Cholesterol and Sphingolipid Enhance the Triton X-100 Insolubility of Glycosylphosphatidylinositol-anchored Proteins by Promoting the Formation of Detergent-insoluble Ordered Membrane Domains*

(Received for publication, May 29, 1997, and in revised form, October 1, 1997)

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Glycosylphosphatidylinositol (GPI)-anchored proteins can be isolated from both cells and sphingolipid and cholesterol-rich liposomes (SCRLs) in association with detergent-insoluble membranes. We found previously that detergent insolubility of lipids was characteristic of phases in which lipid acyl chains are ordered. We presented evidence that GPI-anchored proteins are insoluble because they associate with cholesterol and sphingolipid-rich lipid domains with properties similar to the liquid-ordered phase. Here, this model was tested by a variety of approaches. First, we demonstrated that saponin, which removes cholesterol from cell membranes and allows solubilization of GPI-anchored proteins by Triton X-100, had the same effect on the GPI-anchored protein alkaline phosphatase (PLAP) in SCRLs of appropriate lipid composition. The similarity of saponin action in cells and simple liposomes suggests that the compound disrupts protein-lipid interactions. However, direct interactions between PLAP and cholesterol were not needed for insolubility, because the protein was also insoluble in cholesterol-free liposomes containing lipid in an ordered phase. Instead, cholesterol acted by greatly enhancing the formation of a detergent-insoluble phase by sphingolipids, which have a tendency to form ordered phases. We propose that saponin solubilizes GPI-anchored proteins because the lipid composition of cell membranes (and the SCRLs used above) supports ordered phase formation in the presence but not the absence of cholesterol. Supporting this model, saponin did not promote Triton X-100 solubilization of PLAP in SCRLs with sphingolipid levels high enough to allow ordered phase formation in the absence of cholesterol. We also showed that two additional GPI-anchored proteins are detergent-insoluble in SCRLs and that detergent does not artifically create ordered domains or cause components of solubilized membranes to associate with detergent-resistant membranes present in separate bilayers in the same lysate. We conclude that the ordered domain model explains the behavior of detergent-resistant membranes in liposomes and cells.

A hallmark of GPI-anchored proteins in eukaryotic cells is their insolubility in non-ionic detergents (1–3). This property is surprising because GPI-anchored proteins are restricted to the extracellular leaflet of the lipid bilayer, and it has been generally assumed that most detergent-insoluble membrane proteins bind to the cytoskeleton. We showed earlier that GPI-anchored proteins are present in detergent-resistant membrane (DRM) fragments that can be isolated from cell lysates after extraction (3). A number of other groups have replicated this finding in a wide variety of cell types (4–9). These findings suggested that direct interactions between GPI-anchored proteins and lipids in detergent-insoluble membrane domains might lead to detergent insolubility. Further support for this proposal came from studies in which a GPI-anchored protein, PLAP, was incorporated into sphingolipid and cholesterol-rich liposomes (SCRLs), which have a lipid composition similar to that of detergent-insoluble membrane domains isolated from cells (10). The protein associated with detergent-insoluble membranes derived from SCRLs after extraction. This result showed directly that interactions between PLAP and lipids were sufficient for detergent insolubility of the protein; no other proteins were required. Based on further liposome studies on the lipid requirements for detergent insolubility, we proposed that DRMs are present in cell membranes as domains in a sphingolipid and cholesterol-rich ordered phase.

DRMs are similar in several ways to the liquid-ordered (l o) phase previously described in model membranes (11–15). l o phase domains can co-exist in bilayers with domains in the more familiar gel or liquid crystalline (l c) phases (14). Pure phospholipids exist in a solid, ordered gel phase at low temperatures. Above a melting temperature (T_m) that is characteristic of each lipid, pure lipids form the fluid, disordered l c phase. Most of the phospholipids in cell membranes have low T_m values and are believed to be in the l c phase. The l c phase, which requires sterol to form, has properties intermediate between gel and l c phases. It has been observed in mixtures of cholesterol with lipids whose highly saturated acyl chains gives them a high T_m in the pure state (11–15). Both saturated chain phosphatidylycholines and sphingomyelin can form the l c phase when mixed with cholesterol (13).

* This work was supported by National Institutes of Health Grants GM 47997 (to D. A. B.) and GM 48596 (to E. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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DRMs have several properties in common with the l phase. Both are rich in cholesterol, are detergent-resistant, and contain lipids with similar acyl chain mobility (10). Because of the complexity of cell membranes, further characterization of the physical properties of cellular DRMs is difficult. As a useful working model, we have proposed that DRMs arise from domains in cell membranes that are in the l phase or a phase similar to it. It is likely that the saturated acyl chains generally found in GPI-anchored proteins (16) mediate the association of these proteins with detergent-insoluble domains.

Cerneus et al. (17) showed that after treatment of mammalian cells with saponin, GPI-anchored proteins could readily be solubilized by Triton X-100. Saponin, like other cardiac glycosides, complexes with membrane cholesterol, sequestering this lipid away from other interactions (18). This result suggested that the Triton insolubility of GPI-anchored proteins requires cholesterol. Similarly, Hanada et al. (19) found that the Triton X-100 resistance of PLAP depended on the presence of cholesterol, as well as of sphingolipids. Neither study provided a mechanism for this phenomenon. Based on our model, we propose that the sphingolipid and cholesterol dependence of GPI-anchored protein insolubility does not depend on a specific interaction between the proteins and these lipids. Rather, sphingolipids and cholesterol are required to maintain detergent-insoluble ordered domains in the membranes.

