Differential effects of glycosphingolipids on the detergent-insolubility of the glycosylphosphatidylinositol-anchored membrane dipeptidase

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The insolubility of glycosylphosphatidylinositol (GPI)-anchored proteins in certain detergents appears to be an intrinsic property of their association with sphingolipids and cholesterol in lipid rafts. We show that the GPI-anchored protein membrane dipeptidase is localized in detergent-insoluble lipid rafts isolated from porcine kidney microvillar membranes, and that these rafts, which lack caveolin, are enriched not only in sphingomyelin and cholesterol, but also in the glycosphingolipid lactosylceramide (LacCer). Dipeptidase purified from porcine kidney was reconstituted into artificial liposomes in order to investigate the relationship between glycosphingolipids and GPI-anchored protein detergent-insolubility. Dipeptidase was insoluble in liposomes containing extremely low concentrations of LacCer. In contrast, identical concentrations of glucosylceramide or galactosylceramide failed to promote significant detergent-insolubility. Cholesterol was shown to enhance the detergent-insoluble effect of LacCer. GC–MS analysis revealed dramatic differences between the fatty acyl compositions of LacCer and those of the other glycosphingolipids. However, despite these differences, we show that the unusually marked effect of LacCer to promote the detergent-insolubility of dipeptidase cannot be singularly attributed to the fatty acyl composition of this glycosphingolipid molecule. Instead, we suggest that the ability of LacCer to confer detergent-insolubility on this GPI-anchored protein is dependent on the structure of the lipid molecule in its entirety, and that this glycosphingolipid may have an important role to play in the stabilization of lipid rafts, particularly the caveolin-free glycosphingolipid signalling domains.

Key words: caveolin, cholesterol, lactosylceramide, lipid rafts, sphingomyelin.

INTRODUCTION

The insolubility of multiple glycosylphosphatidylinositol (GPI)-anchored proteins in non-ionic detergents was originally demonstrated in 1988 [1]. This phenomenon appears to be an intrinsic property of the association of the membrane anchor of these proteins with membrane microdomains, often referred to as 'lipid rafts' [2–4]. These structures are enriched in cholesterol, sphingomyelin and glycosphingolipids, and are characterized as being insoluble in certain detergents [3,5,6]. In the original model of lipid rafts, Simons and van Meer [7] proposed that the glycosphingolipids might self-associate through hydrogen bonds between the hydroxy groups of the sphingosine base and the hydroxy fatty acid present on many sphingolipids. Subsequently it was suggested that weak interactions between the glycosphingolipid carbohydrate headgroups might contribute to this interaction [8]. An alternative model postulated that interactions between lipid acyl chains play a key role in raft formation and that rafts exist in membranes as domains in the liquid-ordered (l_o) phase [9]. As lipid rafts are involved in a range of biological processes, including signal-transduction events, sorting and trafficking in the secretory and endocytic pathways, toxin entry into cells and conformational conversion of the prion protein [10–13], understanding the mechanism underlying the clustering of certain lipids and proteins in domains on the membrane is of fundamental importance.

Treatment of a membrane fraction with Triton X-100 and subsequent flotation on a sucrose gradient has now become a widely accepted method for the isolation of lipid rafts or detergent-resistant membranes (DRMs) (reviewed in [13–15]). In addition to cholesterol and sphingomyelin, the DRMs are enriched in a variety of glycosphingolipids, including lactosylceramide (LacCer) [2,16]. Although GPI-anchored proteins are detergent-insoluble in most cell lines, there is one notable exception. In Fischer-rat thyroid (FRT) cells, the GPI-anchored protein gD1-DAF, which comprises the GPI anchor of decay accelerating factor (DAF) fused to the ectodomain of the glycoprotein D1 (gD1), was completely detergent-soluble [16]. This lack of association of gD1-DAF with DRMs appeared to correlate with the lack of expression of the protein caveolin. However, not all cells express caveolin, and DRMs can be isolated that lack caveolin [17–22], suggesting that some additional factor(s) may also be involved in promoting the detergent-insolubility of GPI-anchored proteins. A comparative lipid analysis of Madin–Darby canine kidney (MDCK) cells (in which GPI-anchored proteins are targeted to rafts) and FRT cells, revealed that the latter cells were characterized by a dramatic reduction in the galactose-containing glycolipids galactosylceramide (GalCer), LacCer, Forssman antigen and sulphatide [16]. These observations prompted us to explore the hypothesis that one or more galactose-containing glycosphingolipids may have a critical role to play in promoting the detergent-insolubility of GPI-anchored proteins.

For this study we chose to use the GPI-anchored protein membrane dipeptidase (MDP; EC 3.4.13.19), which has been extensively characterized in our laboratory (reviewed in [23]). Both the glycan core structure and the acyl chain composition of the GPI anchor of porcine kidney MDP have been determined [24]. The GPI anchor of this protein is composed exclusively of diacyl phosphatidylinositol (predominantly C_{18} phosphatidylinositol with only a minor amount of C_{18}–C_{16} phosphatidylinositol). In the present study we show first that MDP in porcine kidney

Abbreviations used: DAF, decay accelerating factor; DMPC, dimyristoyl phosphatidylcholine; DRM, detergent-resistant membrane; FAME, fatty acid methyl ester; FRT, Fischer-rat thyroid; GalCer, galactosylceramide; gD1, glycoprotein D1; GlcCer, glucosylceramide; GPI, glycosylphosphatidylinositol; LacCer, lactosylceramide; MBS, Mes-buffered saline; MDCK, Madin–Darby canine kidney; MDP, membrane dipeptidase.

