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pH-dependent Formation of Membranous Cytoplasmic Body-like Structure of Ganglioside G_{M1}/Bis(Monoacylglycero)Phosphate Mixed Membranes

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ABSTRACT:
Membrane structures of the mixtures of ganglioside G_{M1} and endosome specific lipid, bis(monoacylglycero)phosphate (BMP, also known as lysobisphosphatidic acid, LBPA) were examined at various pH conditions by freeze-fracture electron microscopy and small-angle x-ray scattering (SAXS). At pH 8.5 – 6.5, a G_{M1}/BMP (1/1 mol/mol) mixture formed small vesicular aggregates, whereas the mixture formed closely packed lamellar structures under acidic conditions (pH 5.5, 4.6) with the lamellar repeat distance of 8.06 nm. Since BMP alone exhibits a diffuse lamellar structure at a broad range of pH values and G_{M1} forms a micelle, the present results indicate that both G_{M1} and BMP are required to produce the closely stacked multilamellar vesicles. These vesicles resemble membranous cytoplasmic bodies (MCB) in cells derived from patients suffering from G_{M1} gangliosidosis. Similar to G_{M1} gangliosidosis, cholesterol was trapped in BMP vesicles in G_{M1}- and in a low pH-dependent manner. Studies employing different gangliosides and a G_{M1} analog suggest the importance of sugar chains and a sialic acid of G_{M1} in the pH-dependent structural change of G_{M1}/BMP membranes.

A characteristic feature of endosomes along with the degradative endocytic pathway is the accumulation of vesicles within the organelle (1,2). Recently, it has been shown that the unconventional phospholipid bis(monoacylglycero)phosphate (BMP), also known as lysobisphosphatidic acid, LBPA) can induce the formation of multivesicular liposomes that resemble the multivesicular endosomes (3). BMP is a structural isomer of phosphatidylglycerol with characteristic *sn*-1, *sn*-1' glycerophosphate stereoconfiguration (4,5). This lipid is highly enriched in the specific internal membrane domains of multivesicular late endosomes where the lipid comprises more than 70% of the total phospholipids (6,7). It has been reported that late endosomes/lysosomes change their organization from multivesicular to multilamellar membranes under different pathological conditions and by treatment with certain drugs. These multilamellar vesicles, in which membranes are tightly stacked, are called membranous cytoplasmic bodies (MCB). Although the involvement of BMP domains in late endosomes (8) and lipid-protein interaction (9) have been suggested, the mechanism of the formation of MCB is not well understood. Recently we have shown that a drug which induces multilamellar endosomes alters BMP liposomes from swollen and loosely packed lamellar vesicles to closely stacked multilamellar structures at low pH (10).

Sphingolipidosis is a genetic disease defective in the proteins involved in sphingolipid metabolism (11). Accumulation of MCBs is a characteristic feature of this disease. Different sphingolipids are accumulated depending on the defect. These lipids, such as sphingomyelin and galactosylceramide, themselves form multilamellar structures in aqueous solution. In contrast, in G_{M1}-gangliosidosis, micelle-forming lipid G_{M1} is extensively accumulated and still MCBs are formed. Therefore, it is of interest to investigate the conditions in which the accumulation of G_{M1} induces the formation of closely stacked membranes. In the present study, we examined the membrane structure of ganglioside/BMP mixture in neutral and acidic pH conditions, the latter of which resembles the lumen of late endosomes/lysosomes.

First we examined whether the accumulated G_{M1} colocalize with the BMP-rich membrane domains in intact cells. The addition of exogenous ganglioside to cultured cells mimics the behavior of the cells from gangliosidosis (12). In Fig. 4 (see supplementary material), diffuse fluorescence was observed when cultured human skin fibroblasts were fixed, permeabilized and labeled with fluorescently labeled cholera toxin, which recognizes G_{M1}. In contrast, intracellular compartments were brightly labeled with cholera toxin when cells were grown in the presence of 10 μM G_{M1}. The fluorescence was co-localized with that labeled with anti-BMP antibody. The result suggests the presence of BMP and G_{M1} in the same membrane domains. We next

