

## pH-dependent Formation of Membranous Cytoplasmic Body-Like Structure of Ganglioside G<sub>M1</sub>/Bis(Monoacylglycerol)Phosphate Mixed Membranes

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**ABSTRACT** Membrane structures of the mixtures of ganglioside G<sub>M1</sub> and endosome specific lipid, bis (monoacylglycerol) phosphate (BMP, also known as lysobisphosphatidic acid) were examined at various pH conditions by freeze-fracture electron microscopy and small-angle x-ray scattering. At pH 8.5–6.5, a G<sub>M1</sub>/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<sub>M1</sub> forms a micelle, the results indicate that both G<sub>M1</sub> and BMP are required to produce closely stacked multilamellar vesicles. These vesicles resemble membranous cytoplasmic bodies in cells derived from patients suffering from G<sub>M1</sub> gangliosidosis. Similar to G<sub>M1</sub> gangliosidosis, cholesterol was trapped in BMP vesicles in G<sub>M1</sub>- and in a low pH-dependent manner. Studies employing different gangliosides and a G<sub>M1</sub> analog suggest the importance of sugar chains and a sialic acid of G<sub>M1</sub> in the pH-dependent structural change of G<sub>M1</sub>/BMP membranes.

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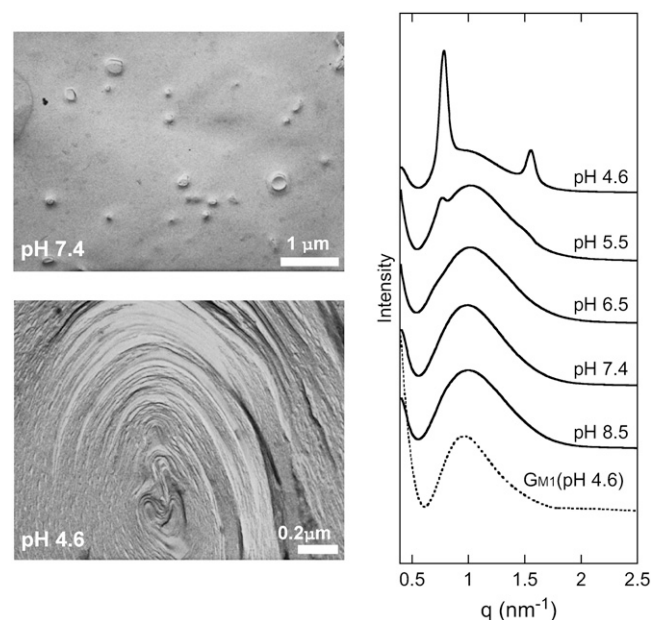
A characteristic feature of endosomes along with the degradative endocytic pathway is the accumulation of vesicles within the organelle (1,2). Recently, it has been shown that the unconventional phospholipid bis(monoacylglycerol) phosphate (BMP), also known as lysobisphosphatidic acid, LBPA can induce the formation of multivesicular liposomes that resemble 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 >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 that 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<sub>M1</sub> gangliosidosis, micelle-forming lipid G<sub>M1</sub> is extensively accumulated and still MCBs are formed. Therefore, it is of interest to investigate the conditions in which the accumulation of G<sub>M1</sub> induces the formation of closely stacked membranes. In our 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<sub>M1</sub> 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). Diffuse fluorescence was observed when cultured human skin fibroblasts were fixed, permeabilized, and labeled with fluorescently labeled cholera toxin, which recognizes G<sub>M1</sub> (see Fig. 4 of the Supplementary Material). In contrast, intracellular compartments were brightly labeled with cholera toxin when cells were grown in the presence of 10  $\mu$ M G<sub>M1</sub>. The fluorescence was colocalized with that labeled with anti-BMP antibody. The result suggests the presence of BMP and G<sub>M1</sub> 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 was  $<10$  nm. The size of vesicles at pH 7.4 was  $\sim 100$ – $300$  nm diameter in contrast to  $\sim 300$  nm– $3$   $\mu$ m 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  $q = \sim 0.55$   $\text{nm}^{-1}$  and a broad bell-shaped peak at  $q = \sim 1$   $\text{nm}^{-1}$ . 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), whereas  $G_{M1}$  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

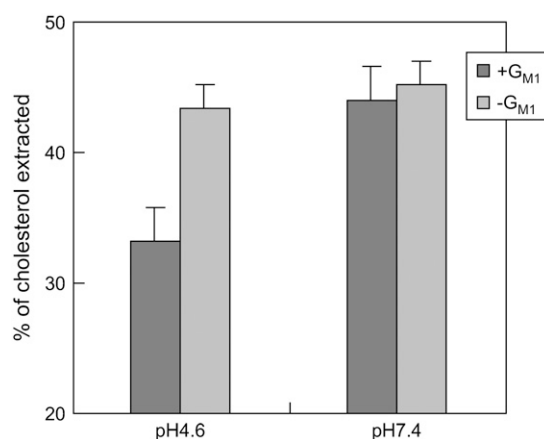


**FIGURE 1** (Left) Freeze-fracture electron micrographs of  $G_{M1}$ /BMP (1:1 mol/mol) mixture at different pH. (Right) SAXS patterns of  $G_{M1}$  at pH 4.6 and  $G_{M1}$ /BMP mixture at different pH.

