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Glycosyl-phosphatidylinositol-anchored membrane proteins can be distinguished from transmembrane polypeptide-anchored proteins by differential solubilization and temperature-induced phase separation in Triton X-114

Nigel M. HOOPER* and Asam BASHIR
Membrane Peptidase Research Group, Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

Treatment of kidney microvillar membranes with the non-ionic detergent Triton X-114 at 0 °C, followed by low-speed centrifugation, generated a detergent-insoluble pellet and a detergent-soluble supernatant. The supernatant was further fractionated by phase separation at 30 °C into a detergent-rich phase and a detergent-depleted or aqueous phase. Those ectoenzymes with a covalently attached glycosyl-phosphatidylinositol (G-PI) membrane anchor were recovered predominantly (> 73 %) in the detergent-insoluble pellet. In contrast, those ectoenzymes anchored by a single membrane-spanning polypeptide were recovered predominantly (> 62 %) in the detergent-rich phase. Removal of the hydrophobic membrane-anchoring domain from either class of ectoenzyme resulted in the proteins being recovered predominantly (> 70 %) in the aqueous phase. This technique was also applied to other membrane types, including pig and human erythrocyte ghosts, where, in both cases, the G-PI-anchored acetylcholinesterase partitioned predominantly (> 69 %) into the detergent-insoluble pellet. When the microvillar membranes were subjected only to differential solubilization with Triton X-114 at 0 °C, the G-PI-anchored ectoenzymes were recovered predominantly (> 63 %) in the detergent-insoluble pellet, whereas the transmembrane-polypeptide-anchored ectoenzymes were recovered predominantly (> 95 %) in the detergent-solubilized supernatant. Thus differential solubilization and temperature-induced phase separation in Triton X-114 distinguished between G-PI-anchored membrane proteins, transmembrane-polypeptide-anchored proteins and soluble, hydrophilic proteins. This technique may be more useful and reliable than susceptibility to release by phospholipases as a means of identifying a G-PI anchor on an unpurified membrane protein.

INTRODUCTION

Eukaryotic cell-surface proteins can be anchored in the membrane by either a transmembrane sequence of hydrophobic amino acids or a covalently attached glycosyl-phosphatidylinositol (G-PI) moiety. Over 60 cell-surface proteins have been identified as having a G-PI anchor attached covalently to the *C*-terminal amino acid (Low, 1989; Cross, 1990). The most commonly used criterion to demonstrate the presence of a G-PI anchor is the susceptibility of a protein to release from the membrane by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) (Hooper, 1991*b*). However, the results obtained using even the most highly purified bacterial PI-PLC must be interpreted with some caution. It is often difficult to rule out the possibility of contaminating protease or phospholipase activities releasing the protein of interest. Also, misleading results can arise if the protein of interest is not itself G-PI-anchored but is tightly associated with a protein that is G-PI-anchored and therefore susceptible to release by PI-PLC. An example of this is lipoprotein lipase, which itself does not possess a G-PI anchor, but which appears to be susceptible to release by PI-PLC due to its tight association with the G-PI-anchored heparan sulphate proteoglycan (Chajek-Shaul *et al.*, 1989). In addition, some G-PI anchors, such as that on human erythrocyte acetylcholinesterase (Roberts *et al.*, 1988), are resistant to hydrolysis by bacterial PI-PLC due to additional acylation of the inositol ring. Also, it has been observed that some G-PI-anchored proteins differ in their susceptibility to release by bacterial PI-PLC depending on the source of the phospholipase or the species or cell type under examination [see Low (1990) for a more comprehensive discussion of these problems]. Thus alternative methods are required to identify or confirm the presence of a G-PI anchor on a protein.