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cortex microvillar membranes is almost completely associated with DRMs which lack caveolin, and that these domains are enriched not only in cholesterol and sphingomyelin, but also in the glycosphingolipid LacCer. Subsequently, affinity-purified amphipathic MDP was reconstituted into artificial liposomes in order to identify any possible differential effects exhibited by glycosphingolipid species on the detergent-insolubility of the protein. LacCer was shown to confer detergent-insolubility on MDP at concentrations far lower than glucosylceramide (GlcCer) or GalCer, and this effect was found to be accentuated by the inclusion of cholesterol in the liposomes. The fatty acyl compositions of the sphingolipid species were determined and shown to be completely different between LacCer and the other glycosphingolipids. However, we also provide evidence that these differences in fatty acyl composition alone are unable to explain the extreme propensity for LacCer to confer detergent-insolubility on the GPI-anchored MDP.

EXPERIMENTAL

Isolation of detergent-resistant membranes

DRMs were isolated from porcine kidney cortex using a protocol similar to that already described [25]. Briefly, microvillar membranes were prepared from 100 g of pig kidney cortex by the method of Booth and Kenny [26] and the membrane pellet was resuspended in Mes-buffered saline (MBS; 25 mM Mes/NaOH, pH 6.5, and 0.15 M NaCl) containing 2% (v/v) Triton X-100. The protein concentration of the resuspended membranes was determined and adjusted to 8 mg/ml using MBS containing 2% (v/v) Triton X-100. Following a 2 h incubation at 4 °C, the solubilized sample was mixed with an equal volume of 80% (w/v) sucrose in MBS containing 2% (v/v) Triton X-100 and an aliquot (4 ml) was layered under a 10 ml continuous gradient of 30–5% (w/v) sucrose in MBS (lacking Triton X-100). The sample was then centrifuged overnight at 140000 ***g*** (4 °C) in an SW28 rotor (Beckman Instruments).

Enzyme and protein assays

MDP was assayed by an HPLC method with glycyl-D-phenylalanine as substrate [27]. Aminopeptidase N was assayed with alanine *p*-nitroanilide as substrate and the product was quantified spectrophotometrically [28]. Protein was quantified using bicinchoninic acid in a microtitre plate assay with BSA as standard.

SDS/PAGE and immunoelectrophoretic blot analysis

Proteins were separated on a 7–17% polyacrylamide gradient gel and transferred to Immobilon P PVDF membranes [29]. MDP was detected using a 1:500 dilution of a polyclonal anti-(porcine MDP) antibody [30] and peroxidase-conjugated secondary antibody (1:10000 dilution) in conjunction with the enhanced chemiluminescence (ECL®) detection method (Amersham). Caveolin was detected with a polyclonal anti-caveolin antibody (1:4000 dilution) (Transduction Laboratories, Lexington, KY, U.S.A.).

Lipid analysis of membrane fractions

Washed membrane resuspensions (0.5 ml) were heated in methanol (3 ml) at 70 °C for 30 min. After cooling, 4 ml of chloroform and distilled water (0.5 ml) were added and the sample was vortex-mixed, followed by centrifugation at 2000 ***g*** for 5 min (4 °C). The upper aqueous phase was removed and discarded and 1 ml of water was added. After repeated vortex-mixing and centrifugation the aqueous layer was again removed and discarded. The water wash step was then repeated twice more. The final organic (lower) phase was concentrated under nitrogen and resuspended in chloroform/methanol/water (30:60:8, by vol.). Lipid extracts were applied to a 1 ml-bed-vol. DEAE-Sephadex A-25 column [31]. Non-acidic lipids were eluted in 10 ml of chloroform/methanol/water (30:60:8, by vol.). Acidic lipids were then eluted with 10 ml of chloroform/methanol/aq.0.8 M sodium acetate (30:60:8, by vol.). Oleyl alcohol was added as an internal standard for the relative quantification of neutral and acidic lipid classes. Individual lipid species were subsequently resolved by TLC. High-performance TLC silica-gel 60 plates (Merck KGaA, Darmstadt, Germany) were chromatographed half-way using a mobile phase consisting of chloroform/methanol/acetic acid/formic acid/water (35:15:6:2:1, by vol.). After drying, the plates were then chromatographed to completion in hexane/di-isopropyl ether/acetic acid (65:35:2, by vol.). Lipids were revealed by submersion of the plates in 3% (w/v) cupric acetate dissolved in 8% (v/v) phosphoric acid in Millipore water followed by charring at 180 °C. Quantification was achieved by extrapolation from standard curves produced by the co-chromatography of known amounts of authentic lipid standards.

