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Effect of Ganglioside G_{M3} on the Erythrocyte Glucose Transporter (GLUT1): Conformational Changes Measured by Steady-State and Time-Resolved Fluorescence Spectroscopy

Haejung Yoon, Minyung Lee and Gil-Ja Jhon*

Department of Chemistry, College of Natural Sciences, Ewha Womans University, Seoul 120-750, Korea
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Abstract : Interactions between ganglioside G_{M3} and glucose transporter, GLUT1 were studied by measuring the effect of G_{M3} on steady-state and time-resolved fluorescence of purified GLUT1 in synthetic lipids and on the 3-O-methylglucose uptake by human erythrocytes. The intrinsic tryptophan fluorescence showed a GLUT 1 emission maximum of 335 nm, and increased in the presence of G_{M3} by 12% without shifting the emission maximum. The fluorescence lifetimes of intrinsic tryptophan on GLUT1 consisted of a long component of 7.8 ns and a short component of 2.3 ns and G_{M3} increased both lifetime components. Lifetime components were quenched by acrylamide and KI. Acrylamide-induced quenching of long-lifetime components was partly recovered by G_{M3}. However, KI-induced quenching of short- and long-lifetime components was not rescued by G_{M3}. The anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH)-probed dimyristoylphosphatidylcholine (DMPC) model membrane was also increased with G_{M3} incorporation. The transport rate of 3-O-methylglucose increased by 20% with G_{M3} incorporation on the erythrocytes. Therefore, G_{M3} altered the environment of lipid membrane and induced the conformational change of GLUT1.
Key words : erythrocyte glucose transporter, fluorescence spectroscopy, ganglioside

Gangliosides are sialylated glycosphingolipids which are asymmetrically located on the outer face of cell membrane (Stults *et al.*, 1989). They are particularly abundant in neurons, comprising 10% of the total lipids (Ledeen, 1978). Gangliosides are well known to possess biological functions that are associated with cell growth, development, differentiation, and transformation (Hakomori, 1981; Hannun, 1989). Exogeneous gangliosides are accumulated in the lipid bilayer of cell membrane and affect the functions of membrane proteins : alterations of enzyme activities (Yates *et al.*, 1988; Chan, 1989), protein phosphorylation (Rafi *et al.*, 1990; Bassi *et al.*, 1991; Weis & Davis, 1991), a second messenger system (Leon *et al.*, 1982), or membrane permeability (Sati *et al.*, 1990). Chatterjee *et al.* (1993) have reported that G_{M3} mediated the activation of protein kinase and phosphatase through G-protein.

Watanabe *et al.* (1979) have reported that G_{M3} was the major ganglioside in the cytosol of human erythrocytes and the third ganglioside in the membrane ghost. G_{M3} is amphiphilic, forms micelles, and interacts with membranes (Bronner, 1987). We have recently reported that exogeneous G_{M3} has an effect on the post-translational carboxyl methylation of human erythrocyte glucose transporter (GLUT1) (Yoon *et al*, 1992). Even though the potent hemolytic effect of G_{M3} has been reported (Hori- kawa *et al.*, 1991), the biological roles of G_{M3} on the human erythrocyte are still unclear.

Our study shows that the membrane structural fluctuation induced by the addition of G_{M3} may be necessary for proteins to carry out their higher activities. We employed steady-state and time-resolved fluorescence techniques to investigate the alteration of conformation on GLUT1. Our results indicate that G_{M3} induces a change in interactions between membrane lipid and GLUT1, and it causes alteration in GLUT1 conformation and function.

Materials and Methods

Materials

[^{3}H]-3-OMG (3-O-methylglucose) was purchased from Amersham (Buckinghamshire, UK). Phloretin, hemoglobin standard, DPH(1,6-diphenyl-1,3,5-hexatriene), DMPC (dimyristoylphosphatidylcholine), egg PC and G_{M3} (monosialosyl ganglioside from bovine brain) were from Sig-

*To whom correspondence should be addressed.
Tel : 82-2-360-2348, Fax : 82-2-360-2384
E-mail : gjjhon@mm.ewha.ac.kr.

ma (St. Louis, USA). Rabbit polyclonal antiserum against the carboxyl-terminal 13 amino acid sequence of GLUT1 was purchased from East Acres Biologicals (Southbridge, MA). Out-dated blood was provided by the Red Cross and used for GLUT1 purification. Fresh blood was donated from healthy students and used for GLUT1 transport assay.