A variety of other techniques are available to identify a G-PI anchor (Hooper, 1991*b*). Amongst these alternative techniques is the selective release of a protein by the G-PI-specific phospholipase D recently purified from mammalian plasma (Davitz *et al.*, 1989; Huang *et al.*, 1990). This phospholipase D is also capable of cleaving the acylated anchor of human erythrocyte acetylcholinesterase (Roberts *et al.*, 1988). Several anti-(cross-reacting determinant) (CRD) antisera are now available which recognize the G-PI anchors of unrelated proteins following endogenous or exogenous digestion by phospholipase C (Zamze *et al.*, 1988; Jager *et al.*, 1990; Hooper *et al.*, 1991). Alternatively, if a suitable cell line is available, the protein of interest can be biosynthetically labelled with components of the anchor structure (e.g. Jemmerson & Low, 1987).

We have previously shown that differential solubilization by a range of detergents can predict the presence of a G-PI anchor on a protein (Hooper & Turner, 1988*a*,*b*). Detergents with low critical micellar concentrations (e.g. Triton X-100, Triton X-114 and Nonidet P-40) were relatively ineffective at solubilizing substantial amounts of G-PI-anchored proteins, whereas detergents with high critical micellar concentrations (e.g. n-octyl β-D-glucopyranoside or CHAPS) solubilized substantial amounts of the G-PI-anchored proteins. In contrast, those proteins anchored by a single membrane-spanning poly-

Abbreviations used: CRD, cross-reacting determinant; G-PI, glycosyl-phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C.
* To whom correspondence should be addressed.

Table 1. Mode of membrane anchorage of the ectoenzymes used in this study

Membrane anchorage	Enzyme	EC no.
G-PI	Acetylcholinesterase	3.1.1.7
	Alkaline phosphatase	3.1.3.1
	5'-Nucleotidase	3.1.3.5
	Aminopeptidase P	3.4.11.9
	Membrane dipeptidase	3.4.13.11
Transmembrane polypeptide	Aminopeptidase N	3.4.11.2
	Aminopeptidase A	3.4.11.7
	Dipeptidyl peptidase IV	3.4.14.5
	Angiotensin-converting enzyme	3.4.15.1

peptide were solubilized efficiently by all of the detergents examined.

Temperature-induced phase separation in Triton X-114 is an extremely powerful technique for distinguishing between amphipathic proteins, integral membrane proteins and hydrophilic soluble proteins (Bordier, 1981). The technique is based on the ability of the non-ionic detergent Triton X-114 to partition into two distinct phases at 30 °C: a detergent-rich phase and a detergent-depleted, or aqueous, phase. Amphipathic membrane proteins, whether anchored by a G-PI moiety or a hydrophobic polypeptide, partition predominantly into the detergent-rich phase, whereas hydrophilic proteins partition predominantly into the aqueous phase (see e.g. Hooper & Turner, 1988*a*). A modification of the original phase separation method has been developed for the fractionation of adrenal chromaffin granule membranes (Pryde, 1986; Pryde & Phillips, 1986). When the membranes were treated with Triton X-114 at 0 °C followed by high-speed centrifugation, about 90% of the membrane protein was recovered in the detergent-solubilized supernatant. However, the remaining 10% of the membrane protein, along with the bulk of the cholesterol and phospholipid, was recovered in the detergent-insoluble, or phospholipid-rich, pellet. The detergent-solubilized supernatant was then further fractionated by phase separation at 30 °C into a detergent-rich phase containing the majority of the integral membrane proteins and an aqueous phase containing the soluble, hydrophilic proteins.

Arising from our earlier observation that those detergents with a low critical micellar concentration are relatively ineffective at solubilizing G-PI-anchored proteins (Hooper & Turner, 1988*a*), we have modified the technique of differential solubilization and temperature-induced phase separation in Triton X-114 (Pryde & Phillips, 1986) to distinguish between those proteins anchored by a G-PI moiety and those anchored by a single membrane-spanning polypeptide. When this technique was applied to pig kidney microvillar membranes, which are abundant in both G-PI-anchored and polypeptide-anchored ectoenzymes (Table 1), the G-PI-anchored proteins were recovered predominantly in the detergent-insoluble pellet, whereas the transmembrane-polypeptide-anchored proteins were recovered predominantly in the detergent-rich phase. Removal of the hydrophobic anchoring domain from the G-PI- and polypeptide-anchored ectoenzymes by phospholipase or protease action respectively resulted in all proteins being recovered predominantly in the aqueous phase. When pig and human erythrocyte membranes were subjected to this technique of differential solubilization and temperature-induced phase separation in Triton X-114, the G-PI-anchored acetylcholinesterase is, in both cases, recovered predominantly in the detergent-insoluble pellet.