examined the membrane structure of BMP/G_{M1} complex. 2,2'-dioleoyl-*sn*-1,*sn*-1'- BMP is a major molecular species of naturally occurring BMP (7,13). We chemically synthesized 2,2'-dioleoyl-*sn*-1,*sn*-1'-BMP (14) and measured the structure of the membranes in the presence of G_{M1} by using electron microscopy and small angle x-ray scattering (SAXS). Fig. 1 shows freeze-fracture electron micrographs of the G_{M1}/BMP (1/1 mol/mol) mixture at pH 7.4 and 4.6. The particles observed at pH 7.4 were mainly unilamellar vesicles, as demonstrated in cross-fracture images, whereas the results at pH 4.6 indicated structures filled with multiple layers or large multilamellar vesicles. Each layer was closely stacked, and the distance between the adjacent layers were less than 10 nm. The size of vesicles at pH 7.4 was ~100 – ~300 nm diameter in contrast to ~300 nm – ~3 μm diameter at pH 4.6. Similar results were observed by negative staining electron microscopy (data not shown). In Fig. 1, pH dependence of the SAXS patterns of the G_{M1}/BMP (1/1 mol/mol) mixture are also shown. At pH 8.5 – 6.5, the SAXS profiles displayed similar curves, exhibiting an evident minimum at q = ~0.55 nm^{-1} and a broad bell-shaped peak at q = ~1 nm^{-1}. These are characteristics of a scattering curve from an assembly of identical small particles. It is reported that dioleoyl BMP forms a diffuse lamellar structure at a pH range of 3.0 – 8.5 (10,15) while G_{M1} forms a stable micellar structure at a pH range of 3.6 – 8.0 (16). Considering the negatively charged bulky headgroup of G_{M1}, which gives a high curvature when inserted into the membrane, it is expected that the G_{M1}/BMP mixture formed such compact aggregates. At pH 5.5, however, the SAXS pattern exhibited two small peaks at q = 0.78 and 1.56 nm^{-1} in addition to the broad peak at q = ~1 nm^{-1}. These two peaks correspond to the first and second order diffraction peaks from a lamellar structure with a 8.06 nm repeat distance. At pH 4.6, the first and second order peaks became much more evident, indicating that the acidic pH condition transformed the G_{M1}/BMP mixture from small aggregates to a planar lamellar structure. The dose response of G_{M1} indicates that the alteration of the membrane structure was inducible by the addition of as low as 10% of G_{M1} (see Fig. 5 in Supplementary Material) at low pH.

One of the consequences of the storage of sphingolipids in MCBs, including G_{M1}, is the accumulation of cholesterol. It is proposed that the preferential association of sphingolipids and cholesterol causes the accumulation of cholesterol in MCBs (8). In Fig. 2, we investigated whether the G_{M1}/BMP membrane traps cholesterol in a pH-dependent manner. Methyl-β-cyclodextrin (MβCD) extracts cholesterol from the membrane. Extraction of cholesterol from BMP and G_{M1}/BMP membranes by MβCD was investigated at pH 7.4 and 4.6. Cholesterol was equally extracted from the BMP liposomes irrespective of pH. The presence of G_{M1} did not affect the extraction at pH

7.4. In contrast, the extraction of cholesterol was significantly reduced in the presence of G_{M1} at pH 4.6. The addition of 10 mol% cholesterol did not alter the gross structure of the G_{M1}/BMP membranes (data not shown). This result suggests that the formation of the closely packed multilamellar structure of G_{M1}/BMP in an acidic environment prevents the cholesterol extraction by MβCD.

Fig. 3 shows the examination of the effects of various gangliosides on the membrane structure of BMP at pH 4.6 (Fig. 3). Similar to G_{M1}/BMP, lamellar diffraction peaks were observed in G_{M2}/BMP membrane. However, the G_{M3}/BMP and G_{D3}/BMP mixtures did not exhibit clear lamellar peaks, suggesting that branched carbohydrate chain is required for the tight packing of the ganglioside/BMP membrane at low pH. The lamellar structure was observed both at pH4.6 and 7.4 when the sialic acid moiety of G_{M1} was substituted for the corresponding sugar alcohol (see Fig. 6 in Supplementary Material), indicating that sialic acid prevents the formation of the lamellar structure of the G_{M1}/BMP membrane at neutral pH.

Although BMP forms a diffuse lamellar structure at broad range of pH values and G_{M1} forms a micelle, the mixture of the two lipids form a closely stacked multilamellar structure at a pH which resembles the lumen of late endosomes/lysosomes. The reported membrane structures of G_{M1}/phospholipid and G_{M1}/cholesterol/Ca^{2+} system suggest that the G_{M1} sugar headgroups of the apposing bilayers are in the distance of direct contact in G_{M1}/BMP membranes at the low pH conditions. Previously Simons and Gruenberg suggested that the accumulation of sphingolipids alters the properties of BMP (LBPA, lysobisphosphatidic acid)-rich membrane domains (8). The present results provide the experimental evidence that the structure of the BMP membrane is indeed altered by G_{M1} and G_{M2} in a pH-dependent manner. This suggests that MCBs in gangliosidosis can be reproduced, at least in part, by gangliosides and BMP in the absence of proteins. The accumulation of cholesterol in MCBs in cells from sphingolipidosis has been believed to be a conquence of the specific interaction of sphingolipids and cholesterol in MCBs (8). The present results suggest that BMP and a low pH are additional players in cholesterol accumulation in MCBs.