In this paper, we used model membranes to test this and other predictions of our model of detergent insolubility. First, we examined the mechanism underlying the requirement of sphingolipids and cholesterol for detergent insolubility of GPI-anchored proteins. We also examined the detergent insolubility of two other GPI-anchored proteins and a transmembrane protein in SCRLs. In addition, we determined how well detergent insolubility reflects membrane properties prior to detergent addition. The results strongly suggest that detergent insolubility is a useful method for investigating cell membrane structure.

EXPERIMENTAL PROCEDURES

Materials—Brain phosphatidylcholine (brain PC), bovine liver phosphatidylcholine (bovine PC), diosyleoylphosphatidylcholine (DOPC), distearoylphosphatidylcholine (DSPC), brain sphingomyelin, and brain cerebrosides were from Avanti Polar Lipids (Alabaster, AL). Cholesterol was from Avanti or Sigma. [3H]Phosphoglycerin (80 Ci/mmol; 1 mCi/ml) was from American Radiolabeled Chemicals (St. Louis, MO). [7-3H]Cholesterol (5–15 Ci/mmol; [3H]cholesterol) and L-3-phosphatidyl[3H]-choline, 1,2-dipalmitoyl (40–85 Ci/mmol; [3H]DPPC) were from American Radiolabeled Chemicals (St. Louis, MO). 

Analysis of Triton X-100 Insolubility—Bacteriorhodopsin from supernatant and pellet fractions was precipitated with 10% trichloroacetic acid in the presence of 10 μg of ovalbumin as carrier, resuspended in gel loading buffer, and subjected to SDS-PAGE. PLAP was either analyzed by the same method or was harvested from supernatant and pellet (after solubilizing in 1 ml of PBS containing 1% Triton X-100 with warming to 37 °C for 5 min) by immunoprecipitation as from cell lysates (25) before SDS-PAGE. PAGE was detected by Western blotting using 1–2 μg/ml antibodies and ECL. Bacteriorhodopsin was detected by silver staining. All bands were quantitated using a Bio-Rad G-670 scanning densitometer.

For acetylated, cholesteralysin, supernatant and pellet fractions were precipitated with 10% trichloroacetic acid in the presence of 10 μg of ovalbumin as carrier, resuspended in gel loading buffer, and subjected to SDS-PAGE. PLAP was either analyzed by the same method or was harvested from supernatant and pellet (after solubilizing in 1 ml of PBS containing 1% Triton X-100 with warming to 37 °C for 5 min) by immunoprecipitation as from cell lysates (25) before SDS-PAGE. PAGE was detected by Western blotting using 1–2 μg/ml antibodies and ECL. Bacteriorhodopsin was detected by silver staining. All bands were quantitated using a Bio-Rad G-670 scanning densitometer.

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that those in the fluid lc phase. At least some lipids (including S
and about 100 ng of protein were incubated with (+Sap) or without
to (-Sap) 0.4% saponin on ice. They were then extracted with Triton and
subjected to ultracentrifugation. Protein in supernatant (S) and pellet (P)
fractons was analyzed by SDS-PAGE and Western blotting after
ultracentrifugation of lysates.

Light Scattering Measurements—DPPC/cholesterol MLVs were pre-
pared and treated with Triton X-100 as described above. The light
scattering of each sample before and after Triton X-100 treatment was
measured in a 1-cm excitation and 4-mm emission path length quartz
cuvette in a Spex 212 Fluorolog spectrofluorimeter, using 0.25-mm
excitation and emission slits. Both excitation and emission wavelengths
were 550 nm. The cuvette was maintained at 45 °C in a Neslab RTE 100
temperature bath. Samples were incubated in the spectrofluorimeter in
the dark for 2 min. Three individual light scattering readings of 1 s each
were then made and averaged. The ratio of post-Triton X-100 to pre-
Triton X-100 light scattering was calculated.

Concentration of solutions is expressed as percent weight/volume for
solids and percent volume/volume for liquids.

RESULTS

Solubilization of PLAP from Sphingolipid and Cholesterol
Containing Liposomes after Saponin Treatment—Cerneus et al.
(17) showed that pretreatment of BeWo cells with a low con-
centration of saponin, which complexes with membrane choles-
terol, resulted in solubilization of PLAP upon addition of Triton
X-100. We confirmed this finding in Madin-Darby canine kid-
ney cells (26). Here, we asked whether saponin had a similar
effect on PLAP in vitro. We have previously shown that PLAP is
insoluble in Triton X-100 when incorporated into SCRLs (10).
For reasons that will be discussed below, in this experiment we
used “low sphingolipid” SCRLs that contained only 10% total
sphingolipid. Liposomes were treated with or without saponin
and then extracted with Triton X-100. Soluble and insoluble fractions were separated by ultracentrifugation. Proteins iso-
lated from each fraction were analyzed by SDS-PAGE and
Western blotting. As shown in Fig. 1A, saponin pretreatment
greatly increased Triton X-100 solubilization of PLAP from
these liposomes, as it does from cells. (Average of two experi-
ments: 91% insoluble without saponin and 2% insoluble with
saponin.) Fig. 1B will be discussed below.