Purification of glucose transporter and reconstitution of GLUT1 into DMPC liposomes

The transporter was purified as described (Baldwin *et al.*, 1982) with a slight modification. All operations were carried out at 4℃. Erythrocyte membranes were prepared by the method of Steck and Kant (1974) and their peripheral proteins were stripped by treating with 1 mM EDTA (pH 10). The membranes were again treated with 0.1 mM EDTA (pH 12) for further removal of the remaining peripheral proteins. The protein-depleted membranes were suspended in 50 mM Tris-HCl/2 mM dithiothreitol, pH 7.4, and octylglucoside was added to give a final concentration of 46 mM. It was then centrifuged and the supernatant was applied to a column (2× 6 cm) of DEAE-cellulose equilibrated in 50 mM Tris-HCl/2 mM dithiothreitol, pH 7.4, containing 34 mM octyl glucoside. The column was eluted with the same buffer at a flow rate of 70 ml/hr. Protein fractions were pooled and dialyzed against 50 mM Tris-HCl/100 mM NaCl/1 mM EDTA (pH 7.4). The purity and the identity of GLUT1 were determined by SDS-polyacrylamide gel electrophoresis (10%) followed by staining with Coomassie blue or immunoblotting. As expected, the native GLUT1 migrated broadly at molecular weight of 55,000 Da.

The GLUT1-containing proteoliposomes were prepared as described (Yoon *et al.*, 1992). Egg PC or DMPC with G_{M3} (10:1, w/w) were mixed in chloroform and the mixture was first dried under a gentle stream of nitrogen to give a thin film, and then 3 ml of GLUT1 solubilized in octyl glucoside (27 μg/ml) was added. Vesicles formed upon vortexing for 30 s were dialyzed against 50 mM Tris-HCl/100 mM NaCl/1 mM EDTA (pH 7.4) for 2 h to remove octylglucoside. The vesicles were filtered again on a 0.22 μm membrane or purified by a Sepharose 4B column (0.8×25 cm). The sizes of the resulting proteoliposomes were 200 nm for GLUT1-G_{M3}-proteoliposomes and 345 nm for GLUT1 proteoliposomes, measured by a particle sizer (Brook Heaven) accommodating a size distribution analysis program (version 2.1).

Steady-state and anisotropy of intrinsic tryptophan fluorescence

Fluorescence measurements were performed by Hitachi F-3010 spectrofluorometer. The excitation wavelength was 295 nm with the emission bandwidth of 10 nm. Fluorescence anisotropy was measured at the emission maximum (335 nm) using two Glan Taylor polarizers in an L-format configuration. The G factor, $G=I_{HV}/I_{HH}$, was determined for each set of measurements. Fluorescence anisotropy (A) is obtained from the following function:

$$A=(I_{VV}-GI_{VH})/(I_{VV}+2GI_{VH}) \quad (1)$$

When specified, 25 μl of DPH solution (4×10^{-4} M in N,N-dimethylformamide) was added to the aqueous dispersion of liposomes with or without G_{M3}. The sample was excited at 360 nm and the steady-state fluorescence anisotropy was measured at 428 nm at the specified temperature. All measurements were made in a thermostat cell maintained at constant temperature. Anisotropy was obtained using the same function as the above equation (1).

Time-resolved fluorescence measurements

Tryptophan fluorescence decay curves were obtained by the time correlated single photon counting (TCSPC) method. The TCSPC system (Edinburgh Instruments Model FL 900CD) consists of a hydrogen flash lamp as an excitation source, two monochromators (f=25 cm), and photon counting electronics. Fluorescence decay profile, f(t), is obtained as a convolution of the true excited state decay function g(t) with the instrument response function i(t):

$$f(t)=\int_0^t i(t-t')\, g(t')\, dt' \quad (2)$$

The instrument response function, i(t), was obtained by replacing the fluorescence sample with colloidal silica and collecting the scattered excitation light. The true decay curves are extracted from the measured emission decay profile and the instrument response function by a lifetime analysis package that are accessible up to four exponential decay fittings.