Purification and reconstitution of amphipathic MDP

Amphipathic MDP was purified to homogeneity by cilastatin–Sepharose affinity chromatography following solubilization of the porcine kidney cortex microvillar membranes with n-octyl β-D-glucopyranoside as described previously [32]. Liposomes were prepared as described previously [33,34] by dissolving 35 mg of dimyristoyl phosphatidylcholine (DMPC) and the desired amount of sphingolipid (bovine brain sphingomyelin, chicken egg-yolk sphingomyelin, bovine brain Type II GalCer, human spleen GlcCer, bovine LacCer; all from Sigma) and/or cholesterol in chloroform and the solution was then dried under nitrogen to produce a film on the interior of a glass vial. Myristic acid (15 mg) was suspended using gentle heating in 10 ml of ethanol and a 100 μl aliquot was transferred to a glass vial. The myristic acid was then dried under nitrogen, resuspended by gentle heating in 4 ml of 10 mM Tris/HCl/0.1 M KCl/1 mM ascorbate, pH 8.0, and transferred to the vial containing the DMPC and/or sphingolipids and cholesterol. Liposomes were produced by sonication of the sample for 30 min at 30 °C and 30% maximum power output. Large multilamellar vesicles were then removed by centrifugation at 140000 ***g*** for 30 min (37 °C) in an SW50 rotor (Beckman Instruments). MDP was then incubated with the liposome preparation at a 1:10 (protein/lipid) ratio for 10 min at 18 °C followed by a further 10 min incubation at 30 °C. Reconstituted protein was isolated by centrifugation on a cushion of 20% (w/v) sucrose in 10 mM Tris/HCl, pH 7.5, for 18 h (27 °C) at 100000 ***g*** in an SW50 rotor (Beckman Instruments).

Solubilization of reconstituted MDP

Reconstituted enzyme was incubated for 1 h at 4 °C with an equal volume of 10 mM Tris/HCl, pH 7.5, containing 2% (v/v) Triton X-100. The sample was then diluted 10-fold with 10 mM Tris/HCl, pH 7.5 (lacking Triton X-100) and centrifuged at 205000 ***g*** for 4 h (4 °C) in an SW50 rotor (Beckman Instruments). The pelleted protein was then resuspended in 500 μl of 10 mM Tris/HCl, pH 7.5, and the amount of insoluble MDP determined by activity assay as described above. The percentage of MDP

that was insoluble could then be determined by relating the amount of enzyme activity in the insoluble pellet to the amount of activity solubilized initially.

GC–MS analysis of the glycosphingolipid fatty acyl compositions

Fatty acid methyl esters (FAMEs) were prepared from sphingolipid samples by acidic transmethylation in 2.5 ml of 2.5 % (v/v) H_2SO_4 in anhydrous methanol for 2 h at 70 °C. Following the addition of 0.9 M NaCl (5 ml), FAMEs were extracted into light petroleum (boiling point 40–60 °C). The light petroleum was then evaporated under nitrogen and the dried FAMEs were resuspended in 50 μl of cyclohexane. Aliquots (1 μl) were resolved on a Hewlett–Packard 5890 gas chromatograph coupled to a VG Quattro 1 mass spectrometer. The column used was a Hewlett–Packard HP20M (Carbowax 20M; 25 m × 0.32 mm internal diameter; 0.3 μm film thickness). The injection was on-column at 50 °C, and the temperature program was 50 °C (1 min) to 230 °C at 8 °C/min. The carrier gas was helium at 55.2 kPa (8 lbf/in²). The MS details were as follows: source temperature, 200 °C; ionization energy, 70 eV; emission, 200 μA; mass range, 40–550 Da; scan time, 2 s.

RESULTS

Association of MDP with microvillar lipid rafts

DRMs were isolated from porcine kidney microvillar membranes by flotation in a sucrose density gradient following solubilization with Triton X-100 as detailed in the Experimental section. A_{620} measurements of each fraction harvested from the sucrose gradient showed a single population of DRMs distributed in fractions 7–10 (Figure 1A). The majority of the total protein was excluded from the DRM region of the sucrose gradient (Figure 1B), with 7 % located in a detergent-insoluble pellet at the base of the centrifuge tube (fraction 0) and a further 74 % in the detergent-solubilized protein region (fractions 1–4 inclusive). SDS/PAGE and immunoelectrophoretic-blot analysis of the individual fractions from the sucrose gradient using a polyclonal antibody raised against porcine MDP [30] gave a single band at 45 kDa (Figure 1E). Subsequent densitometric quantification of the immunoblot (Figure 1C) showed that the vast majority of MDP was located in the DRM region of the sucrose gradient (fractions 7–10). Aminopeptidase N is a type II integral membrane protein which was originally shown to be completely soluble in Triton X-100 [1]. Consistent with this finding, in the present study (Figure 1D), aminopeptidase-N was located exclusively in the detergent-solubilized protein region of the gradient (fractions 1–4), providing evidence that the DRMs isolated by this method were not contaminated with other detergent-soluble membrane fractions. Immunoelectrophoretic blot with an antibody against caveolin failed to detect any immunoreactive protein in any of the fractions from the sucrose gradient (Figure 1F), indicating that the microvillar membranes lack this protein.

Lipid composition of DRMs isolated from the microvillar membrane

The lipid composition of the microvillar membranes and DRMs isolated from them was analysed as described in the Experimental section. The predominant lipid classes in the microvillar membranes were sphingomyelin, phosphatidylcholine, phosphatidylethanolamine and cholesterol (Table 1). The concentrations of the major phospholipid species, phosphatidylcholine and phosphatidylethanolamine, were dramatically reduced, whereas the levels of phosphatidylinositol and phosphatidylserine were not significantly different in the DRMs relative to the microvillar membranes. Sphingomyelin and LacCer were enriched 2.7- and 2.1-fold respectively in the isolated DRMs, whereas cholesterol was only enriched by 1.4-fold.