Cholesterol Is Not Required for Detergent Resistance of
PLAP: Insolubility in Gel Phase Bilayers—The role of choles-
terol in the detergent insolubility of GPI-anchored proteins is
not known. According to our model, PLAP is Triton X-100-
insoluble because it associates with cholesterol-rich ordered
domains. Thus, we predict that the role of cholesterol in insol-
ubility is to maintain lipids in the ordered state. Alternatively,
one might imagine that GPI-anchored proteins must interact
directly and specifically with cholesterol to be insoluble.

Acyl chains in both lc and gel phase lipids are more ordered
than those in the fluid lc phase. At least some lipids (including
DPPC) are detergent-insoluble when present in the gel phase
(10, 29), as is true for the lc phase (10). Thus, if GPI-anchored
proteins partition into an ordered, detergent-insoluble environ-
ment, as we proposed, then they should associate with choles-
terol-free gel phase domains as well as cholesterol-rich ordered
domains. Alternatively, if cholesterol is absolutely required for

insolubility, then Triton X-100 should solubilize PLAP from gel
phase membranes.

Gel phase DPPC liposomes containing PLAP were subjected
to detergent extraction. As shown in Fig. 2A, the protein was
completely insoluble. However, it seemed possible that the
protein might be “forced” into the gel phase as it was the only
phase available. To test this possibility, we incorporated PLAP
into DPPC/DOPC 1:1 liposomes. As the detergent extraction
was performed on ice, DPPC (Tm = 41 °C) was largely present
in the gel phase, and DOPC (Tm = −20 °C) was largely present
in the lc phase (29). PLAP was still completely Triton X-100-
insoluble (Fig. 2B), showing that it associated with the deter-
genolipid-gel phase even when a fluid phase was present.
PLAP was fully soluble in control fluid phase liver PC/chole-
terol 7:1 liposomes (Fig. 2C). As a control for the specificity of
the saponin effect on PLAP in SCRLs (Fig. 1A), DPPC or
DPPC/DOPC liposomes were also treated with saponin before
addition of Triton X-100 (Fig. 2, D and E). As expected, addition
of saponin had no effect on insolubility. This experiment
showed that saponin does not interact with liposomes or with
Triton X-100 in any unexpected cholesterol-independent man-
ner and that 0.4% saponin by itself does not solubilize PLAP.

Together, these results showed that cholesterol is not abso-
lutely required for detergent insolubility of PLAP. Instead,
they support our model that detergent insolubility of GPI-
anchored proteins results from their association with an or-
dered lipid phase.

Cholesterol Enhances GPI-anchored Protein Insolubility by
Promoting Sphingolipid Insolubility—Our previous results
(10) and Fig. 1 (−Sap) show that PLAP is Triton X-100-insol-
uble when it is present in detergent-insoluble cholesterol and
sphingolipid-rich membranes. The results in Fig. 2 show that
the protein is also insoluble when it is in liposomes containing
lipids in the gel phase. Lipids with high Tm, such as sphingo-
lipids, can form a gel phase at or near physiological tempera-
tures (30). Alternatively, they can form an lc phase in the
presence of cholesterol (13). Thus, we might expect that after
removal of cholesterol with saponin, sphingolipids in cell mem-
branes or SCRLs would form a detergent-insoluble gel phase
and that PLAP could associate with this phase. Why then was
PLAP solubilized from SCRLs after saponin treatment (Fig. 1A, +Sap)?

Because formation of an ordered phase by sphingolipids de-
Cholesterol Promotes Insolubility of GPI-anchored Proteins

To test this model for the lipids used in this study, we examined the effect of cholesterol and sphingolipid levels on the solubilization of liposomes by Triton X-100. We made three series of MLVs, each containing \([3H]\)sphingomyelin as a tracer. The first set of liposomes contained graded mixtures of brain PC and sphingolipids (cerebrosides and sphingomyelin 1:1) and a constant 33 mol % cholesterol. The liposomes were subjected to extraction with 1% Triton X-100, followed by ultracentrifugation. Radioactivity in both supernatant and pellet fractions was measured in a scintillation counter. Results (Fig. 3A, squares) showed that the percentage of insoluble sphingomyelin increased with increasing sphingolipid. Results were similar when \([3H]\)cholesterol was substituted for \([3H]\)sphingomyelin (triangles). We next repeated the experiment, using liposomes that lacked cholesterol. As seen in Fig. 3A (circles), the percentage of insoluble \([3H]\)sphingomyelin again increased with increasing sphingolipid. These results are consistent with the idea that high sphingolipid concentration promotes a detergent-insoluble ordered phase. At each concentration of sphingolipids, much less \([3H]\)sphingomyelin was insoluble in liposomes lacking cholesterol (circles) than in cholesterol-replete liposomes (squares), consistent with the promotion of a detergent-insoluble ordered phase by cholesterol.

