Isolation and characterization of two distinct low-density, Triton-insoluble, complexes from porcine lung membranes

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The Triton-insoluble complex from porcine lung membranes has been separated into two distinct subfractions visible as discrete light-scattering bands following buoyant density-gradient centrifugation in sucrose. Both of these detergent-insoluble complexes were enriched in the glycosyl-phosphatidylinositol (GPI)-anchored ectoenzymes alkaline phosphatase, aminopeptidase P and 5'-nucleotidase, and both complexes excluded the polypeptide-anchored ectoenzymes angiotensin-converting enzyme, dipeptidyl peptidase IV and aminopeptidases A and N. The GPI-anchored proteins in both complexes were susceptible to release by phosphatidylinositol-specific phospholipase C. Both complexes were also enriched in cholesterol and glycosphingolipids, and in caveolin/VIP21, although only the higher-density fraction was enriched in the plasmalemmal caveolar marker proteins Ca^{2+} -ATPase and the inositol 1,4,5-trisphosphate receptor.

Among the annexin family of proteins, annexins I and IV were absent from the two detergent-insoluble complexes, annexin V was present in both, and annexins II and VI were only enriched in the higher-density fraction. When the metal chelator EGTA was present in the isolation buffers, annexins II and VI dissociated from the higher-density detergent-insoluble complex and only a single light-scattering band was observed on the sucrose gradient, at the same position as for the lower-density complex. In contrast, in the presence of excess calcium only a single detergent-insoluble complex was isolated from the sucrose gradients, at an intermediate density. Thus the detergent-insoluble membrane complex can be subfractionated on the basis of what appears to be calcium-dependent, annexin-mediated, vesicle aggregation into two distinct populations, only one of which is enriched in plasmalemmal caveolar marker proteins.

INTRODUCTION

The insolubility of glycosyl-phosphatidylinositol (GPI)-anchored proteins in certain detergents was originally demonstrated in 1988 [1], and has now become well established [2,3]. The phenomenon appears to be an intrinsic property of the association of the membrane anchor of these proteins with sphingolipids and cholesterol in membrane microdomains [4,5]. Treatment of a membrane fraction with Triton X-100 and subsequent flotation on sucrose gradients has now become a widely accepted method for the isolation of these membrane microdomains [3,6–10]. Low-density, detergent-insoluble, membrane domains isolated in this way have been shown to be enriched in multiple GPI-anchored proteins, $\text{G}\alpha$ and $\text{G}\beta$ subunits of heterotrimeric GTP-binding proteins, Rap1A/B, Rap 2, annexin II, the Src family non-receptor-type protein kinase c-Yes, actin, glycosphingolipids and cholesterol. In addition, these detergent-insoluble complexes are enriched in a 21–24 kDa integral membrane protein, known as caveolin or VIP21 [8,11]. *In situ*, antibodies raised against this protein [12] have been shown to decorate 50–100 nm vesicles either associated with or in the vicinity of the plasma membrane, termed caveolae [11,13,14]. Caveolae have been implicated in a number of cellular events, including signal transduction [6,15,16], transcytosis [17], potocytosis (the internalization of small molecules from the extracellular medium) [18,19] and interaction with the actin-based cytoskeleton [8,9]. The presence of caveolin and the observation that GPI-anchored proteins appeared to cluster in caveolae [6,20] have led to the assumption that low-density, detergent-insoluble,

membrane complexes represent caveolae-enriched fractions [8,21,22].

While this assumption may be intrinsically correct, caution must be taken in attributing the enrichment of protein constituents in detergent-insoluble membrane complexes to the presence of these molecules in caveolae [23]. Recent studies have indicated that the clustering of GPI-anchored proteins in caveolae may be a consequence of cross-linking with antibodies [24,25], and that detergent treatment causes a redistribution of these proteins into clustered microdomains [26]. The recent introduction of a new method for the separation of caveolae from related, non-caveolar, detergent-insoluble, membrane microdomains has allowed the composition of these structures to be investigated afresh. The isolation of caveolae from rat lung endothelium following perfusion with cationic colloidal silica has shown that only certain proteins are localized in such membrane invaginations [16,27]. In particular, along with caveolin, this isolated membrane fraction was enriched in a Ca^{2+} -ATPase, the inositol 1,4,5-trisphosphate (IP_3) receptor and the ganglioside G_{M1} , all of which have previously been shown by electron microscopy to be localized in caveolae [28–30]. Caveolae isolated in this way appeared to lack detectable amounts of the transmembrane-anchored protein angiotensin-converting enzyme, and the GPI-anchored proteins 5'-nucleotidase and the urokinase/plasminogen activator receptor [31]. These results have served to emphasize the fact that the property of detergent-resistance is not confined to caveolar membranes, prompting a call for a critical re-examination of the relationship between caveolae and detergent-insoluble membrane complexes.

Abbreviations used: GPI, glycosyl-phosphatidylinositol; IP_3 , inositol 1,4,5-trisphosphate; MBS, Mes-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C.

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In the present paper, we show that detergent-insoluble complexes isolated from porcine lung membranes by flotation in sucrose gradients can be subfractionated into two distinct populations whose protein and lipid compositions have been characterized. Although both complexes contain caveolin and multiple GPI-anchored proteins, only the lower-density detergent-insoluble complex is enriched in the caveolar marker proteins Ca^{2+} -ATPase and the IP_3 receptor. We also present evidence to suggest that isolation of this caveolae-enriched subfraction is dependent on the calcium concentration in the isolation buffers, and that the calcium- and phospholipid-binding proteins annexins II and/or VI may be responsible for the separation of these membrane microdomains from the bulk of the detergent-insoluble membrane fraction.