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FIGURE CAPTIONS:

FIGURE 1

(Left), freeze-fracture electron micrographs of G_{M1}/BMP (1/1 mol/mol) mixture at different pH. (Right), SAXS patterns of G_{M1} at pH 4.6 and G_{M1}/BMP mixture at different pH.

FIGURE 2

Cholesterol extraction from BMP/cholesterol and G_{M1}/BMP/cholesterol (10 mol% cholesterol) membranes at different pH.

FIGURE 3

(a), SAXS patterns of G_{M2}, G_{M3} and G_{D3}, and their mixture with BMP (1/1 mol/mol) at pH 4.6. SAXS pattern of G_{M1}/BMP (1/1 mol/mol) mixture at pH 4.6 is also shown. The lamellar distance of G_{M2}/BMP was 9.98 nm. (b), Structures of gangliosides used in this study.

Figure 1

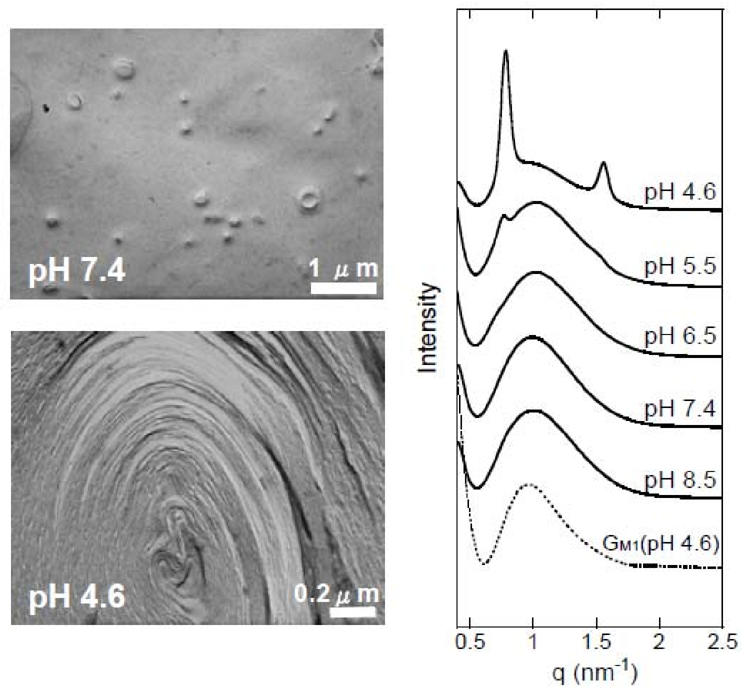

Figure 2

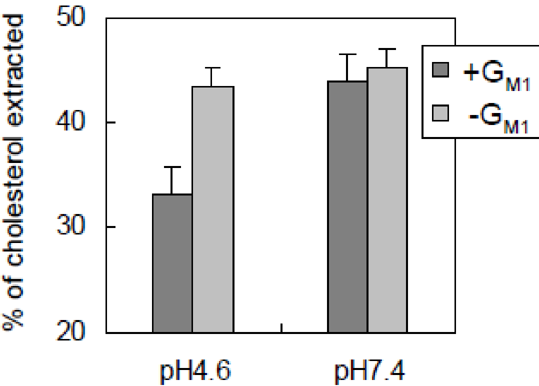

Figure 3

SUPPLEMENTAL MATERIALS

MATERIALS AND METHODS

Materials

Ganglioside G_{M1}, G_{M2} and G_{D3} were purchased from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan), and G_{M3} was from Matreya, Inc. (Pleasant Gap, PA). 2,2'-dioleoyl-*sn*-1,*sn*-1'-BMP was synthesized as described (1). G_{M1}(C1-alcohol) was obtained by the method described previously (2). Cholesterol, [4-^{14}C]-cholesterol and methyl-β-cyclodextrin (MβCD) were obtained from Sigma-Aldrich Co. (St. Louis, MO), American Radiolabeled Chemicals Inc. (St. Louis, MO) and Cyclolab R&D Lab. Ltd. (Budapest, Hungary), respectively.

Immunofluorescence

Cultured human skin fibroblasts were grown as described (3) in the presence or absence of G_{M1}. Cells were then fixed and permeabilized as described (4) and doubly labeled with anti-BMP (anti-LBPA) antibody (5) and Alexa 488-conjugated cholera toxin B-subunit (Molecular Probes, Eugene, OR).