The goodness-of-the-fit was evaluated by the examination of the reduced χ^2 and residual plots for the individual data sets. A fit was considered to be appropriate when residuals and autocorrelation functions showed random deviations of about zero and a χ^2 value of lower than 3 (Beechem & Brant, 1985).

Assays of [^{3}H]-3-OMG uptake by human erythrocytes

Blood was drawn freshly by venipuncture into heparinized tubes. Erythrocytes were obtained by three centrifugation/wash cycles in PBS (Steck & Kant, 1974). Erythrocytes were incubated in 10 volumes of PBS for 1

h at 37°C to deplete intracellular glucose levels, then collected by centrifugation and resuspended in PBS (<50% hematocrit). Aliquots (100 μl) of cell suspension containing G_{M3} in a total volume of 0.2 ml were preincubated at 37°C for 30 minutes. After the preincubation the temperature was lowered to 0°C, and 1.5 ml of [^{3}H]-3-OMG (0.1 Ci/ml, total concentration of 5 mM was used) was added to the mixture to initiate its uptake. Uptake was terminated after the indicated time by rapid addition of 5 ml of ice-cold stop solution (150 mM phloretin/10 mM $HgCl_2$ in PBS, pH 6.0). Zero time value was obtained by adding both the initiation and the stop solutions simultaneously to the cells. The mixtures were centrifuged. Cell pellets were washed again and recentrifuged. Sedimented cells were then haemolysed and radioactivities taken up by the cells were counted by a scintillation counter.

Results and Discussion

Steady-state fluorescence measurement of proteoliposomes containing purified GLUT1

GLUT1 is the best characterized protein of those which catalyze the diffusion of glucose across the membrane. Our previous studies have indicated that GLUT1 was a good substrate for protein carboxyl methyltransferase (EC.2.1.1.77) (Ro *et al.*, 1984) and 30 μM G_{M3} increased the carboxylmethylation of GLUT1 (34%) (Yoon *et al.*, 1992). Carboxylmethylation of GLUT1 increased the affinity of protein to cytochalasin B binding sites (Jhon & Hah, 1991). Interactions between GLUT1 and various active participants through the lipid bilayer affect the transport mechanism (Honkanen *et al.*, 1995). GLUT1 undergoes a large decrease in fluorescence intensity upon glucose binding (Lienhard & Gorga, 1982). It has been shown that this change involves movement of interfacial tryptophan residues towards the membranes surface and that increasing the packing of lipid stabilizes this state. The accumulation of gangliosides in erythrocyte membrane has been reported (Nakakuma *et al.*, 1990). G_{M3} is located in cytosol and membrane bilayer of erythrocyte (Watanabe *et al.*, 1979). We investigated whether G_{M3} affects lipid structure and/or protein structure by fluorescence techniques.

The steady-state fluorescence data shown in Fig. 1 for GLUT1 strongly support the notion that G_{M3} alters GLUT1 conformation. When G_{M3} was incorporated into proteoliposomes containing purified GLUT1, the intrinsic tryptophan fluorescence was increased about 10-15% as shown in Fig. 1. Excitation at 270 nm did not affect the overall shape of the fluorescence spectra indicating that the fluorescence arises mostly from tryptophan residues.

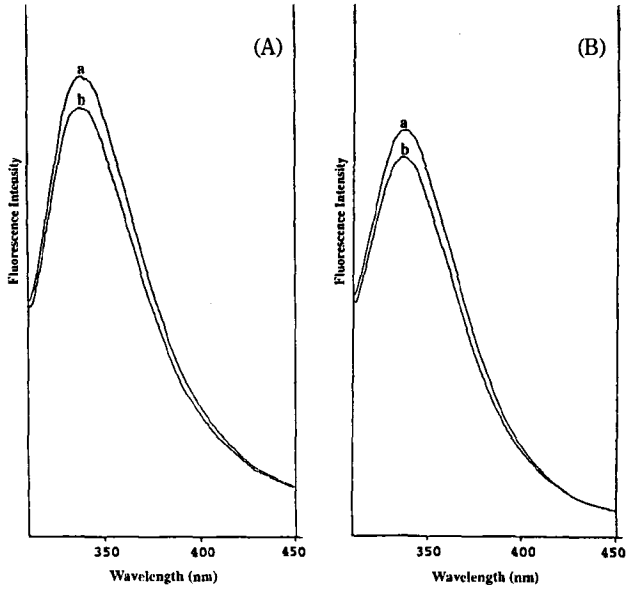

Fig. 1. Steady state fluorescence spectra of GLUT1 proteoliposomes. Purified GLUT1 was reconstituted with G_{M3}- DMPC at 24°C (a) or with DMPC only (b). The excitation wavelengths of 295 nm(A) and 270 nm(B) had been used. Excitation band path was 5 nm.