EXPERIMENTAL

Materials

Antibodies and their sources were as follows: monoclonal antibody to caveolin (Affiniti Research Products Ltd., Exeter, U.K.); donkey polyclonal antibody to c-Yes (Genosys Biotechnologies, Inc., Cambridge, U.K.); monoclonal antibody to β -actin (Sigma Chemical Co., Poole, Dorset, U.K.); rabbit polyclonal antibodies to annexins V and VI, and monoclonal antibodies to annexins I, II (light and heavy chains) and IV (gifts from Dr. J. H. Walker, University of Leeds); monoclonal antibody to plasmalemmal Ca^{2+} -ATPase (Affinity Bioreagents Inc., Neshanic, NJ, U.S.A.); rabbit polyclonal antibody to the IP_3 receptor (Cambridge Biosciences, Cambridge, U.K.). All other materials were from sources previously noted [1,2,32–34].

Isolation of detergent-insoluble complexes by sucrose-gradient centrifugation

Low-density, detergent-insoluble, complexes were prepared routinely from porcine lung by modification of an established protocol [9]. Unless otherwise stated, all procedures were carried out at 4 °C. Porcine lung (200 g) was cut into small pieces with scissors and homogenized with 500 ml of Mes-buffered saline (MBS; 25 mM Mes, pH 6.5, 0.15 M NaCl). Homogenization was performed with three 30 s bursts of a Waring blender. The homogenate was centrifuged at 5000 g for 20 min and the resultant supernatant was filtered through a single layer of cheese-cloth. The filtrate was further centrifuged at 10000 g for 10 min, and the supernatant from this latest spin was used to prepare a microsomal membrane fraction by centrifugation at 40000 g for 90 min. The resulting microsomal membranes were solubilized following resuspension in MBS containing 2% Triton X-100 by repeatedly drawing samples through a Luer 21G needle (approx. 20 times). Samples were then assayed for protein, and sufficient MBS containing 2% Triton X-100 was added to achieve a final protein concentration of 15 mg/ml. Samples were then mixed on a magnetic stirrer for 2 h at 4 °C, following which they were again drawn through a Luer 21G needle (~20 times). The solubilization procedure was completed with 30 strokes of a loose-fitting Dounce homogenizer. Aliquots (2 ml) of solubilized membranes were then layered at the base of ultracentrifuge tubes and adjusted to 40% (w/v) sucrose by the addition of an equal volume of 80% sucrose in MBS containing 2% Triton X-100. A 5–30% linear sucrose gradient (8 ml; in MBS containing 0.5% Triton X-100) was formed above the solubilized membranes. Detergent-insoluble complexes were isolated by ultracentrifugation at 140000 g for 23 h in an SW28 rotor (Beckman Instruments).

Enzyme and protein assays

Aminopeptidase P and angiotensin-converting enzyme were assayed using Gly-Pro-Hyp and benzoyl-Gly-His-Leu respectively as substrate, and the substrate and reaction products were separated and quantified by reverse-phase HPLC as described previously [32,33]. 5'-Nucleotidase, alkaline phosphatase, aminopeptidase N, aminopeptidase A and dipeptidyl peptidase IV were assayed with 5'-AMP, *p*-nitrophenyl phosphate, Ala-*p*-nitroanilide, Glu-*p*-nitroanilide and Gly-Pro-*p*-nitroanilide respectively as substrate, and the products were quantified spectrophotometrically as described previously [1,34]. Protein was quantified using bicinchoninic acid in a microtitre plate assay with BSA as standard [34].

SDS/PAGE and immunoelectrophoretic blot analysis

Proteins intended for immunoblotting with anti-(plasmalemmal Ca^{2+} -ATPase) or anti-(IP_3 receptor) antibodies were separated by SDS/PAGE using 5% gels, and transferred to nitrocellulose membranes using 0.7 M glycine/25 mM Tris/HCl, pH 8.3, as transfer buffer. Separation of protein samples intended for immunodetection with other antibodies was performed on 7–17% polyacrylamide gradient gels followed by transfer to Immobilon P poly(vinylidene difluoride) membranes as previously described [32]. Antigens were detected using peroxidase-conjugated secondary antibodies in conjunction with the enhanced chemiluminescence detection method (Amersham).

Phase separation in Triton X-114

Triton X-114 was precondensed before use [35]. Membrane samples were diluted 3-fold in 10 mM Tris/HCl/150 mM NaCl, pH 7.4, and pelleted by centrifugation at 11600 g for 15 min. Membranes were washed a further three times in the same buffer, following which the pellets were resuspended to a protein concentration of 1 mg/ml (max.). Samples (100 μ l) were incubated in the absence or presence of *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC) (1 unit/ml) for 1 h at 37 °C. Following incubation, the samples were made up to 200 μ l with 10 mM Tris/HCl/150 mM NaCl/1% Triton X-114, pH 7.4, and incubated at 4 °C for 10 min. Samples were then vortexed, incubated at 30 °C for 10 min and then centrifuged at 3000 g for 3 min. The resulting detergent-rich phase was made up to the same volume as the detergent-poor phase by the addition of 10 mM Tris/HCl/150 mM NaCl, pH 7.4.

