EGF receptor (Mr 170 KD) was excised and counted as previously described (4).

Demonstration of GM_3 Derivatives in A431 Cells: A431 cells were extracted with chloroform-methanol (2:1, v/v) followed by three partitions with water according to Folch (16). The Folch upper phase was freed from salt by treatment with C_{18}-silica gel column (17), followed by passage through a column of DEAE-Sephadex (A25) equilibrated with chloroform-methanol-water (30:60:8, v/v/v) and washed extensively with the same solvent. The adsorbed fraction was eluted with chloroform-methanol-0.08 M ammonium acetate (30:60:8, v/v/v), and the eluate was desalted with C_{18}-silica gel column and evaporated to dryness in a rotary evaporator followed by evaporation under nitrogen stream. The dried material was applied on a silica HPLC column packed with Iatrobeads 6RS-8010, which was equilibrated with n-propanol-15% aqueous ammonium hydroxide (92:8, v/v), and eluted with a gradient from the same solvent to n-propanol-15% aqueous ammonium hydroxide (75:25, v/v) during 200 min in Varian 500 HPLC equipment. Eluates were collected over a fraction collector (2 ml/fraction), and each fraction was analyzed on HPTLC. Under these conditions, de-N-acetyl-GM_3 and lyso-GM_3 were clearly separated without overlap. Standard preparations of GM_3, de-N-acetyl-GM_3, and lyso-GM_3 were run under identical conditions, and the elution pattern was highly reproducible. The fractions containing de-N-acetyl-GM_3 and lyso-GM_3 were separately pooled and further analyzed by fluorescamine, ninhydrin, and orcinol reaction. Details of the analytical procedures will be described elsewhere.

RESULTS

The Effect of Triton X-100 on EGF-Dependent Receptor Kinase Activity

Although in our previous study the receptor kinase activity was determined with a constant concentration of 0.2% Triton X-100, we found later that the concentration of the detergent is of crucial importance. The EGF-dependent EGF

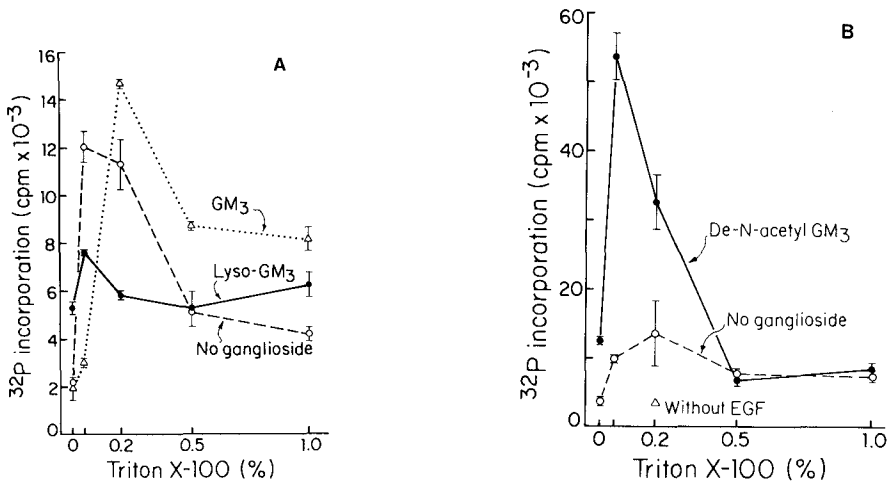

Figure 2. Effects of Triton X-100 on in vitro phosphorylation of EGF receptor (170 KD) in A431 cell membranes. Panel A: Phosphorylation in the presence/or absence of GM_3/or lyso GM_3. Panel B: Phosphorylation in the presence or absence of de-N-acetyl GM_3. A431 cell membranes were incubated in the buffer (20 mM HEPES, pH 7.4, 1 mM $MnCl_2$, 10 μM $ZnCl_2$, 30 μM $NaVO_3$) including 0.33 μM EGF (receptor grade; Collaborative Research, Waltham, MA) plus 1.5 μM carrier BSA and various concentrations of Triton X-100 in the presence of 500 μM GM_3 or 500 μM lyso GM_3, or absence of gangliosides for 10 min at 25°C in Panel A; the same membrane fraction was incubated in the same buffer in the presence or absence of de-N-acetyl GM_3 in Panel B. The reaction was started by the addition of 1.0 μM [r-^{32}P] ATP (10 μCi) for 10 min at 0°C. The total reaction volume was 50 μl, and the amount of membrane protein was 25 μg. The reactions were terminated by addition of 50 μl of Laemmli's sample buffer (20). Aliquots of the incubation mixture were subjected to SDS-polyacrylamide gel electrophoresis. The gel was washed with 1 M NaOH for 15 min at 25°C and treated with 1 M NaOH for 1 hr at 40°C (18) and dried, followed by visualization by autoradiography. The region containing the EGF receptor (170 KD) was excised from the gel and the ^{32}P activity was determined by a liquid scintillation counter.