Reconstitution of MDP into artificial sphingolipid liposomes

The amphipathic form of MDP was purified from porcine kidney cortex by affinity chromatography on cilastatin–Sepharose [32]. This form of the enzyme retains the fatty acids in the GPI anchor and reconstitutes into artificial lipid vesicles [24,32]. In order to investigate the effect of sphingolipids on the detergent-insolubility of MDP, the purified enzyme was reconstituted into liposomes containing DMPC/sphingolipid at various molar ratios. Reconstituted protein was then separated from free protein and aggregated lipid by centrifugation on a sucrose cushion which was harvested in 0.5 ml fractions. Using this method, successfully reconstituted protein remains at the top of the sucrose cushion. Each fraction was then assayed for MDP activity to determine the amount of protein successfully reconstituted into the artificial liposomes. MDP was effectively reconstituted into DMPC/egg-yolk sphingomyelin liposomes with 47–57 % of the total enzyme activity located in the top fraction of the sucrose cushion over a range of DMPC/sphingomyelin ratios (720:1–1:1) (the data for a DMPC/sphingomyelin ratio of 1:1 are shown in Figure 2). When LacCer was incorporated into the liposomes (Figure 3), MDP was effectively reconstituted at molar ratios between 720:1 and 180:1 (DMPC/LacCer). At higher LacCer concentrations the amount of enzyme successfully reconstituted decreased as a function of glycosphingolipid concentration. MDP was successfully reconstituted (> 50 %) into liposomes containing GalCer or GlcCer over the complete range of DMPC/glycosphingolipid molar ratios examined (Figure 3).

Detergent-insolubility of MDP in artificial sphingolipid liposomes

MDP that had been incorporated into the liposomes was then subjected to solubilization at 4 °C with Triton X-100. The detergent-insoluble protein was pelleted by high-speed centrifugation and the percentage of the total enzyme activity in this pellet (i.e. detergent-insoluble) was determined as described in the Experimental section. At DMPC/sphingomyelin molar ratios between 720:1 and 9:1, maximally only 22 % of MDP was detergent-insoluble. Molar DMPC/GalCer/GlcCer ratios from 9:1 to 720:1 did not result in an appreciable amount (maximally 2.8 %) of MDP being detergent-insoluble (Figure 4). In contrast, 44 % of MDP was detergent-insoluble in DMPC/LacCer (9:1) liposomes (Figure 4). Indeed, the amount of detergent-insolubility conferred on MDP by LacCer remained significantly higher than for either of the other two glycosphingolipids, even down to DMPC/LacCer molar ratios as low as 720:1 (at which 22.8 % of MDP was still detergent-insoluble) (Figure 4).

Cholesterol accentuates the detergent-insolubility of MDP

The effect of cholesterol on the detergent-insolubility of purified amphipathic MDP reconstituted into liposomes containing sphingomyelin or LacCer was also examined. Cholesterol increased the amount of detergent-insoluble MDP in DMPC/sphingomyelin/cholesterol (9:1:1) liposomes by 1.6-fold. A similar increase, 1.3-fold, in the detergent-insolubility of MDP was observed when cholesterol was incorporated into liposomes containing LacCer (DMPC/LacCer/cholesterol, 9:1:1). These data indicate that the effect of cholesterol on the detergent-insolubility of MDP was additive rather than synergistic with the sphingolipids, consistent with previous studies where cholesterol