Finally, we repeated the experiment using phospholipid, sphingolipid, and cholesterol-containing liposomes (Fig. 3A, squares) but pretreating the liposomes with saponin before the Triton X-100 extraction. Results are shown in Fig. 3A (diamonds). As expected, after removal of cholesterol with saponin, insolvency was very similar to that seen in liposomes without cholesterol (compare diamonds and circles). In each case, only a small amount of insoluble \([3H]\)sphingomyelin remained after extraction of low sphingolipid membranes.

Saponin would not be expected to affect the solubility of liposomes that do not contain cholesterol. To test this, three sets of liposomes, containing 10, 30, or 50% sphingolipids, were constructed and extracted exactly as in Fig. 3A, circles, except that liposomes were treated with 0.4% saponin before addition of Triton X-100. 10, 21, and 29% of the \([3H]\)sphingomyelin in the three sets of liposomes, respectively, were insoluble (not shown). As expected, these results are very similar to those obtained without saponin treatment (compare relevant points in Fig. 3A, circles).

For technical reasons, in our experiments protein-containing liposomes were LUVs, and protein-free liposomes were MLVs. It was important to ensure that the lipid solubility of MLVs and LUVs was similar, especially when comparing the solubility of lipids and proteins in liposomes of the same lipid composition (i.e., Figs. 1 and 3A). For this comparison, three sets of LUVs were constructed exactly as in Fig. 1, except that no protein was added, and the liposomes contained \([3H]\)sphingomyelin. These LUVs contained 10, 33, or 50% sphingolipid. All contained 33% cholesterol, and the balance was brain PC. Liposomes containing 200 \(\mu\)g of lipid were subjected to Triton X-100 extraction with or without saponin pretreatment, and the fraction of insoluble \([3H]\)sphingomyelin was determined. Results are shown in Fig. 3B. As seen by comparison with Fig. 3A, insolubility of \([3H]\)sphingomyelin in LUVs and MLVs containing 10 and 33% sphingolipid was very similar. Differences in insolubility in liposomes containing 50% sphingolipid probably reflect structural differences between MLVs and LUVs but are less relevant as PLAP was not incorporated into these liposomes.

The Saponin Effect Can Be Overcome at High Sphingolipid Concentrations—These results support the prediction of our model that saponin facilitates Triton X-100 solubilization of PLAP by destroying the detergent-insoluble lipid phase with which insoluble PLAP associates. They also predict that the saponin effect could be overcome if the amount of sphingolipid

![Fig. 3. Effect of cholesterol and saponin on Triton insolubility of sphingomyelin. MLVs (A) or LUVs (B) containing graded ratios of brain PC and sphingolipids (cerebrosides:sphingomyelin 1:1), and 0.1 \(\mu\)C of \([3H]\)sphingomyelin (squares, diamonds, and circles) or \([3H]\)cholesterol (triangles) with (squares, diamonds, and triangles) or without (circles) 33 mol % cholesterol were subjected to Triton extraction with (diamonds) or without (squares, circles, and triangles) pretreatment with 0.4% saponin. Each sample contained 200 \(\mu\)g of total lipid. Insoluble lipids were pelleted by ultracentrifugation, and the radioactivity in supernatant and pellet fractions was measured. The fraction of the total that was pelletable is plotted. Each sample (supernatant + pellet) contained about 50,000 cpm.](image-url)
TABLE I

| Protein             | % Insoluble in SCRLs | % Insoluble (control) |
|---------------------|----------------------|-----------------------|
| Human acetylcholinesterase | 73 ± 4 (n = 3)       | 25 ± 1 (n = 3)        |
| Bovine acetylcholinesterase  | 97 ± 4 (n = 3)       | 61 ± 4 (n = 2)        |
| Bacteriorhodopsin     | 15 ± 6 (n = 2)       |                       |

were high enough to form a detergent-insoluble phase after cholesterol removal. The insoluble lipid observed at higher sphingolipid concentrations in the absence of cholesterol in Fig. 3A, circles, is probably in the gel phase, as gel phase separation can be detected by other methods in similar lipid mixtures (31). To test this prediction, we examined the effect of saponin treatment on Triton X-100-solubilization of PLAP from our usual SCRLs, which contain a relatively high sphingolipid concentration of 33 mol %. As shown in Fig. 1B, 83% of PLAP remained detergent-insoluble even after saponin treatment, whereas 99% of the protein was insoluble in the absence of saponin (average of five experiments). As we know from Fig. 3 and the experiments described above that the lipid insolubility of these saponin-treated liposomes is very similar to that of liposomes that lack cholesterol, we presume that PLAP associated with detergent-insoluble membranes, presumably in the gel phase, that remained after cholesterol removal.

The experiments presented provide a molecular explanation for the effect of saponin on solubilization of PLAP and provide an example of how our model for the structure of DRMs fits data obtained from cells. In the studies described next, we performed important controls that further establish the validity of our in vitro system.