Electron Microscopy

For negative staining images, the samples were absorbed to glow-charged formvar membrane on copper grids, and negatively stained by 2% sodium phosphotungstic acid. Low and neutral pH liposome were stained by different pH solution; one was adjusted to 4.2 and the other to 7.2, respectively. For freeze fracture images, the samples were frozen in liquid propane cooled by liquid nitrogen, fractured in a freeze-etching machine (Balzers BAF400T, Balzers, Liechtenstein) at -110ºC, and replicated by platinum/carbon. Replicated samples were immersed in household bleach to dissolve the lipids, washed in water, and then mounted on formvar-coated copper grids. Both specimens for negative staining and freeze fracture images were examined under a transmission electron microscope (JEOL 1200EX-II, Tokyo, Japan). Electron micrographs recorded on imaging plates were scanned and digitized by an FDL 5000 imaging system (Fuji Photo Film, Tokyo, Japan).

Small-angle x-ray scattering (SAXS)

Lipid film was formed from a chloroform solution of lipids under a stream of nitrogen gas, dried in high vacuum overnight, hydrated and vortexed with buffers. The employed buffers was 200 mM citrate buffer for pH 4.6, 20 mM MES buffer with 150 mM NaCl for pH 5.5 and 6.5, 20 mM HEPES buffer with 150 mM NaCl for 7.4, and 20 mM tricine buffer with 150 mM NaCl for pH 8.5. The lipid concentration of the samples was 10 mM. SAXS measurements were carried out at RIKEN Structural Biology Beamline I (BL45XU) at SPring-8 (6). The SAXS patterns were recorded with 60 s exposure by a beryllium-windowed x-ray image intensifier which is coupled with a cooled CCD camera (1000 × 1018 pixels). Detailed optical settings and data treatments were as described elsewhere (4,7). All the SAXS measurements were performed at 37ºC.

Cholesterol extraction

G_{M1}/BMP (1/1 mol/mol) vesicles containing 10 mol% cholesterol including trace amount of [4-^{14}C]-cholesterol were prepared essentially as describe for x-ray measurements, with the exception that the final concentration of lipids was 40 µM. Dispersions of G_{M1}/BMP/cholesterol at pH 4.6 and 7.4 were incubated with 25 mM MβCD at 37ºC for 30 min and pelleted with one hour centrifugation (4ºC, 53000 × *g*). The extracted cholesterol in the supernatant was measured with liquid scintillation counter. To avoid an artifact due to the difference in precipitation depending on the samples, all samples were pelleted in the absence of MβCD, and the ^{14}C cholesterol value in the supernatant was subtracted from the value obtained in the presence of MβCD.

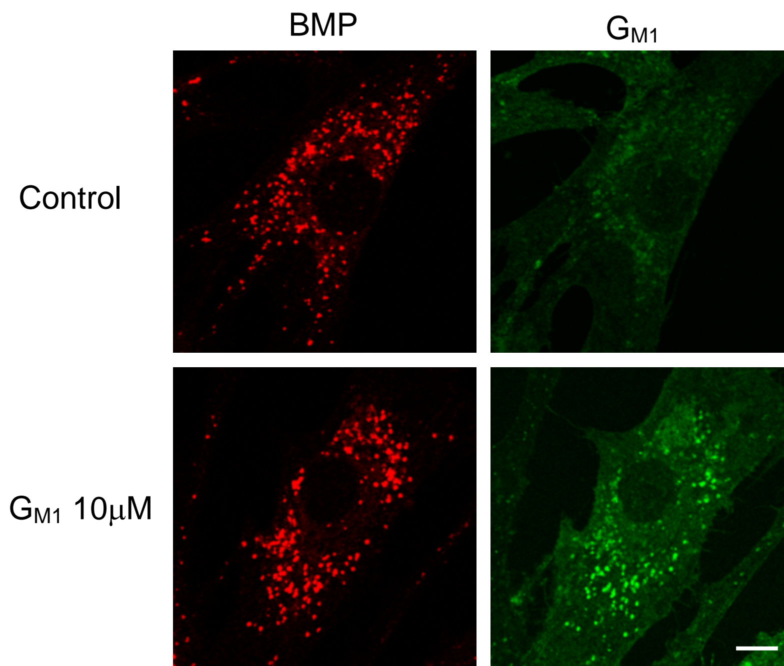

FIGURE 4
G_{M1} accumulates in BMP domains. Human skin fibroblasts were grown in the absence and presence of 10 μM G_{M1} as described in supplemented Materials and Methods. Cells were then fixed and the distribution of internalized G_{M1} and BMP-rich membrane domains was examined. Bar, 10 μm.