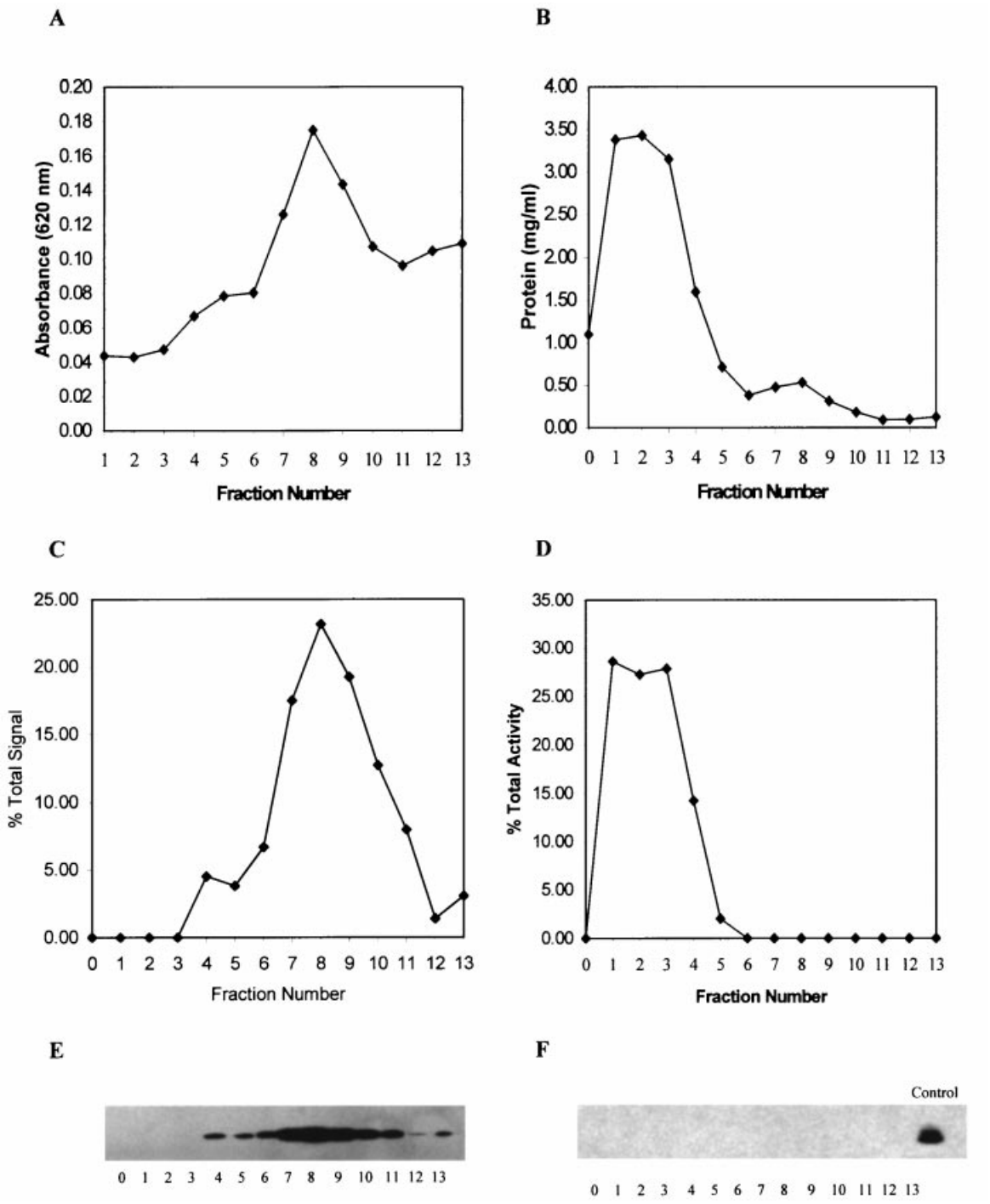

Figure 1 Association of MDP with porcine microvillar DRMs

DRMs were prepared from porcine kidney cortex as described in the Experimental section. Sucrose gradients were harvested in 1 ml fractions (fraction 0, high-density cytoskeletal pellet; fraction 1, base of gradient; fraction 13, top of gradient). (**A**) A_{620}; (**B**) total protein distribution; (**C**) distribution of GPI-anchored MDP expressed graphically following densitometric analysis of the immunoblot shown in (**E**); (**D**) distribution of transmembrane aminopeptidase N; (**F**) immunoblot for caveolin across the sucrose gradient fractions. The positive control was 20 μg of porcine lung membranes.

was shown to enhance the detergent-insolubility of GPI-anchored proteins in both cells and liposomes [4,6].

Determination of sphingolipid fatty acyl compositions

The fatty acyl composition of the sphingolipids was determined following acidic transmethylation of the FAMEs from the individual sphingolipids by GC–MS analysis of the individual peaks on the chromatogram. The egg-yolk sphingomyelin used in all the experiments up to this point consisted primarily of palmitate ($C_{16:0}$) with a lesser amount of stearate ($C_{18:0}$) and trace levels of behenate, lignocerate and nervonate ($C_{22:0}$, $C_{24:0}$ and $C_{24:1}$ respectively) (Figure 5A). In contrast, the predominant acyl species in the LacCer was found to be stearate, with much

Table 1 Lipid composition of microvillar membranes and microvillar DRMs isolated from porcine kidney cortex

Total lipid was extracted from the samples as described in the Experimental section. Individual lipid classes were separated by TLC and revealed by charring with H_2SO_4. Quantification of individual lipid classes was achieved following densitometric analysis by extrapolation from calibration curves produced by the co-chromatography of authentic lipid standards. The results are means ± S.E.M. ($n = 8$ for the microvillar membranes and $n = 6$ for the microvillar DRMs).

	Mass composition (%)	
Lipid class	Microvillar membranes	Microvillar DRMs
Cholesterol	17.44 ± 1.24	23.93 ± 0.04
Sphingomyelin	15.59 ± 1.21	41.77 ± 2.45
LacCer	3.53 ± 1.51	7.26 ± 0.60
Cerebrosides	0.87 ± 0.32	0.14 ± 0.09
Phosphatidylcholine	36.48 ± 2.52	15.50 ± 1.57
Phosphatidylethanolamine	10.99 ± 1.89	4.88 ± 1.54
Phosphatidylinositol	2.18 ± 1.06	1.61 ± 0.05
Phosphatidylserine	6.38 ± 3.25	3.51 ± 1.04
Triacylglycerols	2.86 ± 0.63	Not detected
Sterol esters	3.81 ± 0.63	Not detected

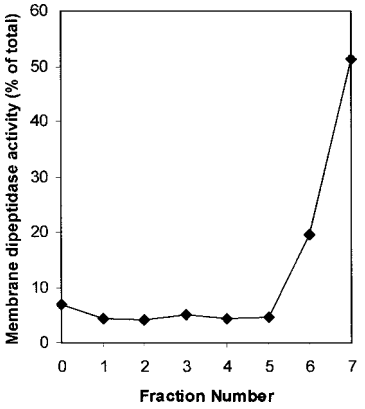

Figure 2 Sucrose-cushion centrifugation of reconstituted MDP

MDP was reconstituted into DMPC/sphingomyelin (1:1) liposomes as described in the Experimental section. The reconstitution mixture was layered on top of a 20%-(w/v)-sucrose cushion and centrifuged for 18 h at 100000 ***g***, after which the tube was fractionated into 0.5 ml aliquots. The percentage distribution of MDP activity throughout the sucrose cushion was determined. Fraction 0, base of tube; fraction 7, top of tube.

lesser amounts of palmitate and oleate ($C_{18:1}$) and trace levels of lignocerate and nervonate (Figure 5B). GlcCer and GalCer had very similar fatty acyl compositions (Figure 5C and 5D). Unlike egg-yolk sphingomyelin and LacCer, lignocerate and nervonate were identified as major constituents of the GlcCer and GalCer. The other major fatty acyl constituents of these two glycosphingolipids were identified as palmitate, stearate and behenate. GlcCer also exhibited substantial levels of arachidate ($C_{20:0}$).