Both Bovine and Human Acetylcholinesterase in SCRLs Are Triton X-100-insoluble—As PLAP is the only GPI-anchored protein we have studied so far, we next examined the detergent extractability of two other GPI-anchored proteins, bovine and human acetylcholinesterase, in SCRLs. These proteins were incorporated separately into preformed SCRLs by dialysis, as described for PLAP (10). Liposomes were then subjected to detergent extraction. Soluble and insoluble fractions were separated by centrifugation and concentrated, and acetylcholinesterase enzyme activity in both pools was measured (27). To ensure that pelletable protein was not simply aggregated, human acetylcholinesterase was also incorporated into liposomes containing liver PC and cholesterol in a 7:1 ratio. Lipids in these liposomes are fully solubilized by Triton X-100 (10). Due to the high detergent concentration in the purified bovine acetylcholinesterase, it could not be incorporated into liver PC/cholesterol 7:1 liposomes. For this reason, to control for aggregation of this protein, it was incorporated into SCRLs, and the liposomes were extracted in Triton X-100 at 37 °C. After this treatment, GPI-anchored proteins from DRMs from model membranes cells (10) and cells (26, 32) remain in the supernatant after high speed centrifugation. Thus, the amount of bovine acetylcholinesterase that could be pelleted after this treatment was taken as a measure of aggregation. Results are shown in Table I. Significant amounts of both proteins pelleted after Triton X-100 extraction of SCRLs. A fraction of both proteins also pelleted under control conditions, when there should have been no association with DRMs. This was especially true for bovine acetylcholinesterase, which thus appeared to be somewhat aggregated. However, it was clear that a substantially higher fraction of both proteins pelleted after incorporation into SCRLs and extraction on ice than under control conditions. We conclude that both proteins can associate with DRMs.

The Transmembrane Protein Bacteriorhodopsin Is Triton X-100-soluble in SCRLs—We wanted to ensure that not all proteins were resistant to Triton X-100 solubilization when incorporated into SCRLs. Most transmembrane proteins in cells are solubilized by Triton X-100 (26). The multiple membrane-spanning protein bacteriorhodopsin was incorporated into SCRLs. After exposure to Triton X-100 or mock extraction, solubilized and insoluble material were separated by ultracentrifugation. Protein was collected by trichloroacetic acid precipitation from supernatant and pellet fractions, analyzed by SDS-PAGE, and detected by silver staining. Protein in each fraction was quantitated by scanning densitometry of the gel. As shown in Table I, although the protein could be pelleted when liposomes were not extracted with detergent, the protein was efficiently solubilized by Triton X-100. Thus, the detergent insolubility of bovine and human acetylcholinesterase and PLAP must reflect a specific association with insoluble membrane domains.

Detergent-resistant Membranes Are Not Formed from Model Bilayers That Do Not Contain l\, Phase Domains—According to our model, DRMs originate from domains in an ordered state that co-exist with fluid l\, phase domains in cell membranes prior to detergent extraction. We were concerned by the alternative possibility that separate phases might not exist in membranes until detergent exposure. DRMs might only be formed after selective solubilization of certain lipids. To test this possibility, we determined whether DRMs could be isolated from liposomes that do not contain ordered domains but that contain lipids with the potential to form such domains. For this experiment, we used a system in which formation of l\, phase domains has been well characterized: liposomes containing mixtures of DPPC and cholesterol. The phase diagram showing the conditions of temperature and lipid composition under which l\, phase domains form in these mixtures has been derived (14). Above the T_m of pure DPPC (41 °C), a uniform l\, phase is present at low concentrations of cholesterol. Above a threshold cholesterol concentration, a separate l\, phase begins to form. (The fact that a threshold lipid concentration of either lipid in a binary mixture is required for phase separation is a well known feature of lipid phase behavior.)

We reasoned that if detergent selectively solubilized some of the DPPC in l\, phase DPPC/cholesterol mixtures, then the cholesterol concentration remaining in the bilayer would rise, leading to the formation of detergent-resistant l\, phase domains. Alternatively, if such detergent-induced reorganization did not occur, then DRMs should only be recovered from liposomes in which l\, phase domains were present before detergent extraction. To test this idea, a series of MLVs containing DPPC and cholesterol in varying ratios was constructed. A small amount of [3H]DPPC or [3H]cholesterol was incorporated into these liposomes as a tracer. (Because both tracers gave very similar results, only those using [3H]DPPC are shown.) Liposomes were subjected to detergent extraction at 45 °C (above the T_m of pure DPPC) and then to ultracentrifugation. Supernatant and pellet fractions were separated, and the radioactivity in each was measured. Results are shown in Fig. 4A (squares). The fraction of the lipid in each set of liposomes that was present in the l\, phase, as determined from the phase diagram (14), is shown in the same figure (diamonds). Formation of the l\, phase occurs at a threshold cholesterol concentration of about 5 mol %. Above about 30 mol % cholesterol, the liposomes are entirely in the l\, phase (14). Comparing the two
Fig. 4. Comparison of detergent insolubility and phase behavior of DPPC/cholesterol mixtures. A and B (squares), MLVs made from graded mixtures of DPPC and cholesterol (totaling 400 μg of lipid) and 0.1 μCi of [3H]DPPC were extracted with Triton at 45 °C. Radioactivity in supernatant and pellet fractions was measured after ultracentrifugation at 4 °C, and the percent of total counts in the pellet was plotted. About 50,000 cpm total counts (supernatant + pellet) were present in each sample. A (diamonds), the fraction of total lipid in the lα phase at 45 °C was determined from a published phase diagram (14). B (diamonds), MLVs made as in A but without [3H]DPPC were extracted with Triton at 45 °C. Light scattering of each sample was measured before and after extraction; the ratio of the two values (after/before extraction) is plotted.

