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The role of protein-linked oligosaccharide in the bilayer stabilization activity of glycophorin A for dioleoyl-phosphatidylethanolamine liposomes

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The importance of the protein-linked carbohydrates for the stabilization of dioleoylphosphatidylethanolamine (DOPE) bilayers has been investigated using glycophorin A, the major sialoglycoprotein of the human erythrocyte membrane, as a stabilizer. Two major types of glycophorin, differing in the sialic acid content, were used in the study. Type MM contains 19.2 ± 2.5 sialic residues per molecule of glycophorin, and type NN contains 10.8 ± 1.2. Type MM could stabilize DOPE bilayers at 0.5 mol%, whereas type NN was unable to do so even at 1 mol%. The importance of the sialic acid content to the stabilization activity of glycophorin was further confirmed by the observation that the neuraminidase-treated type MM showed a lower stabilization activity than the untreated type. Since type NN had no stabilizing activity, we attempted to couple a trisaccharide, NeuNAc → Gal → Glc, to type NN by reductive amination. 2.5 ± 0.8 saccharide chains were added per molecule of type NN. The trisaccharide-attached type NN showed a greater stabilization activity than the parent type NN molecule, indicating again that the sialic acid content of the stabilizer molecule determines the stabilization activity. Addition of wheat-germ agglutinin (WGA), which binds to the sialic acid residues of a glycoprotein, to type MM-stabilized liposomes caused rapid aggregation and destabilization of liposomes, resulting in leakage of an entrapped marker, calcein. The aggregation increased with increasing amount of the lectin; however, the leakage rate was maximum at an optimum concentration of WGA. These results are discussed in terms of the role of sialic acid in the interfacial hydration and charge repulsion which determines the DOPE bilayer stability.

Introduction

Glycophorin A, the major sialoglycoprotein of human erythrocyte membrane, contains 60% (w/w) carbohydrates, in the form of 15 *O*-linked oligosaccharide chains and one *N*-linked chain [1,2]. The amino-terminus of the molecule carrying all the saccharide residues, which is composed of 64 amino acids and 16 oligosaccharide chains, is exposed at the exterior surface of the plasma membrane. The hydrophilic carboxy-terminal domain of 35 amino acid residues extends into the cytoplasm and is linked to the amino-terminus by a sequence of 32 hydrophobic amino acids that span the lipid bilayer [1–5]. A number of methods have been described for reconstitution of glycophorin in lipid bilayers [6–9]. Furthermore, it has been concluded that a large percentage of the protein molecules is incorporated in the bilayer in a way comparable to the native configuration [6,10]. Particularly interesting is the report by Taraschi et al. [6,11] that the incorporation of glycophorin A into DOPE has stabilized the bilayer phase of DOPE. The equilibrium phase of DOPE at physiological pH and temperature is the reverse hexagonal (H_{II}) phase [12]. Apparently, the presence of glycoprotein on the membrane surface has stabilized the L_α phase of this phospholipid. We have previously shown that the glycophorin A-stabilized DOPE bilayer can form stable small unilamellar liposomes entrapping a fluorescent dye, calcein [13,14]. Furthermore, the liposomes can be rapidly destabilized or induced to leak the entrapped calcein if glycophorin A is removed by proteolytic treatment [13] or by aggregation with a multiva-

Abbreviations: NeuNAc, *N*-acetylneuraminic acid; Gal, galactose; GalNAc, *N*-acetyl-galactosamine; Glc, glucose, GlcNAc, *N*-acetyl-glucosamine; GP, glycophorin, GPM, glycophorin type MM; GPN, glycophorin type NN; Neo-GPN, trisaccharide-attached GPN; CFN, *Clostridium perfringens* neuraminidase, β-Gal, β-galactosidase, WGA, wheat-germ agglutinin; G6PDH, glucose-6-phosphate dehydrogenase; PBS, phosphate-buffered saline; DOC, deoxycholate.