Lipid analysis

Lipids from low-density, detergent-insoluble, complexes were analysed essentially by the method of Macala et al. [36]. Samples were diluted in MBS, pelleted by centrifugation at 11600 g for 15 min and washed twice with 1 ml aliquots of MBS. Lipids were extracted as described in [37] and all samples were taken to dryness under nitrogen, following which total lipid was re-suspended in 10 ml of chloroform/methanol/water (30:60:8, by vol.). Acidic and neutral lipid fractions were separated by chromatography on DEAE-Sephadex (A-25) columns. Neutral lipids were eluted with 10 ml of chloroform/methanol/water (30:60:8, by vol.), and acidic lipids were eluted with 10 ml of chloroform/methanol/0.8 M aq. sodium acetate (30:60:8, by vol.). Both lipid classes were evaporated to dryness under a stream of nitrogen. Excess sodium acetate was removed from the acidic lipid fraction by the addition of 3 ml of chloroform/methanol (2:1, v/v) and 0.6 ml of water. Following centrifugation at 4000 g for 5 min, the upper aqueous phase was

removed and discarded. A volume of chloroform/methanol/water (3:48:47, by vol.) equal to that of the discarded aqueous phase was then added to the acidic lipid samples. Following centrifugation at 4000 *g* for 5 min, the upper phase was again removed and discarded. Concentrated acidic and neutral lipid samples were loaded on to C_{18} silica TLC plates, which were developed first in chloroform/methanol/acetic acid/formic acid/water (35:15:6:2:1, by vol.) (to about half-way up the plate), and then in hexane/di-isopropyl ether/acetic acid (65:35:2, by vol.) (to about 1 cm below the top edge of the plate). Lipid standards of known concentration were run on the same TLC plate as the samples, such that the lipid species could be quantified by means of standard calibration curves. Relative proportions of acidic and neutral lipids from the detergent-insoluble complexes were calculated by the inclusion of an internal standard (50 μ g of oleyl alcohol) in the two lipid classes following separation on DEAE-Sephadex (A-25) columns. Lipids on TLC plates were detected by spraying with 3% (w/v) cupric acetate in 8% (v/v) phosphoric acid, followed by heating at 180 °C for 30 min. Quantification was achieved using a scanning densitometer (Scanmaster 3; Howtek) with the Quantity One program (Pdi Software).

RESULTS

Isolation of two detergent-insoluble complexes from porcine lung membranes

Microsomal membranes isolated from porcine lung were solubilized in Triton X-100 at 4 °C, and the detergent-insoluble fraction was subsequently isolated by flotation on sucrose density gradients. When detergent-insoluble complexes were isolated under the experimental conditions described by Lisanti et al. [9], a single light-scattering band was observed within the 5–30% (w/v) sucrose region of the gradient (Figure 1A). Under these

conditions, the final concentration of Triton X-100 in the 40% sucrose region of the gradients was 0.5%, and the detergent was not included in the linear region of the gradient. Increasing the detergent concentration in the 40% sucrose region to 2% and including 0.5% Triton X-100 in the linear region of the gradient gave rise to two, clearly separate, light-scattering bands (Figure 1B). All subsequent experiments were conducted under the experimental conditions described in Figure 1(B), resulting in the separation of two light-scattering bands. The 'lower' band was concentrated around fractions 4 and 5 (just above the 40% sucrose interface), while the 'upper' band was located mainly in fractions 8 and 9. The majority (~50%) of the microsomal membrane protein remained in the 40% sucrose region of the gradient, with a further 16% being located in the insoluble pellet at the bottom of the centrifuge tube. The lower detergent-insoluble band contained ~18% of the total membrane protein, while the upper band contained only about 7%.

Characterization of the lipid and protein profiles of the two detergent-insoluble complexes

The lipid compositions of the two detergent-insoluble complexes isolated from porcine lung were determined (Table 1). On a mass-proportionate basis, both of the detergent-insoluble bands from the sucrose gradient were enriched ~2-fold in cholesterol and sphingomyelin (compared with the starting microsomal membranes). Conversely, the amounts of phosphatidylcholine and phosphatidylethanolamine were significantly reduced in these bands. Only trace levels of the acidic phospholipids, phosphatidylserine and phosphatidylinositol, could be detected in the detergent-insoluble bands, and the predominantly mitochondrial phosphoacylglycerol, diphosphatidylglycerol, could not be detected at all in either band. The only significant difference observed between the lipid compositions of the lower and upper detergent-insoluble bands was a significantly higher proportion of cerebrosides in the upper band as compared with the lower band.

The protein profiles of the two detergent-insoluble complexes were also investigated (Figure 2). The soluble membrane protein fraction in the 40% sucrose region of the gradient exhibited a similar Coomassie Blue staining pattern to that of the starting microsomal membranes, indicating that a large proportion of the microsomal membrane protein was effectively solubilized by Triton X-100. The protein staining patterns of the two detergent-insoluble bands were distinct from the microsomal membrane and solubilized protein profiles. A strong similarity could be observed between the protein profiles of the lower and upper detergent-insoluble bands, but several differences could be detected. Specifically, proteins of 140, 69, 35.5 and 13 kDa were more abundant in the lower band, and proteins of 56, 41 and 18 kDa were more abundant in the upper band.