receptor kinase activity was very low in the absence of Triton X-100, increased greatly with addition of Triton X-100 up to 0.05%, and decreased greatly at concentrations higher than 0.2% (see Fig. 2A and 2B; open circle, dotted line). Inhibition of EGF receptor kinase activity by Triton X-100 detergent alone has been reported (18). This basic activity was compared with addition of various types of gangliosides.

The Effect of Modified and Native GM_3 on EGF Receptor Kinase Activity

Based on the above background values of EGF-dependent kinase activity at various concentrations of detergent, the effects of GM_3 and its two derivatives (lyso-GM_3 and de-N-acetyl-GM_3) on EGF-dependent kinase activity were compared. The inhibitory effect of GM_3 was observed only at relatively low concentrations of Triton X-100, whereas lyso-GM_3 showed continuous inhibition at

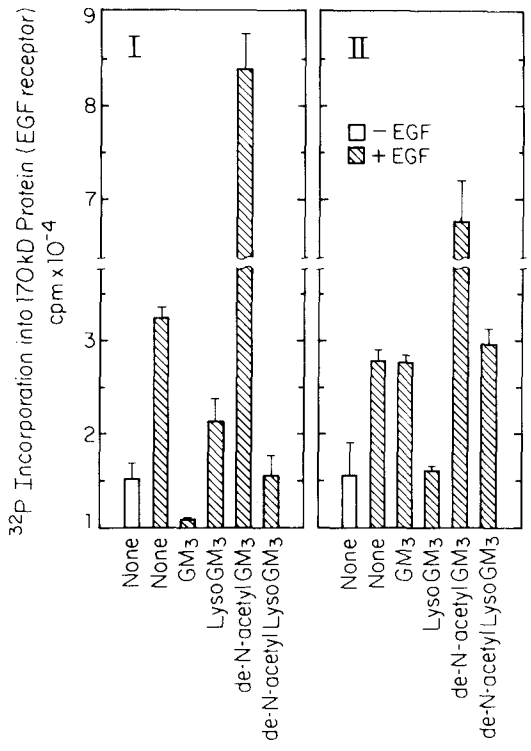

Figure 3. Modulatory effects of various GM_3 derivatives on in vitro phosphorylation of EGF receptor (170 KD) in A431 cell membranes. In vitro phosphorylation assay was performed in the presence of GM_3, lyso-GM_3, de-N-acetyl GM_3, or de-N-acetyl lyso-GM_3 (500 μM). The assay method is detailed in the legend of Fig. 2. Triton X-100 concentration was 0.05% (experiment I) or 0.2% (experiment II).

concentrations up to 0.2% of Triton X-100, i.e., lyso-GM_3 showed a wider range of inhibitory activity than GM_3 (Fig. 2A). It should be noted, however, that lyso-GM_3 itself has strong detergent activity and shows enhanced kinase activity in the absence of Triton X-100, as shown in Fig. 2A (solid circle, solid line). Interestingly, a very strong enhancement of activity was demonstrated with addition of de-N-acetyl-GM_3 (neuraminyl GM_3) over a wide range of detergent concentrations (Fig. 2B, solid circle, solid line). Thus, this derivative is opposite to GM_3 and lyso-GM_3 in its modulation of the receptor kinase under physiological ATP concentration and under low Triton X-100 detergent concentration. Modulation of EGF-dependent receptor kinase activity by various GM_3 derivatives was compared, as shown in Fig. 3. Interestingly, lyso-GM_3 and GM_3 showed a similar inhibitory activity, while de-N-acetyl GM_3 showed strikingly opposite reactivity.

Demonstration of De-N-Acetyl-GM_3 and Lyso-GM_3 in A431 Cells

The fraction eluted from DEAE-Sephadex column and further separated by

HPLC on Iatrobeads 6RS-8010 column showed two components, one with the same mobility as de-N-acetyl-GM_3 and the other with the same mobility as lyso-GM_3. The compounds were detected by HPTLC with fluorescamine and ninhydrin reaction. Further details of the chemical characterization of both de-N-acetyl-GM_3 and lyso-GM_3 will be described elsewhere.