The lipid phase transition temperature of DMPC vesicles was measured by the anisotropy of the membrane-inserted DPH as a function of temperature ranging from 19 to 33°C. The increases in the anisotropy of the G_{M3}-DMPC model membrane has been observed depending on the concentration of G_{M3} (Fig. 2). The changes in flu-

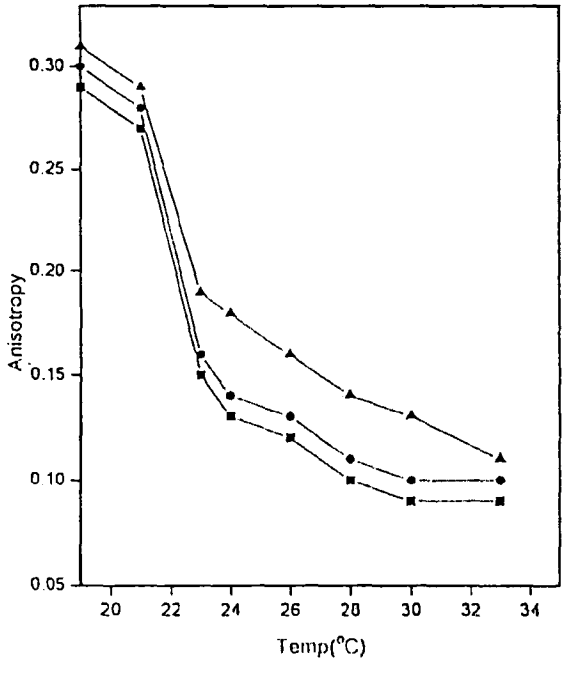

Fig. 2. Fluorescence anisotropy of DPH in DMPC model membrane as a function of temperature. DMPC:G_{M3} are used as 1: 0 (square), 1: 0.03 (circle), and 1: 0.2 (triangle).

orescence by G_{M3} may be due to increased lipid packing caused by G_{M3} incorporation. It is likely that physical interaction between G_{M3} and membrane lipid altered the ordering of membrane lipids and increased the membrane rigidity. Therefore, it appears that G_{M3} changed the structure of membrane lipid, and the interactions between tryptophan residues inside of protein core and membrane lipid subsequently increased.

Time-resolved fluorescence measurements

The fluorescence decays for GLUT1 were measured and analyzed by a reconvolution fit. The Marquardt search algorithm yielded reliable fits. The measured decay curves can be fitted into either two or three lifetime components. The three component fitting gives a better χ^2. However, the third lifetime component was too large (>25ns) and its amplitude was negligible compared to those of two other components (data not shown). Representative tryptophan fluorescence decays of proteoliposomes and G_{M3}-proteoliposomes are shown in Fig. 3. The statistical effectiveness of limiting the nonlinear least-square analysis of a multi-tryptophan protein to multi-exponentials is illustrated by χ^2 values and plotted residuals. The values of χ^2 were in the range of 1.45 to 4.03 for three exponential fittings and the residuals were randomly distributed regarding time as shown in Fig. 3. Previous interpretations are in close agreement with the observed changes in fluorescence decay data summarized in Table 1. We used DMPC-proteoliposomes for lifetime measurement at 19°C and egg PC-proteoliposomes at 35°C measurement. G_{M3} increased lifetimes of tryptophans. This agreed with the general trend shown in the steady-state intensity at 19°C (Fig. 1A). In the case of DMPC-proteoliposomes at 19°C, GM3 increased 28% (7.8 ns to 10 ns) of the long lifetime component and 13% of the short lifetime component (2.3 ns to 2.6 ns). The variation of the two components in the fluorescence decay suggests that six tryptophan residues on GLUT1