Characterization of multiple polypeptide- and GPI-anchored ectoenzymes in the two detergent-insoluble complexes

Individual fractions from the sucrose gradient were also assayed for a variety of known ectoenzymes that are anchored by a single membrane-spanning polypeptide (Figure 3A). Aminopeptidases A and N and dipeptidyl peptidase IV, which are type II integral membrane proteins, and angiotensin-converting enzyme, which is a type I integral membrane protein, were all present to a slight extent (up to ~17% of total activity across the gradient) in the lower detergent-insoluble fraction. However, when the lower band was harvested from the gradient and washed extensively, 100% of the activities of aminopeptidases A and N and dipeptidyl

Figure 1 Effect of experimental conditions on the isolation of detergent-insoluble complexes from porcine lung membranes

Low-density, Triton-insoluble, complexes were prepared as described in the Experimental section. Sucrose gradients were harvested in 1 ml fractions (fraction 1, base of gradient; fraction 12, top of gradient), the absorbances of which were subsequently measured at 620 nm. (A) Membranes were solubilized in 1% Triton X-100 at a protein concentration of 15 mg/ml. The detergent concentration was then adjusted to 0.5% by the addition of an equal volume of 80% (w/v) sucrose. Samples were overlaid with a 5–30% linear sucrose gradient lacking Triton X-100. (B) Membranes were solubilized in 2% Triton X-100 at a protein concentration of 15 mg/ml. The detergent concentration was maintained at 2% by the addition of an equal volume of 80% sucrose containing 2% Triton X-100. Samples were overlaid with a 5–30% linear sucrose gradient containing 0.5% Triton X-100.

Table 1 Lipid composition of the detergent-insoluble complexes isolated from porcine lung

The two detergent-insoluble complexes obtained following ultracentrifugation in sucrose gradients were harvested and diluted 3-fold with MBS. Membranes were pelleted by further centrifugation at 11 600 *g* for 15 min and washed twice with MBS. Lipids from the microsomal membranes and the detergent-insoluble complexes were extracted and analysed by TLC as described in the Experimental section. Results are expressed on a mass-proportionate basis and are means $\pm$ S.E.M. ($n = 4$). n.d., not detectable.

Lipid class	Lipid composition (% by mass)		
	Microsomal membranes	Lower detergent-insoluble band	Upper detergent-insoluble band
Sphingomyelin	13.6 $\pm$ 1.9	27.5 $\pm$ 6.8	28.6 $\pm$ 5.5
Phosphatidylcholine	20.0 $\pm$ 0.9	12.1 $\pm$ 3.2	9.9 $\pm$ 1.6
Phosphatidylethanolamine	30.5 $\pm$ 1.4	21.5 $\pm$ 5.1	18.8 $\pm$ 5.5
Cerebrosides	n.d.	0.1 $\pm$ 0.2	5.9 $\pm$ 1.4
Cholesterol	17.7 $\pm$ 1.3	34.9 $\pm$ 1.3	35.1 $\pm$ 1.4
Phosphatidylinositol	6.3 $\pm$ 1.2	Trace	Trace
Phosphatidylserine	6.0 $\pm$ 2.2	Trace	Trace
Diphosphatidylglycerol	0.9 $\pm$ 0.4	n.d.	n.d.
Non-esterified fatty acids	4.1 $\pm$ 2.0	Trace	Trace

Figure 2 SDS/PAGE analysis of detergent-insoluble complexes isolated from porcine lung membranes

Low-density, Triton-insoluble, complexes were prepared as described in the Experimental section. The lower and upper detergent-insoluble bands were harvested separately from the sucrose gradient, diluted 3-fold with MBS, and pelleted by centrifugation at 11 600 *g* for 15 min. The fractions were washed a further three times with MBS, and 40 μ g of protein from each fraction was separated by SDS/PAGE. The gel was stained with Coomassie Blue. Lanes: M, microsomal membranes; SP, soluble protein taken from the 40% sucrose region; L, lower detergent-insoluble band; U, upper detergent-insoluble band. The arrows indicate protein bands which differed significantly in intensity between the two detergent-insoluble bands. Molecular mass markers (kDa) are shown.

peptidase IV remained in the supernatant (Table 2), indicating that the lower detergent-insoluble band is slightly contaminated by solubilized protein from the 40% sucrose region of the gradient. Only in the case of dipeptidyl peptidase IV could any activity be detected in the upper detergent-insoluble band (Table 2).

The activities of three GPI-anchored ectoenzymes, aminopeptidase P, alkaline phosphatase and 5'-nucleotidase, were also assayed in the fractions from the sucrose density gradient (Figure 3B). Regions of the gradient containing the lower detergent-insoluble band (fractions 4 and 5) retained 24, 21 and 17% of the

total activities across the gradient of aminopeptidase P, alkaline phosphatase and 5'-nucleotidase respectively. The amounts of the activities of these enzymes in the upper detergent-insoluble band (fractions 8 and 9) were generally higher than those in the lower band (27, 33 and 40% of total activity across the gradient respectively). Measurements of the GPI-anchored enzyme activities in the harvested and washed detergent-insoluble fractions (Table 2) indicated that alkaline phosphatase was enriched 3- and 9-fold in the lower and upper detergent-insoluble complexes respectively, relative to the starting microsomal membranes. The distribution of 5'-nucleotidase followed a similar but less pronounced trend, being 4- and 6-fold enriched in the lower and upper detergent-insoluble complexes respectively.