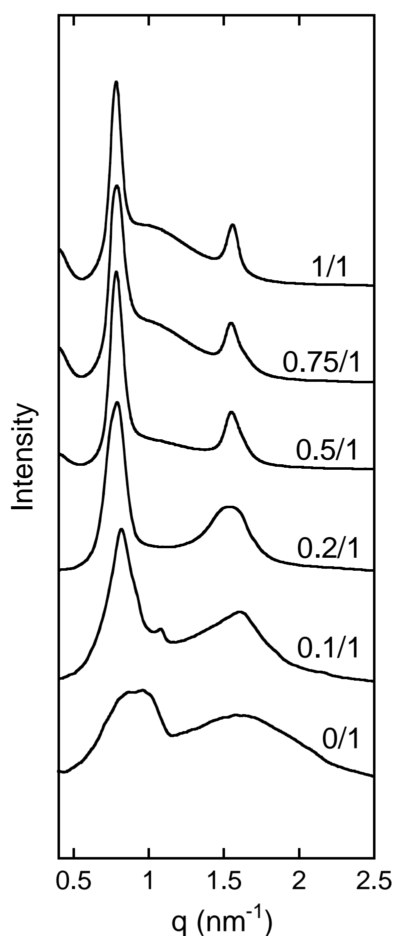

FIGURE 5
SAXS patterns of G_{M1}/BMP mixture with different G_{M1} content at pH 4.6. G_{M1}/BMP molar ratios for the respective SAXS pattern were shown in the figure. BMP alone forms diffuse lamellar structure with the lamellar repeat distance of ~7 nm. At G_{M1}/BMP = 0.1/1 (mol/mol), the first and second order lamellar peaks became more evident and shifted to the small angle region (q = 0.82 nm^{-1}) and another set of first and second lamellar peaks were observed at q = 1.08 nm^{-1} and 2.16 nm^{-1}, corresponding to the 5.82 nm lamellar repeat distance. The appearance of these two lamellar distances suggests that G_{M1} distributed heterogeneously in the membranes and G_{M1}-rich and -poor regions were phase separated. At molar ratio of 0.2/1, the lamellar peaks shifted further to the small angle region. The first lamellar peak was observed at q = 0.78 nm^{-1} which corresponds to 8.06 nm lamellar distance.

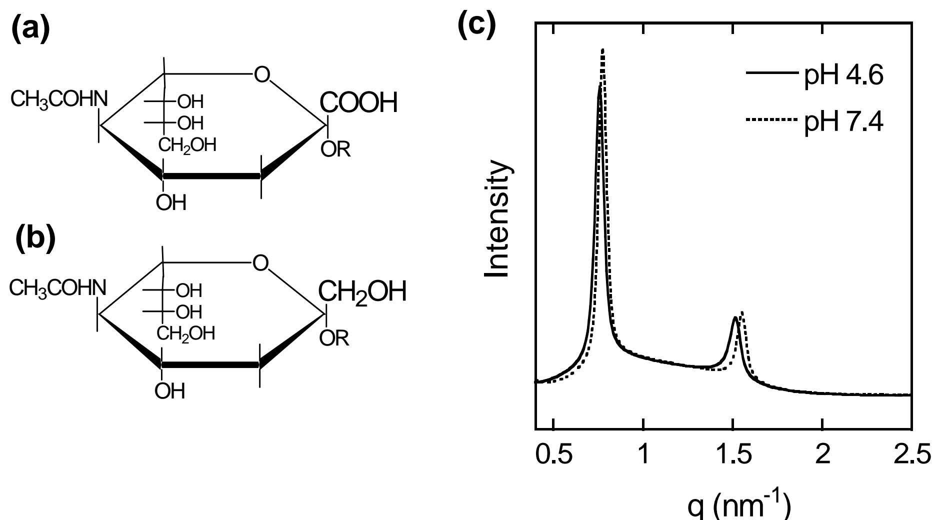

FIGURE 6
Structure of NeuAc (sialic acid) (a) and its derivative (b). For (b), carboxyl group attached to the C1-carbon was converted to the primary alcohol (2). (c), SAXS pattern of G_{M1}(C1-alcohol)/BMP = 1/1 (mol/mol) mixture at pH 4.6 and 7.4. At both pH conditions, diffraction peaks derived from the lamellar structure were observed with the lamellar repeat distance of 8.28 nm for pH 4.6 and 8.10 nm for pH 7.4.

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