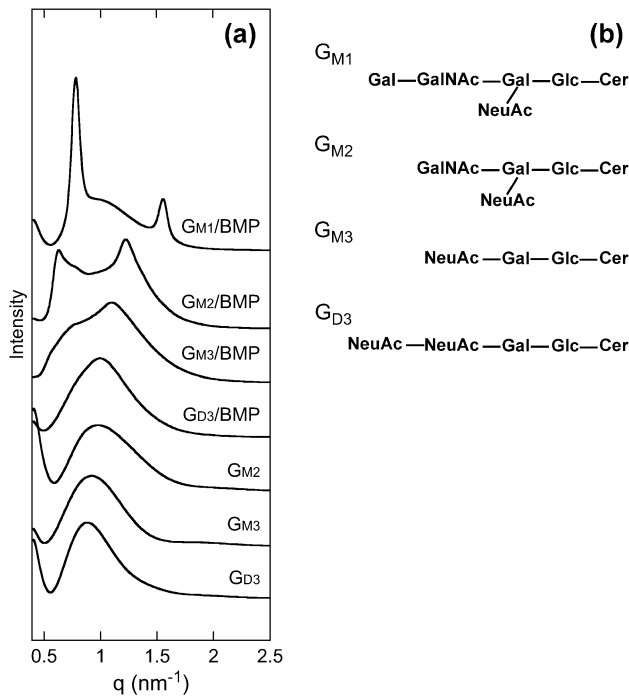
$q = 0.78$  and  $1.56$   $\text{nm}^{-1}$  in addition to the broad peak at  $q = \sim 1$   $\text{nm}^{-1}$ . These two peaks correspond to the first- and second-order diffraction peaks from a lamellar structure with an 8.06 nm repeat distance. At pH 4.6, the first- and second-order peaks became much 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 the 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). We investigated whether the  $G_{M1}$ /BMP membrane traps cholesterol in a pH-dependent manner (Fig. 2). Methyl- $\beta$ -cyclodextrin ( $M\beta$ CD) extracts cholesterol from the membrane. Extraction of cholesterol from BMP and  $G_{M1}$ /BMP membranes by  $M\beta$ CD 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  $M\beta$ CD.

Fig. 3 shows the examination of the effects of various gangliosides on the membrane structure of BMP at pH 4.6. 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 a branched carbohydrate chain is required for



**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  $\text{GM}_2$ ,  $\text{GM}_3$ , and  $\text{GD}_3$ , and their mixture with BMP (1:1 mol/mol) at pH 4.6. SAXS pattern of  $\text{GM}_1$ /BMP (1:1 mol/mol) mixture at pH 4.6 is also shown. The lamellar distance of  $\text{GM}_2$ /BMP was 9.98 nm. (b) Structures of gangliosides used in this study.

the tight packing of the ganglioside/BMP membrane at low pH. The lamellar structure was observed both at pH 4.6 and 7.4 when the sialic acid moiety of  $\text{GM}_1$  was substituted for the corresponding sugar alcohol (see Fig. 6 in the Supplementary Material), indicating that sialic acid prevents the formation of the lamellar structure of the  $\text{GM}_1$ /BMP membrane at neutral pH.

Although BMP forms a diffuse lamellar structure at broad range of pH values and  $\text{GM}_1$  forms a micelle, the mixture of the two lipids forms a closely stacked multilamellar structure at a pH that resembles the lumen of late endosomes/lysosomes. The reported membrane structures of  $\text{GM}_1$ /phospholipid and  $\text{GM}_1$ /cholesterol/ $\text{Ca}^{2+}$  system suggest that the  $\text{GM}_1$  sugar headgroups of the apposing bilayers are in the distance of direct contact in  $\text{GM}_1$ /BMP membranes at the low pH conditions. Previously, Simons and Gruenberg suggested that the accumulation of sphingolipids alters the properties of BMP (LBPA)-rich membrane domains (8). Our results provide the experimental evidence that the structure of the BMP membrane is indeed altered by  $\text{GM}_1$  and  $\text{GM}_2$  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 consequence of the specific interaction of sphingolipids and cholesterol in MCBs (8). Our results

suggest that BMP and a low pH are additional players in cholesterol accumulation in MCBs.

## SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at <http://www.biophysj.org>.

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