In order to verify the mode of membrane anchorage exhibited by aminopeptidase P, alkaline phosphatase and 5'-nucleotidase in the two detergent-insoluble complexes isolated from porcine lung membranes, samples were incubated with bacterial PI-PLC and then subjected to phase separation in Triton X-114 (Table 3). All three enzyme activities were released efficiently into the detergent-poor phase from the starting microsomal membranes and from the lower and upper detergent-insoluble complexes by the action of PI-PLC. Although significant amounts of the three GPI-anchored enzymes were released from the microsomal membranes at 37 °C in the absence of PI-PLC (Table 3), negligible release occurred at 4 °C, implicating the action of an endogenous hydrolase that did not co-isolate with the GPI-anchored enzymes in either of the two detergent-insoluble fractions.

Characterization of caveolar marker proteins in the two detergent-insoluble complexes

The sucrose gradient fractions were also examined for the presence of a number of proteins which have been identified in caveolae. Immunoelectrophoretic blot analysis revealed that both the lower and upper detergent-insoluble bands contained significant amounts of the 21–24 kDa integral membrane protein caveolin/VIP21 (Figure 4A). When the fractions from the sucrose gradient were subjected directly to immunoblot analysis with antibodies against the IP₃ receptor and the plasmalemmal Ca²⁺-ATPase, no signal was detected. The fractions corresponding to the detergent-insoluble bands were pooled and concentrated, and then immunoblotted with antibodies against caveolin, the IP₃ receptor and the plasmalemmal Ca²⁺-ATPase (Figures 4A

Figure 3 Distribution profiles of polypeptide- and GPI-anchored enzyme activities across the sucrose gradient

Low-density, Triton-insoluble, complexes were prepared as described in the Experimental section. **(A)** Polypeptide-anchored enzyme activities: angiotensin-converting enzyme, dipeptidyl peptidase IV, aminopeptidase A and aminopeptidase N. **(B)** GPI-anchored enzyme activities: aminopeptidase P, alkaline phosphatase and 5'-nucleotidase. Fraction 0 represents the insoluble pellet, fractions 1–4 the 40% sucrose region and fractions 5–12 the 5–30% linear sucrose gradient. The lower and upper bands were located in fractions 4–5 and fractions 8–9 respectively.

Table 2 Specific activities of polypeptide- and GPI-anchored ectoenzymes in detergent-insoluble complexes isolated from porcine lung

The two detergent-insoluble complexes obtained following ultracentrifugation in sucrose gradients were harvested and diluted 3-fold with MBS. Complexes were pelleted by further centrifugation at 11 600 *g* for 15 min and washed twice with MBS. Enzyme activities in the microsomal membranes and the detergent-insoluble complexes were assayed as described in the Experimental section. Results are means $\pm$ S.E.M. (*n* = 4). n.d., not detectable.

Enzyme	Specific activity (nmol of product/min per mg of protein)		
	Microsomal membranes	Lower detergent-insoluble band	Upper detergent-insoluble band
Polypeptide-anchored			
Dipeptidyl peptidase IV	52.2 $\pm$ 2.8	n.d.	5.6 $\pm$ 0.4
Aminopeptidase A	5.0 $\pm$ 0.1	n.d.	n.d.
Aminopeptidase N	12.2 $\pm$ 0.6	n.d.	n.d.
GPI-anchored			
Alkaline phosphatase	47.1 $\pm$ 1.2	139.1 $\pm$ 2.6	436.9 $\pm$ 7.4
5'-Nucleotidase	65.2 $\pm$ 1.1	270.1 $\pm$ 6.9	413.7 $\pm$ 30.3

and 4B). Although caveolin was strongly enriched in both of the detergent-insoluble bands as compared with the starting microsomal membranes, both the IP₃ receptor and the Ca²⁺-ATPase

were detected only in the lower detergent-insoluble band and not in the upper detergent-insoluble band. Fractions taken at 1 ml intervals from the sucrose gradient were subjected to SDS/PAGE

Table 3 Effect of PI-PLC treatment on the phase separation in Triton X-114 of GPI-anchored enzyme activities present in membranes isolated from porcine lung

Low-density, Triton-insoluble, complexes were prepared as described in the Experimental section. Fractions were washed extensively in 10 mM Tris/HCl/150 mM NaCl, pH 7.4, followed by incubation in the absence or presence of *B. thuringiensis* PI-PLC (1 unit/ml) for 1 h at 37 °C. Samples were then subjected to phase separation in Triton X-114 as described in the Experimental section. Enzyme activities were assayed in the resultant detergent-rich and detergent-poor phases, and the results are expressed as a percentage of the total enzyme activity present in the detergent-poor phase (means $\pm$ S.D., $n = 4$). n.d., not detectable.