Table 1. Lifetime data of GLUT1-proteoliposomes calculated by deconvolution analysis

proteoliposomes	two fittings						
	T(°C)	τ_1(ns)	α	τ_2(ns)	α	<τ>	χ^2
proteoliposomes	19	2.3	0.64	7.8	0.36	4.3	3.63
	35[a]	1.3	0.71	7.0	0.29	3.0	1.55
G_{M3}-proteoliposomes	19	2.6	0.74	10	0.27	4.6	4.03
	35[a]	1.4	0.72	7.8	0.28	3.2	1.45
proteoliposomes wih acrylamide	19	1.9	0.66	6.8	0.34	3.5	3.18
G_{M3}-proteoliposomes with acrylamide	19	2.2	0.74	8.6	0.26	3.9	3.02
proteoliposomes with KI	19	1.7	0.56	5.6	0.44	3.5	1.67
G_{M3}-proteoliposomes with KI	19	1.7	0.56	5.5	0.44	3.4	1.66

[a]Egg PC was used.
[b]200 mM KI and 200 mM acrylamide were used.
[c]Average lifetimes were calculated from the individual lifetime τ_i and corresponding steady-state fraction at 335 nm as $\langle\tau\rangle = \Sigma_i f_i \tau_i$

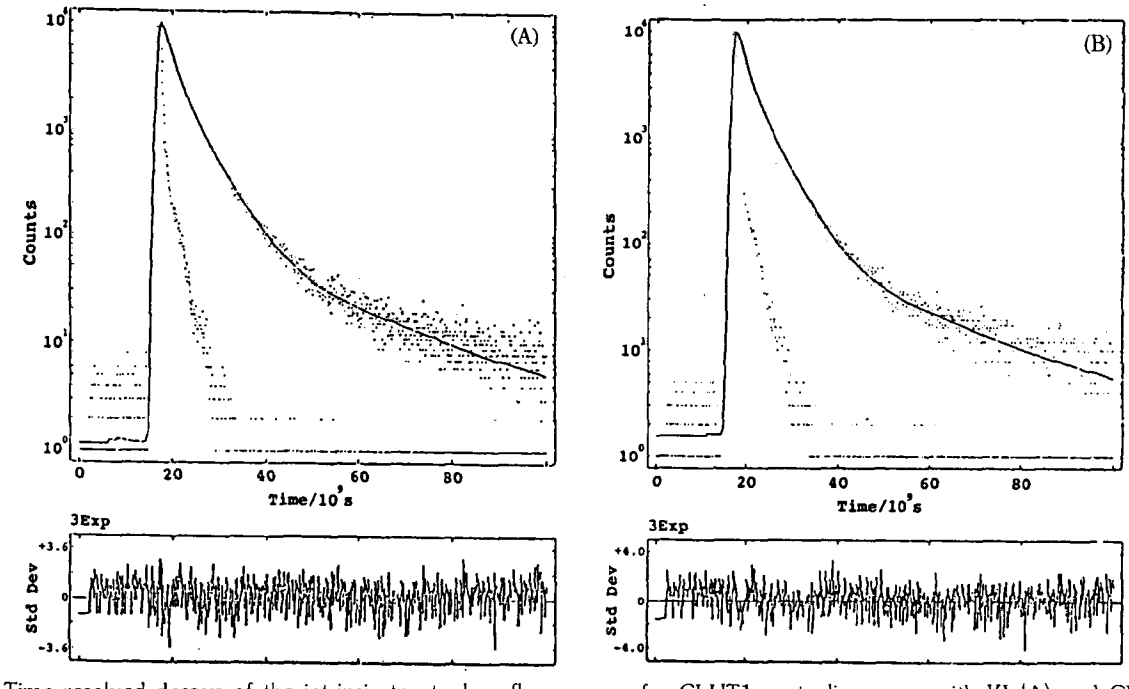

Fig. 3. Time resolved decays of the intrinsic tryptophan fluorescence for GLUT1 proteoliposomes with KI (A) and GLUT1-G_{M3} proteoliposeomes with KI (B) at 19°C. The excitation and emission monochromators were set at 295 and 335 nm, respectively.

are located in at least two different chemical environments. The long-lifetime component is presumably originated from a conformational change due to the interaction between tryptophan residues and their environment, while the short-lifetime component is a result of dynamic quenching by the solvent or protein matrix (Vos & Engelborghs, 1995). The increase of the long-lifetime component of G_{M3}-proteoliposomes probably originated from a relaxed conformation with low solvent accessibility. Demchenko *et al.* (1993) reported that the red shift is caused by relaxation of the protein structure around the excited state in many proteins. However, tryptophan residues in G_{M3}-proteoliposomes are located in a more rigid and nonpolar environment than those in G_{M3}- free proteoliposomes. Therefore, they are not expected to be susceptible to these relaxation processes, displaying a negligible shift (Fig. 1) .