DISCUSSION

Previously, GM_3 but not GM_1 or other gangliosides exogenously added to culture medium was shown to inhibit human ovarial epidermoid carcinoma A431 and oral epidermoid carcinoma KB cells, whereby EGF-dependent receptor kinase activity was strongly inhibited. The inhibited kinase activity was solely directed to tyrosine phosphate (4). In those studies, however, the concentration of ATP used was 10 nM, which is much lower than the physiological concentration of ATP, and GM_3 clearly inhibited the receptor kinase activity over a wide range of concentrations in the presence of 0.2% Triton X-100 (4). Therefore, the physiological significance of GM_3 inhibition of the receptor kinase activity was ambiguous. As shown in this study, when ATP concentration was adjusted closer to physiological, i.e., 1.0 μM, the inhibitory activity of GM_3 was only found at very low concentrations of Triton X-100. While searching for conditions to reproduce the effect of GM_3 at physiological ATP concentration and low Triton X-100 concentration, we found that two GM_3 derivatives showed pronounced effects on EGF receptor kinase activity. A strong inhibition of the activity was demonstrated with GM_3 at a low detergent concentration, while the inhibition with lyso-GM_3, in which the amino group of sphingosine is free, occurred at a wider range of detergent concentrations.

In contrast to GM_3 and lyso-GM_3, a strong enhancement of the receptor kinase activity was observed with de-N-acetyl-GM_3, in which the amino group of sialic acid is free. The enhancing activity with de-N-acetyl GM_3 was extremely strong over a wide range of concentrations of Triton X-100 with physiological concentration of ATP. The ganglioside fraction prepared from A431 cells and from rat brain showed the presence of a similar compound with the same HPTLC

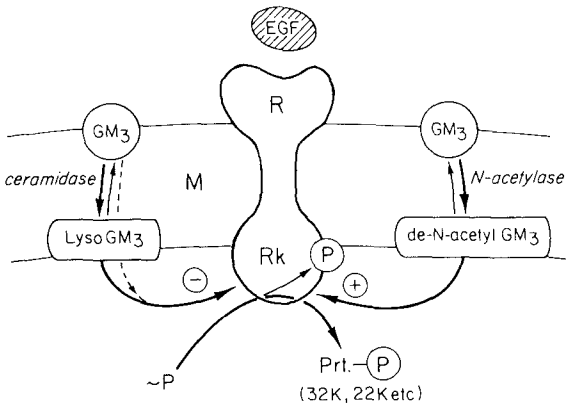

Figure 4. De-N-acetyl GM_3 and lyso-GM_3 as positive and negative modulators of transmembrane signaling mechanism through EGF receptor kinase. Transmembrane signal through EGF receptor (designated R) can be modulated by modified GM_3 on EGF stimulation. Since EGF-dependent kinase activity was greatly stimulated by de-N-acetyl GM_3 but was inhibited by both GM_3 and lyso-GM_3 under physiological conditions, a possible mechanism modulating the EGF dependent-receptor kinase activity through modification by GM_3 and its derivative is illustrated in this scheme. Binding of EGF to its receptor (R) promotes kinase activity (Rk) triggering phosphorylation of various functional proteins (32K, 22K, etc.) as well as the receptor itself. The mechanism leads to internalization of EGF and triggers a cascade mechanism of protein phosphorylation. GM_3, an ubiquitous and fundamental ganglioside component of membranes (M), provides both positive and negative modulatory function. A positive signal is provided by de-N-acetylation of the sialic acid moiety of GM_3 (probably by N-acetylase) and a negative signal is provided by de-N-fatty acylation of the ceramide moiety of GM_3 (probably by ceramidase). The presence of de-N-acetyl GM_3 or lyso-GM_3 is transient, and they may be re-synthesized to GM_3 after their function is accomplished.

mobility and reactivity separated by DEAE-Sephadex chromatography followed by HPLC on Iatrobeads in an n-propanol-ammonia system. Although further extensive chemical characterization of these GM_3 derivatives is needed, the presence of such compounds in the naturally-occurring ganglioside fraction strongly suggests that formation of these modified GM_3 compounds is instrumental in modulating transmembrane signals through receptor kinase activity. A working hypothesis is presented in Fig. 4, in which positive and/or negative signals are provided by removal of an N-acetyl group from the sialic acid moiety, or removal of a fatty acid from the ceramide moiety by their respective specific enzymes. These modified GM_3 compounds, though found in small quantities, are highly effective as promotors or inhibitors of EGF-dependent receptor kinase activity.

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