Effect of fatty acyl composition on the detergent-insolubility of MDP using egg-yolk sphingomyelin and brain sphingomyelin liposomes

We attempted to determine whether a simple change of fatty acyl composition from predominantly palmitate to stearate was sufficient to increase the propensity for a sphingolipid to promote the detergent-insolubility of MDP. For this purpose MDP was reconstituted into liposomes containing either egg-yolk sphingomyelin (predominantly palmitate residues) or

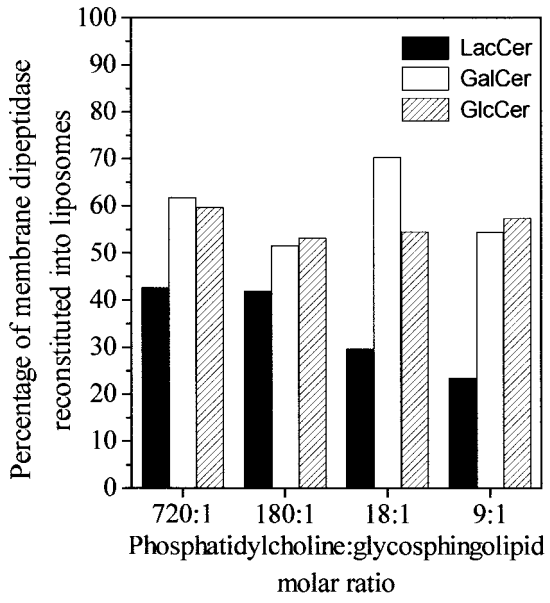

Figure 3 Reconstitution of MDP in DMPC/glycosphingolipid liposomes

MDP was reconstituted into liposomes with various glycosphingolipid contents as described in the Experimental section. Reconstituted protein was isolated by centrifugation through a sucrose cushion (see Figure 2) and the results shown are expressed in terms of the percentage of MDP activity recovered in the top fraction of the sucrose cushion.

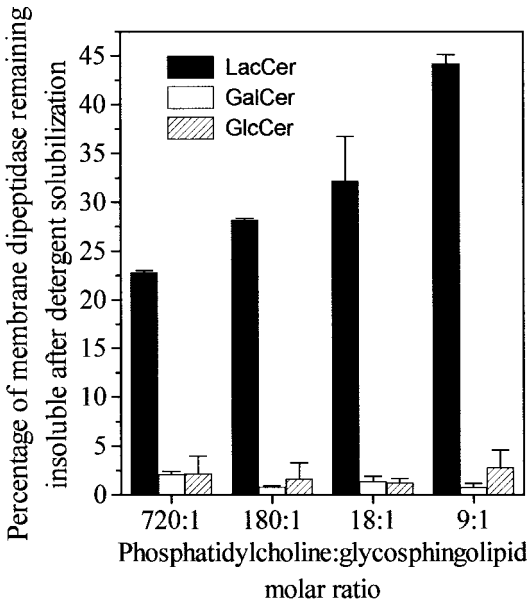

Figure 4 Detergent-insolubility of MDP in DMPC/glycosphingolipid liposomes

MDP was reconstituted into liposomes with various glycosphingolipid contents as described in the Experimental section. Reconstituted protein was isolated by centrifugation through a sucrose cushion and then solubilized in Triton X-100 at 4 °C. The results show the percentage of MDP activity that remained insoluble and could be pelleted by high-speed centrifugation. Values are means ± S.E.M. ($n = 3$).