Curves showed that insolubility paralleled lα phase formation, although the former was shifted slightly to the right (i.e. to higher cholesterol concentrations) of the latter. Thus, in this system insoluble membranes were only obtained from liposomes that contained lipids in the lα phase before extraction, and detergent did not create insoluble membranes where lα phase domains did not exist.

We have previously shown that completely fluid liposomes are fully solubilized by Triton, whereas completely lα phase membranes are fully insoluble (10). For this reason, it is likely that lα phase domains present in the DPPC/cholesterol mixtures were preferentially solubilized and that most of the remaining insoluble lipid was in the lα phase. (However, our data do not prove this point.) If lα phase domains were actually solubilized preferentially, then detergent insolubility could be taken as an indication of the amount of membrane in the lα phase in the two-phase mixture. However, the shift between the two curves in Fig. 4A showed that at least some lα phase membrane was solubilized. For this reason, detergent insolubility is at best an imperfect measure of the amount of lα phase in two-phase mixtures. Even if lα phase domains are preferentially solubilized, detergent insolubility will underestimate the amount of lα phase present.

We were concerned that our only measure of lipid insolubility was pelleting during ultracentrifugation. Although control experiments have shown that similar liposomes pellet quite efficiently (10), the possibility remained that some liposomes might not pellet completely, especially under conditions close to solubilization. Incomplete pelleting would lead to an underestimation of the amount of insoluble membrane. To validate our findings using a second independent measure of solubilization, we took advantage of the fact that liposomes scatter light much more efficiently than detergent-lipid micelles. Thus, turbidity can be used as a measure of detergent solubilization (33, 34).

We repeated the extraction experiments shown in Fig. 4A, measuring the light scattering of each sample before and after extraction. The turbidity of each sample, expressed as the ratio of the scattering intensity after detergent extraction to the scattering intensity before extraction, is plotted in Fig. 4B (diamonds). Liposomes containing less than 15% cholesterol showed a low and fairly constant turbidity, probably reflecting the light scattering of detergent-lipid micelles. As the cholesterol concentration was raised to ≥20 mol %, the turbidity of detergent-extracted samples increased, reflecting the presence of insoluble membranes. The ultracentrifugation results shown in Fig. 4A are replotted for comparison in Fig. 4B (squares). The concentration of cholesterol required for insolubility (15–20 mol %) was strikingly similar in both cases. Thus, very similar results were obtained using two independent techniques.

PLAP Must Be in a Bilayer That Contains Detergent-resistant Domains to be Detergent-resistant—It seemed possible that detergent extraction might artificially induce GPI-anchored proteins to associate with lα phase domains. To test this possibility, we asked whether GPI-anchored proteins must be present in bilayers that contain detergent-insoluble domains to associate with DRMs during solubilization. If detergent extraction drives proteins into DRMs during solubilization, then they might be expected to “hop” into DRMs present in separate bilayers in the same lysate. We performed a mixing experiment to address this question. PLAP was incorporated into LUVs containing liver PC and cholesterol in a 7:1 ratio. These liposomes are fully solubilized by Triton X-100 (10). The PLAP-containing 7:1 liposomes were mixed with protein-free PC/cholesterol 7:1 LUVs (Fig. 5, left) or with SCRLs (Fig. 5, right), and the mixtures were subjected to detergent extraction and ultracentrifugation. As expected, no detectable pellet was observed after extraction and ultracentrifugation of PC/cholesterol 7:1 liposomes. A pellet of the expected size was observed in the tube containing SCRLs mixed with PLAP-containing PC/cholesterol 7:1 after detergent extraction and ultracentrifugation, indicating that DRMs derived from the SCRLs were present. Nevertheless, as shown in Fig. 5, the protein was...
Insolubility of Proteins in Liposomes Mimics That in Cell Membranes—Phase behavior of membrane lipids is much better understood in model membranes than in cells. This is because many of the techniques used to study phase separation in liposomes are not feasible in cells, due to the greater complexity of biological membranes. Detergent insolubility is an exception. We have proposed that DRMs exist in cell membranes as lipid domains that are more ordered than the conventional, fluid state. We have also suggested that GPI-anchored proteins are detergent-resistant because their acyl chains interact preferentially with these domains. The data provided here, including a straightforward molecular explanation for the importance of cholesterol and sphingolipid in the detergent insolubility of GPI-anchored proteins in cells, provide strong additional support for several aspects of our model.