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lent anti-glycophorin A antibody [14]. These studies indicate the importance of the glycoprotein for the liposome stability. However, it is not clear whether the stabilization activity is associated with the protein or the carbohydrate moiety of the glycoprotein. We have taken advantage of two naturally occurring glycophorin A types which differ in the oligosaccharide structure. *O*-Linked oligosaccharide chains present in human glycophorins contribute to the expression of many blood group antigens, including MN antigens. In particular, the sialic acid residues have been shown to be an integral part of MN blood-group antigens [15–18]. Erythrocytes of phenotype M contain a small amount of N antigen and neuraminidase treatment of type M erythrocytes produces a transient increase in N activity before both activities are destroyed [15–17], suggesting that the sialic acid is involved in the antigenic structures. We have used glycophorin A type MM and NN, which differ in sialic acid content, to study the role of the protein-linked oligosaccharide in the DOPE bilayer stabilization activity of glycophorin A. The data clearly indicate that the sialic acids on the glycophorin molecule play an important role in this activity. Furthermore, we report here a contact-induced destabilization of the DOPE liposome containing glycophorin A, as induced by a sialic acid-binding lectin, WGA.

Materials and Methods

Materials

Dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids. Glycophorin type MM (GPM) (cat. No. G7389, lot No. 57F 4089), Glycophorin type NN (GPN) (cat. No. G9266, lot No. 46F 4013), WGA, WGA-agarose gel, RCA I-agarose gel, trisaccharide NeuNAc → Gal → Glc (cat. No. A3001, lot No. 87F0799), $NaCNBH_3$, neuraminidase (*Clostridium perfringens*) and β-galactosidase were purchased from Sigma Chemical Co. All other chemicals were of analytical grade. GP was radioiodinated with ^{125}I by using Iodo-beads [19].

Enzyme treatments

To remove the negatively charged sialic acid residues on GP, it was treated with *Clostridium perfringens* neuraminidase (CFN) at 37°C for 4 h. Briefly, 40 units of enzyme and 1 mg of protein (GPM) in sodium acetate buffer pH 5.6 was incubated at 37°C for 3–4 h. The amount of SA released was quantitated by the method of Warren (TBA assay) [20].

Terminal galactose residues of the desialylated GPM were cleaved using *Escherichia coli* β-galactosidase in 0.1 M citrate-phosphate buffer (pH 6.0) or PBS (pH 7.0) [21].

Affinity chromatography

WGA-Sepharose. 5 ml WGA-Sepharose gel was packed into a small column and equilibrated with 50 mM Tris-HCl (pH 8.0) containing 0.02% NaN_3. A known quantity of the radioactively labeled GP was applied and allowed to incubate for about 15 min. The column was eluted with the same buffer followed by 0.1 M *N*-acetylglucosamine (GlcNAc) in the same buffer. In the same experiments, the GlcNAc concentration was increased up to 1 M [22–24].

RCA I-agarose. The column (0.5 × 3 cm) was equilibrated with PBS and eluted with 0.2 M lactose in the same buffer [25].

Conjugation of trisaccharide NeuNAc → Gal → Glc to glycophorin-NN by reductive amination

Conjugation of trisaccharide, NeuNAc → Gal → Glc, was carried out in 10 mM sodium borate buffer containing 60 mM NaCl (pH 8.5) [26,27]. Routinely, GPN containing a trace amount of iodinated protein, and trisaccharide (molar ratio 1 : 150) in borate buffer, were mixed with a 10^3-fold excess of $NaBH_3CN$, also dissolved in the same buffer. Reaction mixture was incubated at 37°C for 48–72 h prior to separation. Excess reagents were removed by column chromatography on a Bio-Gel P6 column equilibrated in PBS (pH 7.8). Fractions were analyzed for radioactivity and for total sialic acids by Svennerholm's resorcinol assay [28]. Fractions containing the conjugate were pooled and concentrated. The total sialic acid content was quantitated by TBA assay [20] after releasing the bound sialic acids by CFN treatment, as described above.

Incorporation of glycophorin into phospholipid vesicles

DOPE:glycophorin liposomes were prepared by a sonication method. In routine experiments, 2 μmol of DOPE or DOPC was mixed with a trace amount of hexadecyl [^{3}H]cholestanyl ether (final specific activity 1×10^{12} cpm (mol lipid)$^{-1}$) and dried under a stream of N_2 and vacuum-desiccated for no less than 2 h. The lipid mixture was then hydrated with PBS (200 μl) containing 310 μg (0.5 mol%) GP [6,13,14] and 50 mM calcein. In some experiments, glucose 6-phosphate dehydrogenase (G6PDH) (10^3 U) in 10 mM glucose 6-phosphate (pH 7.8) was used as an entrapped marker, replacing calcein. The mixture was allowed to incubate at 4°C for 12–24 h with occasional mixing. The lipid mixture was then vortexed and allowed to incubate at 4°C for an additional 10–18 h. Then the suspension was sonicated in a bath sonicator for 5 min and incubated at 4°C overnight. Two additional cycles of sonication were performed with a 6–12 h resting interval. Unentrapped calcein was removed by chromatography on a Bio-Gel A 0.5 M column. Unentrapped G6PDH was removed by column chromatography on Sepharose 4B. The fractions were measured for ^{3}H-cpm