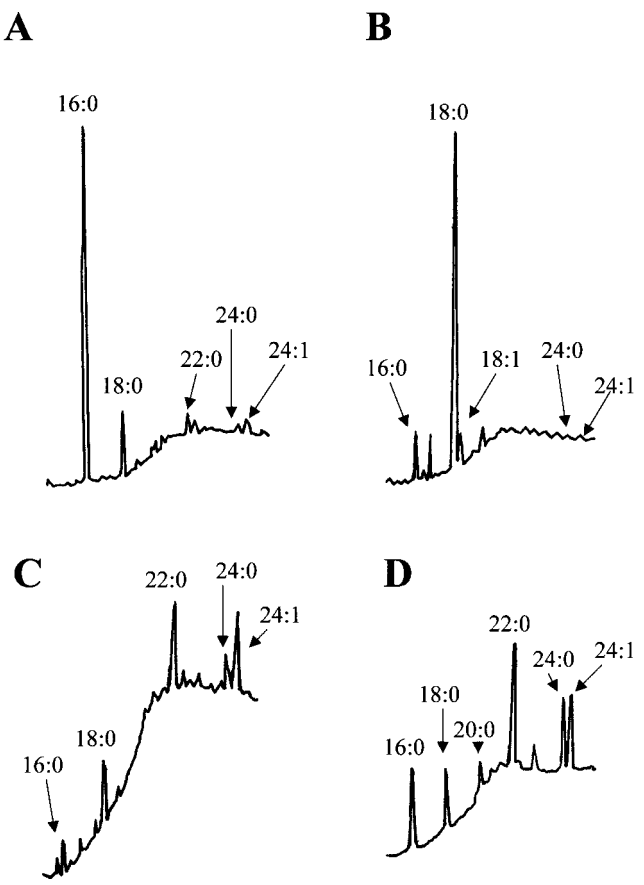

Figure 5 GC–MS analysis of sphingolipid fatty acyl compositions

FAMEs of sphingolipid samples were prepared by acidic transmethylation and resolved by GC on a Hewlett–Packard HP20M (Carbowax 20M) column in a Hewlett–Packard 5890 gas chromatograph linked to a VG Quattro 1 mass spectrometer. The identities of the peaks shown were confirmed by comparison with the retention times and mass spectra of authentic FAME standards. (**A**) Egg-yolk sphingomyelin; (**B**) LacCer; (**C**) GalCer; (**D**) GlcCer. Abbreviation: 16:0 etc., $C_{16:0}$ fatty acid etc.

brain sphingomyelin (predominantly stearate residues; Avanti Polar Lipids Inc., Alabaster, AL, U.S.A.) at a concentration of sphingomyelin (DMPC/sphingomyelin, 9:1) just below that which had previously been shown to confer detergent-insolubility on MDP for the egg-yolk sphingomyelin. MDP was reconstituted effectively [$75.6 \pm 0.3\%$ and $67.8 \pm 1.4\%$ ($n = 3$) respectively] into liposomes containing either egg-yolk sphingomyelin or brain sphingomyelin. However, the detergent-insolubility of MDP was not altered on changing the length of the acyl chains in the sphingomyelin, with only $10.5 \pm 2.9\%$ ($n = 3$) of MDP being detergent-insoluble in the egg-yolk sphingomyelin liposomes and $9.3 \pm 1.3\%$ ($n = 3$) in the brain sphingomyelin liposomes.

DISCUSSION

In the present study, we isolated DRMs from porcine kidney microvillar membranes using the technique of buoyant sucrose-density-gradient centrifugation in the presence of Triton X-100, which has become a widely accepted method for the isolation of these membrane microdomains [2,35–37]. The $MgCl_2$ precipitation method used to isolate the microvillar membranes effectively removes other internal membranes from this subsection of the plasma membrane [26,38]. The vast majority of the GPI-anchored MDP was found to be associated with the microvillar membrane DRMs, residing in a single low-density peak within the sucrose gradient. Two other GPI-anchored proteins, alkaline phosphatase and aminopeptidase P, co-migrated with MDP in the sucrose gradient (results not shown). Immunoelectrophoretic-blot analysis failed to detect caveolin either in the microvillar membranes or in the DRMs isolated from these membranes. Although the detergent-insolubility of GPI-anchored proteins in FRT cells could be restored on transfection and expression of caveolin [16], in the absence of this protein, multiple GPI-anchored proteins are detergent-insoluble in the kidney microvillar membrane [1,39]. This reinforces the fact that membrane components other than caveolin can promote and/or maintain the detergent-insolubility of GPI-anchored proteins.

Consistent with our earlier observation [1] we show that the transmembrane protein aminopeptidase N was completely excluded from the kidney microvillar DRMs. We have also shown that this protein is excluded from DRMs isolated from porcine lung [25] and human brain [40]. This contrasts with other reports in which aminopeptidase N, along with other transmembrane anchored peptidases, has been localized to DRMs isolated from intestinal enterocytes [41], monocytes [42] and synoviocytes [43]. This difference in apparent raft localization may reflect cell/tissue-specific differences in the protein and/or lipid composition of the various membranes, but clearly indicates that rafts are not 'peptidase-rich hot-spot regions' [43] of the plasma membrane in all cell types.

Lipid analysis revealed that the microvillar DRMs were enriched in sphingomyelin and cholesterol, as well as in the glycosphingolipid LacCer. Originally both sphingomyelin and cholesterol were identified as being enriched in rafts, while LacCer was reported to be 100% detergent-insoluble in rafts isolated from MDCK cells [2]. A comparison of the lipids in the detergent-insoluble pellet following extraction of MDCK cells with either Triton X-100 or CHAPS revealed that, although sphingomyelin, cerebrosides and the ganglioside G_{M3} were similarly enriched in the insoluble pellets, LacCer and the Forssman antigen, although enriched in the Triton X-100-insoluble fraction, were depleted from the CHAPS-insoluble pellet [44]. In this context it is noteworthy that we originally reported that multiple GPI-anchored proteins in the porcine kidney microvillar membrane were more efficiently solubilized by the detergent CHAPS than by Triton X-100 [1,39]. This similarity in behaviour upon detergent extraction provides circumstantial evidence for the close association of LacCer and GPI-anchored proteins in lipid rafts, and that their interaction may be important in maintaining the integrity of such domains.