**Insolubility of Proteins in Liposomes Mimics That in Cell Membranes**—Phase behavior of membrane lipids is much better understood in model membranes than in cells. This is because many of the techniques used to study phase separation in liposomes are not feasible in cells, due to the greater complexity of biological membranes. Detergent insolubility is an exception. In this and another study (31), we have found a good correlation between detergent insolubility and the presence of ordered domains. This suggests that detergent insolubility provides a valuable tool for studying phase behavior in membranes. In this study, we found striking parallels between the insolubility behavior of both GPI-anchored proteins and lipids in cells in our model system and that previously reported for cells. Insolubility was observed both in vivo and in vitro. In addition, in both cases, insolubility depends on membrane sphingolipid and cholesterol. Saponin treatment abolishes Triton X-100 insolubility of both lipids and GPI-anchored proteins in model membranes, as shown here, and in cells (17, 26).

Clearly, however, liposomes model cell membranes only imprecisely. The asymmetry of the lipid bilayer and the presence of proteins in cell membranes are probably among the most important differences between the two systems. In addition, it is possible that some biological phospholipids may participate in the formation of ordered domains. Phospholipids containing highly unsaturated acyl chains would be unlikely to do so. However, there is evidence that palmitoyloleoyl-PC, which is one of the most abundant phospholipids in mammalian cell membranes, may sometimes form an l, like phase with cholesterol (15). This may explain why some insoluble membranes were isolated in mixtures of bovine PC and cholesterol, even in the absence of sphingolipids (Fig. 3A), although no insoluble membranes could be isolated from similar mixtures of DOPC and cholesterol (10).

For these reasons, although our liposome system is useful in providing a model for the phase behavior of lipids in cell membranes, it cannot be used to predict the exact lipid composition required for formation of ordered domains in vivo. Nevertheless, the fact that saponin abolishes Triton X-100 insolubility in cells suggests that they, like low sphingolipid SCRLs, have a lipid composition that is sufficient for formation of ordered domains in the presence but not the absence of cholesterol.

**Cholesterol Promotes Formation of a Detergent-insoluble Phase, Explaining the Cholesterol Requirement for Triton X-100 Insolubility of GPI-anchored Proteins**—There has been no explanation for the observation that cholesterol is required for the Triton X-100 insolubility of GPI-anchored proteins in cells (17, 19). Our finding that PLAP was insoluble in gel phase liposomes showed that insolubility does not strictly require cholesterol and suggests that the role of cholesterol in insolubility is to promote DRM formation. The fact that saponin treatment did not lead to complete Triton X-100 solubilization of PLAP or lipids in SCRLs that contained high amounts of sphingolipids is consistent with this model.

These findings can be explained by the phase behavior of phospholipid, sphingolipid, and cholesterol mixtures. In another study (31), we analyzed phase separation using a detergent-free fluorescence quenching assay. As expected, in mixtures of low \(T_m\) phospholipid and sphingolipid, phase separation and formation of a gel phase occurred above a threshold concentration of sphingolipid. Similarly, an \(l_p\) phase formed above a threshold concentration of sphingolipid in ternary mixtures containing phospholipids, sphingolipids, and a constant amount of cholesterol. However, phase separation occurred more readily in the presence of cholesterol. Thus, cholesterol was required for phase separation at low but not at high sphingolipid concentrations. These results agree with the recent demonstration by Silvius et al. (35) that cholesterol can promote phase separation in ternary mixtures of low and high \(T_m\) PC and cholesterol.

Because the effects of saponin are so similar in low sphingolipid SCRLs and in cells, our finding that cholesterol promotes the formation of insoluble domains is very likely to be true in cells as well as liposomes. Together, our data suggest that the plasma membranes of eukaryotic cells contain enough sphingolipids to form ordered domains in the presence of sterols but not enough to form gel phase domains in the absence of sterols.
Cholesterol Promotes Insolubility of GPI-anchored Proteins

This is likely to be an important evolutionary adaptation, as the presence of gel phase domains in membranes can greatly slow the lateral diffusion of membrane proteins and can even lead to a loss of permeability barrier function.

It is interesting to note from comparing Figs. 1 and 3A that PLAP is more insoluble than [3H]sphingomyelin. This is most noticeable in “high sphingolipid” SCRLs after saponin treatment; 83% of the PLAP, but only about 25% of the [3H]sphingomyelin, was insoluble. This difference probably stems from structural differences between the proteins and lipids, which allow the protein to partition more completely into the ordered domains than the lipid. This will be a very interesting area for future examination.

Insolubility Behavior of Other Proteins Fits the Model—In addition to PLAP, two other GPI-anchored proteins, human and bovine erythrocyte acetylcholinesterase, were insoluble in Triton X-100 when incorporated into SCRLs. As detergent insolubility is a general feature of cellular GPI-anchored proteins, this finding provides further evidence that SCRLs are a good model for DRMs in cell membranes. Bacteriorhodopsin, a transmembrane protein, was almost fully solubilized from SCRLs by Triton X-100. This reflects the behavior of most transmembrane proteins in cells and shows that detergent insolubility of the three GPI-anchored proteins we have examined is specific.