and enzyme activity or calcein fluorescence in the presence or absence of 0.15% deoxycholate (DOC).

Fluorescence quenching measurements

The integrity of the liposomes was tested by measuring the calcein fluorescence quenching. The peak fractions from the gel filtration column containing ^{3}H-cpm were pooled and the fluorescence was measured using a Perkin-Elmer LS5 spectrofluorometer with λ_{ex} = 490 nm and λ_{em} = 520 nm. The lipid concentration in the cuvette was 1 μM in PBS containing 1 mM EDTA. Percent fluorescence quenching was calculated from the formula:

$$\% \text{ quenching} = (1 - F_0/F_t)100$$

where F_0 and F_t are the fluorescence of the liposome samples before and after addition of 0.15% DOC, respectively.

G6PDH activity assay

G6PDH activity was measured as described previously [29]. Briefly, liposomes containing G6PDH in PBS were incubated with 7 mM G6P and 2 mM NAD^+. The reaction was followed by monitoring the absorbance at 340 nm with a Perkin-Elmer Lambda 3A spectrophotometer. The total enzyme activity was measured after disrupting the liposome membrane with 0.15% (final concentration) Triton X-100. The percentage of enzyme latency is calculated as follows:

$$\% \text{ latency} = (1 - E_0/E_t)100$$

where E_t and E_0 are the enzyme activity measured in the presence and absence of Triton, respectively.

Light-scattering measurements of liposomes in the presence of WGA

Liposome aggregation in the presence of WGA was measured by monitoring the light scattering at 90° using a Perkin-Elmer LS5 spectrofluorometer with both the excitation and emission wavelengths set at 660 nm, and a slit width of 5 nm.

WGA-induced release of calcein from liposomes

Liposomes (50 μM) containing entrapped calcein were incubated at room temperature with WGA (0–50 μg/ml) for various intervals (0–60 min). The reaction mixture was diluted to 2 ml PBS containing 1 mM EDTA for the fluorescence measurement as described above. The percentage of calcein released was calculated using the formula:

$$\% \text{ release} = \frac{(F - F_0)}{(F_t - F_0)} \times 100$$

where F_0 and F are the calcein fluorescence before and after the addition of WGA, respectively, and F_t is the total fluorescence after addition of DOC.

Results

Glycophorin A molecules with different sialic acid content

In order to elucidate the role of protein-linked oligosaccharide, especially the sialic acid, in the DOPE bilayer-stabilizing activity of glycophorin A, we used two naturally occurring, antigenically different glycophorins A, i.e., GPM and GPN. It should be noted that different batches of GPM and GPN may differ in sialic acid content. Similar variations are known to occur in blood type determinants and also in serum glycoproteins. These variations arise due to the microheterogeneity of the oligosaccharide chains among the individuals [30]. Thus, it is important to carry out all experiments with the same lot of GPM and GPN. Since the exact structural differences between GPM and GPN are not known, we decided to directly measure the sialic acid content of the two glycophorin types. Table I shows that GPM contained 19.2 ± 2.5 sialic residues per molecule of GP, and GPN contained only 10.8 ± 1.2. Therefore, GPN contains about 56% of the sialic acid of GPM. In an effort to increase the sialic acid content of GPN, we conjugated a trisaccharide NeuNAc → Gal → Glc to the NH_2 groups of GPN by a reductive amination reaction. The product of the reaction, termed Neo-GPN, contained 13.3 ± 1.8 sialic residues per molecule of GP (Table I), which represents an increase of 2.5 ± 0.8 sialic residues per molecule of GPN, or a 23% increase in sialic acid content. The sialic acid content of GP was also measured indirectly by the binding of GP to a lectin affinity column containing WGA. WGA binds strongly with terminal sialic acid residues of glycoproteins. Bound GP was eluted from the column by 0.1–1 M GlcNAc. Table I shows that GPM bound quantitatively to the column, while only about 70% and 80% of GPN and Neo-GPN was retained by the WGA column, respectively. Thus, the relative affinity of GP to WGA is directly proportional to the sialic acid content of the glycoprotein.