The susceptibility of the fluorescence lifetime decays quenched by acrylamide and KI used for probe of the solvent accessibility of GLUT1 tryptophan residues. Quenching of the tryptophan fluorescence by KI showed that more than 75% of the total GLUT1 tryptophan fluorescence is inaccessible (Pawagi & Deber, 1990). As shown in Table 1, KI, an ionic quencher, quenched tryptophan fluorescence : average lifetime was quenched by 19%, while short-lifetime component and long-lifetime component were quenched by 26% and 28%, respectively. In the case of GLUT1-proteoliposomes, acrylamide quenched 17% of short-lifetime component and 13% long-lifetime component. However, in the case of G_{M3}-proteoliposomes, quenched fluorescence by acrylamide was partially recovered while that by KI was not. The changes of acrylamide partitions into the lipid bilayer decreased due to G_{M3}. The incorporation of G_{M3} into the lipid membrane suggests that G_{M3} may affect the microviscosity of membrane lipid, resulting in conformational change of GLUT1 that is in a more relaxed form, thus preventing collision between the quencher and some of the tryptophan residues, possibly Trp 388 and Trp 412. Trp 388 and Trp 412 are believed to be located near or at the active site of the glucose transporter (Chin *et al.*, 1992).

3-OMG uptake by human erythrocytes

Recent evidence suggests that the glucose transporter, pre-existing in the plasma membrane, can be activated under a number of conditions (Harrison *et al.*, 1991; Shett *et al.*, 1993). The specific mechanism for the presumed activation of glucose transporter in the plasma membrane is not known. However, it has been proposed that increasing the glucose binding affinity of GLUT1 could result in an enhancement of transport rate under appropriate conditions (Harrison *et al.*, 1991). The measurement of [^{3}H]-3-OMG uptake showed that G_{M3} affects the transport activity of GLUT1 (Fig. 4). G_{M3} may alter the GLUT1 conformation so that the opening of the channel is oriented to have external binding site(s) for glucose. G_{M3} is the major ganglioside in whole erythrocyte, even though G_{M3} is the third ganglioside present in the erythrocyte membrane. In conclusion, our data strongly suggest that G_{M3} alters the structure of erythrocyte bilayer, and induces conformational fluctuation of GLUT1 to carry out its higher activity.

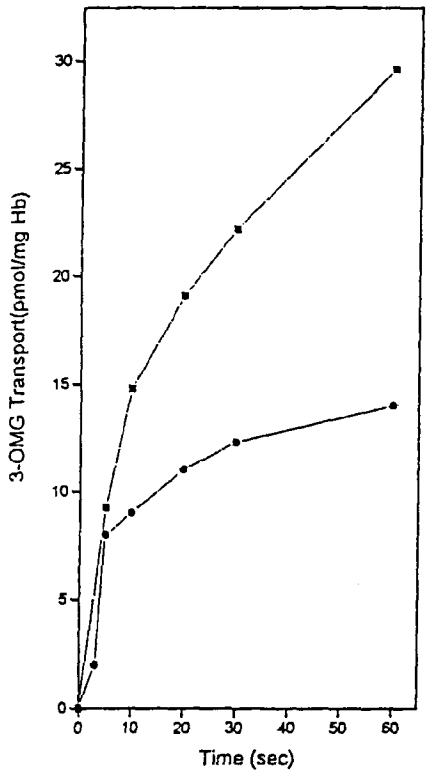

Fig. 4. Time course of unidirectional uptake of 3-OMG on human erythrocytes. The assays were held after preincubation of 30 minutes with ganglioside at 37°C. 3-OMG uptake measured at 0°C in the presence of 30 μM G_{M3} (square) or in the absence of G_{M3} (circle).

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