For the first time we report the effect of individual glycosphingolipids on the detergent-insolubility of a GPI-anchored protein in artificial liposomes. Neither GalCer nor GlcCer was able to cause more than 3% of MDP to become detergent-insoluble at any of the DMPC/glycosphingolipid ratios studied. In contrast, even extremely low concentrations of LacCer (DMPC/LacCer, 720:1) conferred significant (>20%) detergent-insolubility on the reconstituted MDP. While at the concentration found in the microvillar membrane rafts (equivalent to a DMPC/LacCer molar ratio of 18:1), LacCer caused 32% of MDP to be detergent-insoluble in the liposomes. Thus, this detergent-insolubility-promoting effect does not appear to be a general property of glycosphingolipids, but rather is limited to LacCer.

Schroeder et al. [3] demonstrated that the acyl-chain composition of a lipid affects its ability to confer detergent-insolubility on GPI-anchored placental alkaline phosphatase in liposomes,

and, more recently, Benting et al. [45] showed that the acyl- and alkyl-chain-length composition of the GPI anchor is important for raft association in artificial liposomes. Unlike GalCer and GlcCer, LacCer exhibited a very high content of stearate. As the GPI anchor of porcine MDP primarily contains stearoyl residues [24], an acyl-chain-length match between the stearoyl residues in the MDP GPI anchor and the predominating stearoyl residues in LacCer may explain why this glycosphingolipid was so effective in promoting the detergent-insolubility of MDP. In order to test this hypothesis we reconstituted MDP into liposomes containing either egg-yolk sphingomyelin or brain sphingomyelin at molar ratios just below those known to promote detergent-insolubility in the case of egg-yolk sphingomyelin (i.e. 9:1, DMPC/sphingomyelin). Whereas egg-yolk sphingomyelin contains predominantly palmitate residues, brain sphingomyelin contains predominantly stearate (like LacCer). However, MDP was almost entirely solubilized by detergent, irrespective of which sphingomyelin species was used to make the liposomes, indicating that a simple increase in the length of the acyl chains is not sufficient to significantly alter the detergent-insolubility of this GPI-anchored protein.

The supporting phosphatidylcholine matrix in the liposomes used in the current study contains myristoyl residues, whereas MDP contains mostly stearoyl residues, suggesting a potential acyl-chain-length mismatch. However, more than 70% of the MDP is effectively reconstituted into the lipid vesicles (recovered in fractions 6 and 7 in Figure 2), despite this mismatch. There is also a difference in the acyl chain length between LacCer (C18:0) and the other two glycosphingolipids used. However, the inability of brain sphingomyelin ($C_{18:0}$) to increase detergent-insolubility over egg-yolk sphingomyelin ($C_{16:0}$) indicates that it is more than a simple acyl chain match between the sphingolipids and the acyl chains on MDP that is involved in the increased detergent-insolubility seen with LacCer.

The hydroxy groups of the sugar residues provide the possibility for additional intermolecular hydrogen-bonding between individual glycosphingolipids [46], and there is evidence for attractive interactions between uncharged lipid bilayers containing LacCer [47]. In addition, exogenous administration of gangliosides to MDCK cells was shown to displace GPI-anchored proteins from lipid rafts, although it is unclear whether this was a function of the acyl chains or the headgroup of the glycolipids [48]. These observations raise the possibility that the sugar headgroup of LacCer interacts with the glycan chain of the GPI anchor on MDP (which is substituted on the reducing terminal mannose residue with Gal or GalNAc residues on 71% of the total population [24]) and that such interactions may contribute to the detergent-insolubility-promoting effect of this glycosphingolipid. Although addition of lactose to the liposomes containing LacCer had no effect on the amount of MDP that was detergent-insoluble (results not shown), this could be due to the inability of the free sugar to compete effectively with the lipid-bound sugar on the surface of the liposomes.

The GPI-anchored alkaline phosphatase was shown to acquire its detergent-insolubility in or before the medial or *trans* cisternae of the Golgi [2]. As GalCer is synthesized in the endoplasmic reticulum [49] and does not promote the detergent-insolubility of MDP, this glycolipid is unlikely to be involved in the clustering of GPI-anchored proteins. However, both sphingomyelin and GlcCer are synthesized in early compartments of the Golgi [49], but only sphingomyelin promoted the detergent-insolubility of MDP, indicating that sphingomyelin probably has a major role to play in the formation of lipid rafts, as proposed previously [3,4]. In contrast, the synthesis of LacCer has recently been shown to occur in the late Golgi [50]. Thus the detergent-insolubility of GPI-anchored proteins cannot rely entirely on the presence of LacCer, but our data imply that this lipid may have a role in stabilizing the association of GPI-anchored proteins with sphingomyelin and other raft components. This conclusion would be consistent with a recent report that, in a glycosphingolipid-deficient cell line, glycosphingolipids are not essential for the formation of DRMs [51].

In summary, we have shown that MDP behaves as a typical GPI-anchored protein in terms of its association with lipid rafts in a biological membrane, and that caveolin is not required for this interaction. For the first time we have examined the relationship between different glycosphingolipid species and the detergent-insolubility of a GPI-anchored protein. In so doing we have identified LacCer as a potentially crucial component of lipid rafts in mammalian cells. The extreme detergent-insolubility-promoting effect of LacCer cannot be explained solely on the basis of molecular fatty acyl composition. We suggest that the role of LacCer in promoting the detergent-insolubility of GPI-anchored proteins is dependent upon the structure of this molecule in its entirety, and that this glycosphingolipid may have an important role to play in the stabilization of lipid rafts, particularly the caveolin-free glycosphingolipid signalling domains [22,52].

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