TABLE I

Sialic acid content and interaction with WGA-Sepharose of GP

Glycophorin	Sialic acid content [a]		% GP bound to WGA-Sepharose [b]
	mol (mol GP)$^{-1}$	nmol (μg GP)$^{-1}$	
GPM	19.2 ±2.5	0.6 ±0.08 (8)	100
GPN	10.8 ±1.2 (4)	0.35 ± 0.04 (12)	71
Neo-GPN	13.28 ± 1.8 (12)	0.45 ± 0.06 (12)	82

[a] Determined by method of Warren [20]. Data expressed as mean ± S.D. (number of determinations).

[b] [^{125}I]GP (10 μg) was applied to a WGA-Sepharose column. Bound GP was eluted with 0.1–1 M GlcNAc in PBS.

We next decided to prepare GP with a low sialic acid content. This was done by treating GPM with neuraminidase. The resulting product, termed CFN-GPM, contained no detectable sialic acid (data not shown). GP contains 15 *O*-linked, mucin-type, sugar chains and one *N*-linked sugar chain [1,2]. The majority of the *O*-linked chains are tetrasaccharides containing two sialic acid residues per chain (structure B). A few *O*-linked chains are composed of a trisaccharide with only one sialic acid (structure A)

structure A
NeuNAcα2 → 6(3) Gal → GalNAc-Ser/Thr

structure B
NeuNAcα2 → 3 Gal → GalNAc-Ser/Thr
6
↑
2
α
NeuNAc

Removal of sialic acid from either structure by neuraminidase will expose the penultimate sugar residue, galactose. To further confirm the completeness of the neuraminidase reaction, the GPs were tested on another lectin affinity column, RCA I-agarose. RCA I binds to the terminal β-galactose residues of sugar chains [31]. Binding to RCA I primarily reflects the number of terminal galactose residues, as the sialic acid residues are removed from a mucin-type oligosaccharide chain [32]. Ricin immobilized on Sepharose beads has been used extensively for the isolation, purification and structure determination of glycoconjugates. As shown in Fig. 1a, GPM did not bind to the RCA I-agarose column; all ^{125}I-GP was eluted in the flow-through fractions. CFN-GPM, on the other hand, showed a strong affinity to the column with approximately 95% bound and 70% eluted with 0.2 M lactose (Fig. 1b).

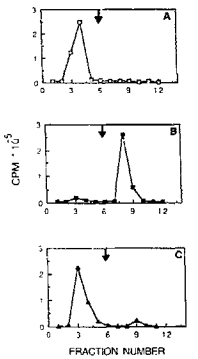

Fig. 1. Binding of glycophorin to the RCA I-agarose column. (A) ^{125}I-labeled; (B) CFN-treated glycophorin-type MM, CFN-GPM; (C) CFN-β-Gal-GPM. Elution was with PBS (pH 7.9) followed by 0.2 M lactose in the same buffer (indicated by the arrow).

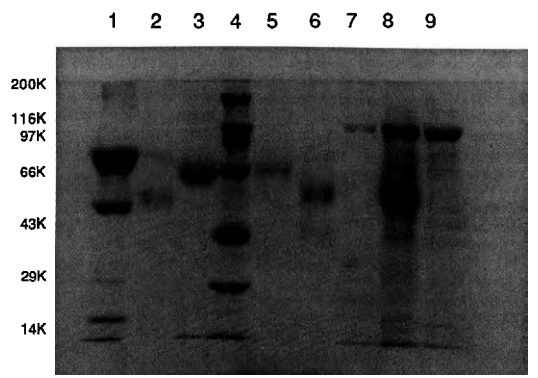

Fig. 2. SDS-polyacrylamide gel electrophoresis of different types of glycophorin in the presence of β-mercaptoethanol. Lane 1, Neo-GPN; lane 2, CFN-GPN; lane 3, GPN; lane 4, molecular weight standards; lane 5, GPM; lane 6, CFN-GPM; lane 7, β-galactosidase (3 μg); lane 8, CFN-β-Gal-GPM; lane 9, β-galactosidase (12 μg).