Fraction	PI-PLC	Activity (% of that in detergent-poor phase)		
		Alkaline phosphatase	Aminopeptidase P	5'-Nucleotidase
Microsomal membranes	—	31.1 $\pm$ 1.4	31.4 $\pm$ 3.6	44.9 $\pm$ 0.9
	+	72.2 $\pm$ 6.3	78.5 $\pm$ 3.2	84.8 $\pm$ 4.6
Lower detergent-insoluble band	—	0.0	n.d.	0.0
	+	92.1 $\pm$ 1.1	n.d.	72.8 $\pm$ 2.2
Upper detergent-insoluble band	—	8.1 $\pm$ 1.2	0.0	0.0
	+	74.7 $\pm$ 1.9	46.3 $\pm$ 4.4	66.5 $\pm$ 0.8

Figure 4 Immunodetection of proteins in detergent-insoluble complexes isolated from porcine lung membranes

Low-density, Triton-insoluble, complexes were prepared as described in the Experimental section. (A) Caveolin; (C) β -actin. In the left-hand set of plates, a 20 μ l aliquot of each 1 ml fraction extracted from the sucrose gradient was separated by SDS/PAGE and transferred to poly(vinylidene difluoride) membranes (fraction 0 represents the insoluble pellet, fractions 1–4 the 40% sucrose region and fractions 5–12 the 5–30% linear sucrose gradient). In the right-hand set of plates, the lower and upper detergent-insoluble bands were harvested from the sucrose gradient, diluted 3-fold with MBS and pelleted by centrifugation at 11 600 g for 15 min. The complexes were washed a further three times with MBS and 20 μ g of protein from each fraction was separated by SDS/PAGE and immunodetected with the relevant antibody. (B) IP_3 receptor and plasmalemmal Ca^{2+} -ATPase. Protein (30 μ g) from each washed fraction was separated by SDS/PAGE, transferred to nitrocellulose and immunoblotted with the relevant antibody, as described in the Experimental section. Lanes: M, microsomal membranes; L, lower detergent-insoluble band; U, upper detergent-insoluble band.

and subsequent immunodetection using a monoclonal antibody against β -actin (Figure 4C). No antigen was detected in either the upper or lower detergent-insoluble band, even after prolonged

overexposure of the X-ray film. In fact, the cytoskeletal protein could only be detected in the 40% sucrose region of the sucrose gradient. However, subsequent harvesting and concentration of the detergent-insoluble complexes from the sucrose gradient, followed by immunoblotting, showed that β -actin was present in both of the detergent-insoluble complexes (Figure 4C).

Characterization of annexins in the two detergent-insoluble complexes

The presence in the two detergent-insoluble complexes of various members of the annexin family of proteins was also investigated (Figure 5). Immunoblotting with specific antibodies revealed that both the heavy and light chains of annexin II were associated solely with the lower detergent-insoluble band. The distribution of annexin VI paralleled that of annexin II, with the majority of the protein being located within the lower detergent-insoluble band. Annexins I and IV were absent from both the upper and lower detergent-insoluble bands (annexin IV appeared to be loosely associated with the detergent-insoluble complexes but was removed by washing in MBS). Finally, annexin V was present in both the upper and lower detergent-insoluble bands.

Effect of calcium concentration on the isolation of the two detergent-insoluble complexes

The detergent-insoluble complexes were isolated in the presence of either excess calcium or the metal chelator EGTA (Figure 6). Absorbance profiles of the resultant sucrose gradients (Figure 6A) showed that the inclusion of EGTA (10 mM) in the isolation buffers prevented formation of the lower detergent-insoluble band, which was clearly visible in fraction 4 in control experiments (no added calcium or EGTA). The addition of calcium (1 mM) to the isolation buffers resulted in a major light-scattering band at fractions 6–9 (in between the two bands observed under control conditions). The distribution of total protein throughout the sucrose gradients was altered very little by the absence or presence of calcium in the isolation buffers (Figure 6B), although the changes in the protein content of the upper band did follow the variations observed in the absorbance measurements. Similarly, the inclusion of either excess calcium or EGTA in the isolation buffers had a negligible effect on the distribution of the polypeptide-anchored ectoenzyme dipeptidyl peptidase IV (Figure 6C). In contrast, the distribution of the GPI-anchored ectoenzyme aminopeptidase P was radically altered by the inclusion of either excess calcium or EGTA (Figure 6D). Amino-

Figure 5 Immunodetection of annexins in detergent-insoluble complexes isolated from porcine lung membranes

Low-density, Triton-insoluble, complexes were prepared as described in the Experimental section. In the left-hand set of plates, a 20 μ l aliquot of each 1 ml fraction from the sucrose gradient was separated by SDS/PAGE and transferred to poly(vinylidene difluoride) membranes (fraction 0 represents the insoluble pellet, fractions 1–4 the 40% sucrose region and fractions 5–12 the 5–30% linear sucrose gradient). In the right-hand set of plates, the lower and upper detergent-insoluble bands were harvested from the sucrose gradient, diluted 3-fold with MBS and pelleted by centrifugation at 11 600 g for 15 min. The complexes were washed a further three times with MBS, and 20 μ g of protein from each fraction was separated by SDS/PAGE followed by transfer to poly(vinylidene difluoride) and immunodetection with the relevant antibody. Lanes: M, microsomal membranes; L, lower detergent-insoluble band; U, upper detergent-insoluble band.

peptidase P was present in both detergent-insoluble bands under control conditions, but the presence of EGTA in the isolation buffers resulted in this enzyme being distributed exclusively in the upper band, corresponding to the disappearance of the lower band as determined by absorbance measurements. The presence of excess calcium in the isolation buffers resulted in an exclusive distribution of aminopeptidase P in a detergent-insoluble band located at a density intermediate between the two bands detected in the control experiment. The change in the distribution of

caveolin upon isolation of the detergent-insoluble complexes in the presence of excess calcium was identical with that observed for aminopeptidase P (Figure 6E). In the presence of EGTA, caveolin and annexins II and VI dissociated from the lower detergent-insoluble band, with the bulk of the signal being detected in the 40% sucrose region of the gradient (fractions 1–4) for all three proteins (Figures 6E, 6F and 6G). The inclusion of excess calcium in the isolation buffers caused the association of both annexins II and VI with a single major detergent-insoluble band in fractions 7, 8 and 9 from the sucrose gradient, while the distribution of caveolin was not drastically altered.