The acyl chains of many GPI-anchored proteins are largely saturated (16). We have previously proposed that these proteins partition preferentially into ordered domains, which are rich in saturated chains, and that detergent insolubility results from this association. The structure of the lipid anchors of both human and bovine erythrocyte acetylcholinesterase proteins has been determined (36–38), allowing us to directly examine the effect of acyl chain saturation on detergent insolubility. A large fraction (69%) of bovine acetylcholinesterase molecules contains 18:0 chains at both positions (36). As we would have predicted, this protein was largely insoluble in SCRLs. Human acetylcholinesterase, in contrast, appears to be unusual among GPI-anchored proteins in that it contains predominantly not only 18:0 alkyl but also 22:4 and 22:5 acyl groups (38). However, this protein is also modified with a palmitate chain linked to the inositol ring (37). (This acyl chain is present in immature GPI-anchored proteins in that it contains predominantly not saturated (16). We have previously proposed that these proteins have several of the properties of l_o phase membranes to insoluble domains in separate membranes present in the same lysate. Together, these studies provide substantial evidence that exposure to detergent does not rearrange membrane lipids to create insoluble domains from previously uniform l_i bilayers.

Acknowledgments—We are grateful to Dr. T. Rosenberg for purified bovine and human acetylcholinesterase and to Drs. T. Rosenberg and R. Haltiwanger for insightful comments on the manuscript.

REFERENCES
1. Hooper, N. M., and Turner, A. J. (1988) Biochim. Biophys. Acta 988, 427–454.
2. Brown, D. A., and Rose, J. K. (1992) Cell 88, 533–544.
3. Cinek, T., and Horvai, V. (1995) J. Immunol. 154, 2282–2270.
4. Fiedler, K., Kobayashi, T., Kurachal, T. V., and Simons, K. (1993) Biochemistry 32, 6635–6673.
5. Sargiacomo, M., Sodini, M., Zhang, T., and Lisanter, M. P. (1993) J. Cell Biol. 122, 789–797.
6. Hennies, A., Timson Gauzon, K. L., Kwon, J., Shaw, A. S., and Lublin, D. M. (1990) Mol. Cell. Biol. 13, 6363–6382.
7. Chang, W.-J., Yang, Y.-S., Rothberg, K. G., Hooper, N. M., Turner, A. J., Gambiel, H. A., De Gunzburg, J., Mumby, S. M., Gilman, A. G., and Anderson, R. G. W. (1994) J. Cell Biol. 126, 127–138.
8. Sevinsky, J. R., Rao, L. M. V., and Ruf, W. (1996) J. Cell Biol. 133, 293–304.
9. Schroeder, R., London, E., and Brown, D. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12130–12134.
10. Ipsen, J. H., Carlstron, G., Murotsus, O. G., Wennström, H., and Zuckermann, M. J. (1987) Biochim. Biophys. Acta 905, 162–172.
11. Almeida, P. F. F., Vaz, W. L. C., and Thompson, T. E. (1992) Biochemistry 31, 5677–6747.
12. Sankaram, M. B., and Thompson, T. E. (1990) Biochemistry 29, 10670–10765.
13. Sankaram, M. B., and Thompson, T. E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8686–8690.
14. Mateo, C. R., Acuna, A. U., and Brochon, J.-C. (1995) Biochemistry 34, 978–987.
15. McConville, M. J., and Ferguson, M. A. J. (1993) Biochem. J. 294, 305–324.
16. Cernus, P. V., Ueffel, H., Tsuchana, G., Srou, G. J., and van der Eede, A. (1993) J. Biol. Chem. 268, 3150–3155.
17. Elias, P. M., Goerke, J., and Friend, D. S. (1978) J. Biol. Chem. 253, 8668–8675.
18. Hanada, K., Nishijima, K., Akamatsu, Y., and Pagano, R. E. (1995) J. Biol. Chem. 370, 6523–6526.
19. Roberts, W. L., Mair, R. L., and Rosenberry, T. L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7817–7821.
20. Oesterhelt, D., and Stoeckenius, W. (1974) J. Biol. Chem. 249, 10944–10953.
21. Arreaza, G., and Brown, D. A. (1995) J. Biol. Chem. 270, 19123–19127.
22. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 651–652, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
23. Arreaza, G., and Steedkens, W. (1974) Methods Enzymol. 31, 677–678.
24. Dencher, N. A., and Heyn, M. P. (1978) FEBS Lett. 96, 322–326.
25. Arreaza, G., Melkonian, K. A., Laosse-Beur, M., and Brown, D. A. (1994) J. Biol. Chem. 269, 19123–19127.
26. Lamb, D. A., Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 651–652, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
27. Reutter, M.-T., Roux, M., and Rigaud, J.-L. (1988) Biochemistry 27, 2668–2677.
28. Silvius, J. R., del Guidice, D., and Lafluer, M. (1996) Biochemistry 35, 15198–15208.
29. Roberts, W. L., Myher, J. J., Kukis, A., and Rosenberry, T. L. (1988) Biochim. Biophys. Res. Commun. 160, 271–277.
30. Roberts, W. L., Salkitkam, S., Reinhold, V. N., and Rosenberry, T. L. (1988) J. Biol. Chem. 263, 18776–18784.
31. Roberts, W. L., Myher, J. J., Kukis, A., Low, M. G., and Rosenberry, T. L. (1988) J. Biol. Chem. 263, 18766–18775.
32. Doring, T. L., Masterson, W. J., Hart, G. W., and Englund, P. T. (1990) J. Biol. Chem. 265, 611–614.