These results indicate that the neuraminidase treatment of GPM was complete; CFN-GPM was an asialoglycoprotein. We have also prepared GP with the galactose residues removed by a β-galactosidase treatment of CFN-GPM. The resulting GP, termed CFN-βGal-GPM, also did not bind with the RCA I-agarose column (Fig. 1c); approximately 92% was eluted in the flow-through fractions.

SDS-PAGE of glycophorin A molecules with different sialic acid content

SDS-PAGE of the various GP preparations was done under reducing conditions to examine the integrity of the protein. An accurate estimate of molecular weight of glycophorin was not possible with SDS-PAGE, due to the large content of saccharide. Although the reported molecular weight for glycophorin A is 31000 [3], the estimated molecular weight (Fig. 2) for GPM was 73800 (lane 5), compared to 62000 for GPN (lane 3). This difference may be due to the presence of negative charge arising from sialic acid. However, the treatment with neuraminidase gave rise to a CFN-GP of molecular weight 57000 for either GPM or GPN (lanes 6 and 2, respectively). Lane 1 of Fig. 2 shows the electrophoretic behavior of Neo-GPN on SDS-PAGE. The apparent molecular weight of the major band has increased to 69000 due to the addition of 0.10 nmol of NeuNAc per μg of GPN (Table I). CFN-GPM which has been further treated with β-galactosidase to remove the terminal galactose residues showed an electrophoretic mobility (lane 8) only slightly different from that of the desialylated GPM (lane 6). These results clearly demonstrate that the enzyme treatments had removed appropriate saccharide residues from the glycoprotein without grossly altering the integrity of the molecule. Furthermore, the anomolous electrophoretic mobility of glycophorin is due to its abundance in sialic acid. When the sialic acids are removed, the mobilities of GPM and GPN are identical. Further removal of galactose residues changed the mobility only slightly, indicating that the neutral sugar contributes very little to the abnormal electrophoretic mobility in SDS-PAGE.

Stabilization of DOPE liposomes with GP

We have tested the ability of these different GP molecules (0.5 mol%) to stabilize DOPE in bilayer form. In contrast to DOPC, DOPE does not form stable bilayers by itself at physiological temperature and pH [12,37]. Formation of stable bilayer liposomes can be monitored by encapsulating a water-soluble marker such as an enzyme (e.g., G6PDH) or a fluorescent marker (e.g., calcein). DOPE by itself forms large lipid aggregates (H_{II} phase) when hydrated in PBS (pH 8.0); hence, the lipid recovery from the Sepharose 4B column was low (Table II). Also, since it does not form stable bilayers, there was no encapsulation of the water-soluble marker. However, in the presence of a stabilizer such as GP, DOPE forms stable bilayer vesicles with entrapped water-soluble marker [6,11,13,14]. Therefore, the amount of enzyme or dye marker entrapped in the DOPE-stabilizer vesicles directly reflects the stabilizing activity or capacity of that particular stabilizer.

TABLE II

Stability of GP-liposomes

Glycophorin	% Lipid recovery [a]	% Enzyme latency [b]		
		16 h	7 days	28 days
GPM	82	80	72	60
CFN-GPM	10	12	0	0
CFN-βGal-GPM	4–8	21	5	0
GPN	13	20	8	0
Neo-GPN	61	61	61	20
None (DOPE) [c]	8	1	0	0
None (DOPC) [d]	88	89	80	70

[a] Liposomes were prepared by sonication with 0.5% (m/m) GP in DOPE entrapping G6PDH and chromatographed on a Sepharose 4B column. Percent of lipid eluted in the void volume fraction is shown.

[b] Latent G6PDH activity in liposomes was measured at indicated time.

[c] No GP was included in liposomes composed of DOPE.

[d] No GP was included in liposomes composed of DOPC.

Liposomes were prepared by sonication with encapsulated G6PDH. Non-entrapped G6PDH was removed by column chromatography on a Sepharose 4B column. Even after this purification step, some non-latent enzyme activity was usually detected, which may have been due to a non-specific association of the enzyme with the outer surface of the bilayer membrane. Enzyme latency was also measured over a period of 28 days with liposomes stored at 4°C. The rate of decay of enzyme latency is also a good indication of liposome stability, with stable liposomes showing a low rate and vice versa.