DISCUSSION

In the present study, we have isolated detergent-insoluble complexes from porcine lung, a tissue which has been shown to be particularly abundant in caveolae [9,38]. By altering both the detergent concentration used to solubilize the lung microsomal membranes and that present in the sucrose gradient, we have been able to subfractionate detergent-insoluble complexes into two populations: a more dense fraction migrating at a lower region in the sucrose gradient, and a less dense fraction which achieves its buoyant density at a point further up the gradient (Figure 1B). Neither of the two detergent-insoluble complexes were enriched in angiotensin-converting enzyme, dipeptidyl peptidase IV, aminopeptidase A or aminopeptidase N (Figure 3A), consistent with our previous results showing that these polypeptide-anchored ectoenzymes are soluble in Triton X-100 [1]. However, both detergent-insoluble complexes were enriched in the GPI-anchored ectoenzymes alkaline phosphatase, aminopeptidase P and 5'-nucleotidase (Figure 3B), although the lower detergent-insoluble complex exhibited lower GPI-anchored ectoenzyme specific and total activities as compared with the upper complex (Table 2). All three of the GPI-anchored proteins were essentially equally sensitive to release by PI-PLC from both of the detergent-insoluble complexes (Table 3).

Membrane microdomains isolated on the basis of their insolubility in non-ionic detergents are enriched in sphingolipids and cholesterol, and it has been suggested that the glycolipids in such domains self-associate, giving rise to the property of detergent-insolubility [3,6,39,40]. Both of the detergent-insoluble complexes isolated from porcine lung membranes were found to be enriched ~2-fold in cholesterol and sphingomyelin (Table 1). The only significant difference in lipid composition between the two detergent-insoluble complexes was a significantly higher proportion of cerebroside in the upper as compared with the lower fraction.

Caveolae isolated from rat lung endothelium following *in situ* coating with cationic colloidal silica are enriched in caveolin, Ca^{2+} -ATPase and the IP_3 receptor [16]. Although both detergent-insoluble complexes isolated from porcine lung contained caveolin (Figure 4A), only the lower detergent-insoluble band contained the caveolar marker proteins Ca^{2+} -ATPase and the IP_3 receptor (Figure 4B), clearly indicating, along with the differences in the gross protein profiles (Figure 2), that the protein compositions of the two detergent-insoluble bands are distinct. Annexin II and annexin VI have also recently been detected in endothelial caveolae purified to homogeneity from rat lung tissue using cationic colloidal silica [27]. In the present study, both annexins II and VI were also found to be enriched in the lower detergent-insoluble complex isolated from porcine lung (Figure 5), again indicating that this fraction may represent a purer caveolar fraction than a bulk detergent-insoluble preparation. The presence of several calcium-binding proteins, as well as Ca^{2+} -ATPase

Figure 6 Effect of calcium on the isolation of detergent-insoluble complexes from porcine lung membranes

Low-density, Triton-insoluble, complexes were prepared as described in the Experimental section. Membranes were separated by sucrose gradient centrifugation under control conditions (●), in the presence of 1 mM CaCl_2 (○) or in the presence of 10 mM EGTA (△). Gradients were harvested in 1 ml fractions (fraction 0 represents the insoluble pellet, fractions 1–4 the 40% sucrose region and fractions 5–12 the 5–30% linear sucrose gradient). Relative amounts of caveolin, annexin II and annexin VI in each fraction were determined by immunoblotting, and the results are expressed as a percentage of the total signal detected following autoradiography. Dipeptidyl peptidase IV and aminopeptidase P activities were assayed as described in the Experimental section (the distribution of aminopeptidase P in EGTA-containing fractions was determined by immunoblotting with a polyclonal antibody to the protein).

and the IP_3 receptor, might suggest a role for caveolae in some Ca^{2+} -signalling events.

Fluorescence microscopy using a probe specific for G-actin has previously indicated that this cytoskeletal component is present in plasmalemma-associated 'beaded' structures [41]. On the assumption that these structures may have represented caveolae, Lisanti et al. [9] examined the binding of radio-iodinated Gc-Globulin to isolated detergent-insoluble complexes from mouse lung. Subsequent flotation of the detergent-insoluble membrane complexes revealed that the radiolabel did associate with the caveolin-rich membrane fraction. In addition, the cytoplasmic surface of caveolae can be labelled *in situ* with gold-conjugated myosin II subfragment 1, an actin-binding protein [42]. In the present study the concentration of actin in the fractions isolated directly from the sucrose gradient was below the level of detection (Figure 4C). However, following concentration of the fractions from the sucrose gradient corresponding to the upper and lower detergent-insoluble complexes, immunoblotting revealed the presence of β -actin in both complexes (Figure 4C). Caveolae isolated from rat lung endothelium by the use of cationic colloidal silica were also found to contain a small amount (approx. 10% of the total) of β -actin [16].