As shown in Table II, GPM was a good DOPE bilayer stabilizer as indicated by 82% column recovery and an excellent level of enzyme latency in the liposomes, which also lasted for a long time upon storage. The stability of GPM-stabilized DOPE liposome was comparable to that of the liposomes composed of DOPC, a bilayer-forming phospholipid (Table II). The stabilization activity of GPN was much less than that of GPM. The lipid recovery was low (13%) and enzyme latency was only 20% initially, decaying to zero in 4 weeks. Increasing the GPN concentration to 1 mol% gave similarly poor stabilization results. The stabilization activity of GPM was greatly reduced when its sialic acid was removed with a neuraminidase treatment. The lipid recovery and enzyme latency were both very low for CFN-GPM; almost the same as pure DOPE without any glycoprotein (Table II). Further removal of galactose residues from the desialylated

GPM, as indicated by the activity of CFN-βGAL-GPM, still showed poor stabilization activity. Interestingly, GPN treated with trisaccharide by reductive amination showed a much higher activity than GPN itself. DOPE liposomes stabilized with Neo-GPN showed 61% lipid recovery and an initial enzyme latency of 61%, which decayed slowly to 20% after 4 weeks in storage. Although the stabilization activity of NeoGPN was not quite as high as that of GPM, it was certainly much higher than that of GPN or CFN-GPM.

The above experiment was done using a standard protocol with 2 days of hydration (see Materials and Methods). It was noted that a prolonged hydration period improved liposome stability. For example, if DOPE was hydrated for 7 days in PBS at 4°C in the presence of GPN, more stable liposomes were made, with 75% lipid recovery and about 70% enzyme latency. Thus, longer periods of incubation with buffer containing protein followed by sonication of the lipids improves the liposome stability. However, these liposomes were only stable for 2–3 days at 4°C; the enzyme latency decayed to zero during this period. Thus, the hydration protocol affects the liposome stability, but a poor stabilizer (e.g., GPN) still does not perform well, even with a prolonged hydration period. Furthermore, we have noticed that GPN from different lots had different stabilization activity, probably related to the variation in its sialic acid content. All data concerning GPN presented here came from experiments using GPN of the same lot number to minimize experimental variations.

Thus, we conclude that the DOPE bilayer stabilization activity of GP is closely related to its sialic acid content. Both the stabilization activity and the sialic acid content follow the same order: GPM > Neo-GPN > GPN > CFN-GPM ≈ CFN-βGal-GPM (Tables I and II). Interestingly, artificially adding sialic acid residues to the NH_2 groups of GP, as in the case of Neo-GPN, also helped to improve the DOPE bilayer stabilization activity as the naturally occurring sialic acid which is mainly attached to the Ser/Thr residues of the glycoprotein.

Destabilization of GP-containing DOPE liposomes by WGA

The stabilization activity of GP can also be measured by the entrapment of calcein. This method is particularly convenient for studying the kinetics of liposome destabilization, because the fluorescence enhancement due to calcein leakage can be measured continuously with a fluorometer. We have used this method to study the lysis of GP-containing DOPE liposomes induced by proteolytic cleavage [13] or binding to a multivalent antibody [14]. We now use the same method to study the WGA-induced liposome destabilization.

Calcein was entrapped in the liposomes at a self-quenching concentration of 50 mM. Liposome destabilization, at various concentrations of WGA, was measured by the fluorescence enhancement of calcein as it was released from liposomes. Increased turbidity, as measured by 90° light scattering, of the resulting liposome-WGA suspension was taken as an indicator of liposome aggregation. Fig. 3A shows the % calcein release from liposomes stabilized with GPM with increasing concentrations of WGA. No release of calcein was observed in the absence of WGA. Initially, increasing amounts of WGA resulted in an increased destabilization of liposomes shown as enhanced fluorescence. However, after 20 $\mu g \cdot ml^{-1}$ of WGA, the % calcein release from the liposomes started to decrease. Although increasing incubation times increased the leakage of calcein from liposomes at all concentrations of WGA, the rate of release was maximum at 20 $\mu g \cdot ml^{-1}$ WGA. However, increasing WGA concentration resulted in increased liposome aggregation as shown in Fig. 3B; no optimal WGA concentration was observed.