The annexins are a family of structurally related proteins that exhibit Ca^{2+} -dependent binding to phospholipids (see [43] for a recent review). These proteins have been implicated in a number of cellular processes, including membrane trafficking, transmembrane channel activity, phospholipase A_2 inhibition, transduction of mitogenic signals and mediation of cell-matrix interactions, as well as in the aggregation of biological membranes [44]. In particular, the annexin II-p11 complex aggregates chromaffin granules at much lower free Ca^{2+} concentrations ($\sim 1 \mu M$) than those required by other members of the annexin family ($> 100 \mu M$) [45]. Based on the observation that the lower detergent-insoluble complex isolated from porcine lung was enriched in annexin II, it seemed conceivable that certain membranous components may undergo annexin-mediated aggregation, giving rise to this denser lower band. In order to test this hypothesis, we isolated detergent-insoluble complexes in the presence of either excess calcium or the chelator EGTA, on the assumption that, as calcium is required for the association of annexins with phospholipid molecules, sequestration of the cation should release the protein from membranes, preventing annexin-mediated aggregation. The presence of low concentrations of the bivalent cation calcium seems to be a prerequisite for the formation of the lower band. In its absence both annexins II and VI, along with caveolin, dissociated from the detergent-insoluble complex and were recovered in the soluble part of the sucrose gradient, concomitant with a loss of two distinct detergent-insoluble bands (Figure 6). In contrast, excess calcium appeared to cause a gross aggregation of the two detergent-insoluble complexes into a single complex with an intermediate density.

Annexin II has been identified as a major component of detergent-insoluble complexes isolated from MDCK cells [46]. These authors discussed the apparent discrepancy between their observation and previous studies, which either failed to detect annexin II or only identified low levels of this protein in such complexes, as being due to the presence of chelators in the isolation buffers. Our results confirm this suggestion and highlight the importance of the relative concentration of calcium in both the isolation and solubilization buffers used in the preparation of detergent-insoluble membrane microdomains. As both annexins II and VI are present in pure caveolae preparations [27], it is possible that, when a sufficient (but not excessive) calcium concentration is maintained at the point of tissue homogenization or membrane solubilization, these molecules form a protein-

protein bridge between adjacent caveolar membranes, producing a membrane aggregate that migrates at a higher density in the sucrose gradient, separate from the bulk of the detergent-insoluble membrane material.

A survey of the literature indicates that the majority of researchers have isolated detergent-insoluble complexes in the absence of chelating agents in the buffers [9,10,21,22], and in most cases have observed only a single light-scattering band on sucrose gradient fractionation. In the light of the results of the present study, this could be explained if the calcium concentration rose above a certain point and caused a gross aggregation of the detergent-insoluble complexes. Interestingly, in a few cases the existence of subpopulations of detergent-insoluble complexes has been observed. For example, the GPI-anchored urokinase-type plasminogen activator receptor was observed to have a broader distribution on sucrose gradient fractionation of detergent-insoluble membrane material than caveolin [10], leading the authors to suggest 'that under certain thus far undefined conditions, GPI-linked molecules exist in Triton X-100 insoluble, low density complexes that differ from caveolin-coated caveolae'. The observation in the present study that, in the presence of EGTA, caveolin is found in the soluble fraction, whereas the GPI-anchored aminopeptidase P remained in a detergent-insoluble fraction (Figure 6), would support this suggestion. These results also reinforce the observation that the property of association of GPI-anchored proteins in detergent-insoluble domains with cholesterol and sphingolipids is an intrinsic property of this type of protein [4,5] that is independent of the presence of caveolin.

Recently, several GPI-anchored proteins (F3, NCAM-120 and Thy-1) were observed to reside in two distinct detergent-insoluble complexes isolated from rat brain membranes [47]. In contrast to the results of the present study (see Table 3), the GPI-anchored F3 in the lower detergent-insoluble band isolated from rat brain membranes was found to be resistant to release by bacterial PI-PLC, whereas that in the upper detergent-insoluble band was susceptible to release. In addition, sucrose density-gradient fractionation of silica-coated rat lung endothelial cell membranes solubilized with Triton X-100 revealed the presence of a broad band of caveolin-containing fractions, only a proportion of which were pooled and characterized further [16]. Similarly, upon isolation of caveolae in the absence of detergent, a broad distribution of caveolin was observed on sucrose density-gradient fractionation, of which only a subpopulation was harvested and used for further study [48]. Attempts to isolate membrane microdomains in the absence of detergent and without EGTA in the isolation buffers have been unsuccessful due to aggregation of the membranes, possibly mediated by annexins (E. T. Parkin, A. J. Turner and N. M. Hooper, unpublished work). The calcium chelators EGTA and EDTA have sometimes been included in the buffers used to isolate detergent-insoluble complexes [3,7,8] which, from the results of the present study, would lead to the removal of annexins II and VI, with subsequent isolation of a single detergent-insoluble complex.

In conclusion, we have subfractionated the detergent-insoluble complex isolated from porcine lung membranes into two fractions, only one of which is enriched in the caveolar marker proteins Ca^{2+} -ATPase and the IP_3 receptor. The enrichment of annexins II and VI in this fraction, and the observation that the subfractionation into two distinct detergent-insoluble complexes can be altered by a change of the calcium concentration in the isolation and solubilization buffers, suggests that annexins and Ca^{2+} may be critically involved in the formation of certain detergent-insoluble membrane microdomains, and further that they may be involved in the formation and function of caveolae.

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