A close examination of the calcein release kinetics revealed an initial lag period immediately after the addition of WGA at the optimal concentration, i.e., 20 $\mu g \cdot ml^{-1}$ (Fig. 4). This was the case for both GPM- and Neo-GPN-stabilized liposomes. Interestingly, the aggregation of GPM-liposomes showed a rapid rise during

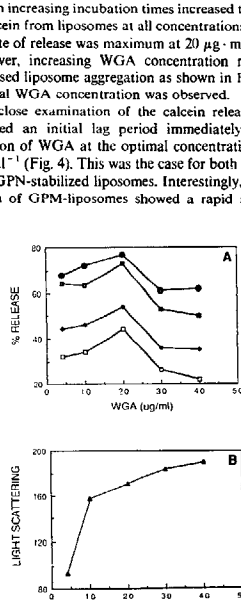

Fig. 3. WGA-induced destabilization of GPM-stabilized DOPE liposomes. (A) liposomes (50 μM) were incubated with varying amounts of WGA for 15 min (□), 25 min (◆), 40 min (■) or 60 min (●), and % calcein release was measured. (B) Liposomes (50 μM) incubated with varying amounts of WGA for 7 min and 90° light scattering was determined as described under Materials and Methods.

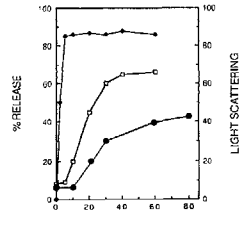

Fig. 4. Percent calcein release as a function of time. DOPE liposomes (50 μM) containing 0.5 mol% of GPM (□), or Neo-GPN (●) incubated with 20 μg ml^{-1} of WGA. At various time intervals, the sample was diluted in PBS-EDTA buffer and % release (□, ●) or light scattering (◆) was determined.

this period (Fig. 4). The leakage of calcein did not start until liposome aggregation had reached the maximal level. Such temporal correlation strongly suggests that the leakage of calcein from liposomes is a result of liposome aggregation. Furthermore, comparison of the steady-state level of calcein release between the two types of liposomes shows that WGA had induced more calcein release from the GPM-liposomes (60–75%) than the Neo-GPN-liposomes (40–45%) (Fig. 4). This may be accounted for by the lower affinity of Neo-GPN to WGA as compared to GPM (Table I).

Discussion

The purpose of this study was to establish the role of the protein-linked oligosaccharide, particularly sialic acid, in DOPE bilayer stabilization. To do so, it was necessary to use a series of GP isoforms which differ in the carbohydrate structure, specifically the sialic acid content. The two antigenic variants of GP, types MM and NN, were very useful for this purpose. Our study has demonstrated a large difference in the sialic acid content of the two types. However, more GP isoforms with lower or intermediate levels of sialic acid content were also needed. This was achieved by removing sialic acid residues from GP by neuraminidase treatment to obtain CFN-GPM and CFN-GPN, which had identical electrophoretic mobility on SDS-PAGE and showed no detectable sialic acid content. We also chemically attached more sialic acid to GPN by conjugating a trisaccharide, NeuNAc → Gal → Glc, to the NH_2 groups of GPN. The resulting neoglycoprotein (Neo-GPN) showed an intermediate level of sialic acid content between GPM and GPN (Table I). There are only five lysine residues in GP which are potentially available for conjugation with the trisaccharide [1]. We managed to add 2.5 ± 0.8 sialic residues per GP molecule, taking up half of the total available sites. Prolonging the reaction time and/or increasing trisaccharide concentration in the reaction did not result in higher level of conjugation (data not shown). We suggest that not all lysine residues are equally accessible for the reaction. The two on the N-terminal side of the molecule (Lys-18 and Lys-30) are probably more accessible than the others located on the C-terminal side. Thus, the sialic acid content of GP was varied from an undetectable level (CFN-GPM and CFN-GPN) to 19.2 ± 2.5 (GPM) residues per GP molecule. These molecules were used to study the DOPE bilayer stabilization activity of GP.

DOPE liposome stabilization was assayed by the latency of an entrapped enzyme, G6PDH, and by the recovery of lipid from a gel filtration column (Table II). The data strongly indicate the important role of sialic acid of a glycoprotein in stabilizing the bilayer phase of unsaturated PE. The more sialic acid in the molecule, the higher the stabilization activity. The stabilization activity of GP could be enhanced by chemically conjugating sialic acid-containing trisaccharides to the molecule with a reductive amination reaction; i.e., Neo-GPN. This result indicates that the location of the sialic acid on the protein, whether linked to Ser/Thr as the naturally occurring *O*-linked oligosaccharides or to Lys as in the Neo-GPN, does not make a difference in terms of the stabilization activity.

There are potentially two different ways in which the protein-linked oligosaccharide can contribute to the DOPE bilayer stabilization. The first is by contributing to the interfacial hydration level of the DOPE bilayer. The head group of PE is poorly hydrated compared to, e.g., that of phosphatidylcholine. There are only approximately 7–10 H_2O molecules associated with a given head group of PE [33–35], whereas there are 31–34 molecules of H_2O associated with a PC head group [33,36]. Such a low level of hydration is one of the primary forces which drive PE into the formation of H_{II} phase, which only requires a minimal level of hydration per head group [37]. Carbohydrates are excellent H-bonding molecules. The presence of carbohydrate on the bilayer surface will presumably increase the hydration level by H-bonding with H_2O, thereby stabilizing the bilayer phase of DOPE. The second factor which may contribute to the stability of the GP-containing liposomes is the surface charge and steric hindrance. Bilayer-to-H_{II} phase transition requires close apposition of the neighboring bilayers [38]. The negative charge of the sialic acid may prevent such apposition by charge repulsion when the liposomes collide. Thus, the liposomes containing GP may be kinetically trapped in the bilayer state, even when the equilibrium phase is the H_{II} phase. This latter possibility is the more likely, because even the liposomes stabilized with GPM were

slowly destabilized over a week period and lost a portion of the enzyme latency (Table II). Also, the bilayer stabilization may result from intervesicle steric hindrance by the bulky hydrophilic carbohydrate moieties of glycophorin, which presents a barrier to fusion.

The results shown in Figs. 3 and 4 also indicate that the GP-stabilized liposomes can be rapidly destabilized by binding with a bivalent lectin, WGA. Binding of the lectin to the sialic acid residues may decrease the hydration at the membrane surface, leading to a transition to the non-bilayer phases. However, the primary driving force for the bilayer destabilization must be related to the fact that WGA has brought the liposomes into close contact with each other. WGA may also shield the negative charge from the liposome surface and reduce the charge repulsion between liposomes. Alternatively, WGA may cross link the glycophorin molecules within the individual vesicles, resulting in a lateral segregation of the protein [6]. The aggregated liposomes rapidly undergo a phase transition to the non-bilayer phase, e.g., the H_{II} phase, probably as a result of close apposition of the protein-free domains of the liposome membranes. This hypothesis is supported by the observation that the content leakage of the liposomes did not occur until the liposomes were maximally aggregated (Fig. 4). Furthermore, the model also predicts that at high WGA concentrations, the close bilayer apposition between the aggregated liposomes would be inhibited, because the excess WGA sterically hinders such close contact. This is in fact observed in the experiment described in Fig. 3. The rate of calcein leakage from the GPM-stabilized liposomes was optimal at 20 $\mu g\ ml^{-1}$ of WGA; higher WGA concentrations induced lower rates. We have previously shown that GP-stabilized DOPE liposomes can be destabilized by proteolytically removing GP from the liposomes [13]. The destabilization rate is proportional to the square of the liposome concentration, indicating a collision-dependent reaction. Liposome destabilization can also be induced by binding the liposomes to anti-GP antibody molecules immobilized on glass beads [14]. Again, the liposomes are brought to a close contact on the surface of the bead. Thus, the stabilization of the DOPE liposomes can be established by preventing the liposomes from coming into close contact, and the destabilization can be induced by bringing the liposomes into a close contact.

In conclusion, we have demonstrated that the DOPE bilayer stabilization activity of GP is mainly associated with the sialic acid residues and not with the protein moiety. This is probably due to the negative charge of sialic acid which prevents the close contact of liposomes by charge repulsion. Destabilization of the liposomes can be induced by bringing the liposomes together with a lectin, WGA. This study should be useful for the future design of liposomes for the purpose of drug delivery and immunodiagnosis.

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