MATERIALS AND METHODS

Materials

PI-PLC from *Bacillus thuringiensis* was a gift from Dr. M. G. Low. Units of PI-PLC activity are μmol/min. Triton X-114 was pre-condensed before use (Bordier, 1981). Bicinchoninic acid, trypsin and papain were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Pig tissues and blood and all other materials were from sources previously noted (Hooper *et al.*, 1991). Human blood was obtained from healthy volunteers.

Membrane preparation

All operations were carried out at 4 °C. Microvilli were prepared from pig kidney cortex by the method of Booth & Kenny (1974), except that the 15000 *g* centrifugation steps were each extended from 12 min to 15 min. Crude microsomal membranes were prepared from pig lung by homogenization in 10 vol. of 0.32 M-sucrose/50 mM-Hepes/NaOH, pH 7.4. The homogenate was centrifuged at 8000 *g* for 15 min, and the supernatant was centrifuged at 26000 *g* for 2 h. The resulting microsomal pellet was resuspended in 10 mM-Hepes/NaOH, pH 7.4. Erythrocyte ghosts were prepared by the method of Hanahan & Ekholm (1976).

Differential solubilization and temperature-induced phase separation in Triton X-114: three phase system

The procedure described is a modification of the method of Pryde & Phillips (1986). In a 1.5 ml microcentrifuge tube the membrane protein sample (final concentration 4 mg of protein/ml) was diluted with 10 mM-Hepes/NaOH, pH 7.4 (see Fig. 1). Pre-condensed Triton X-114 was added to a final

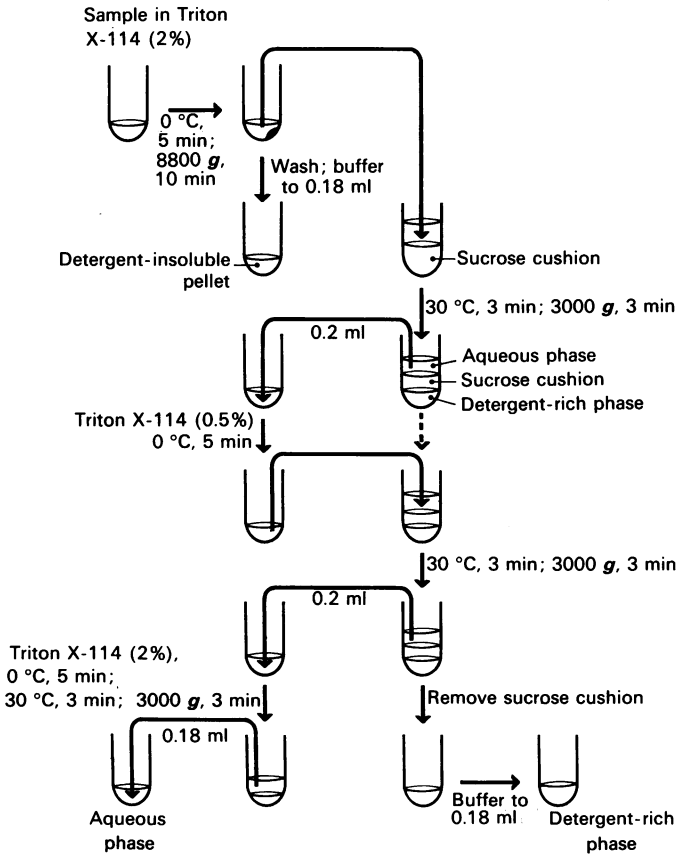

Fig. 1. Schematic representation of the differential solubilization and temperature-induced phase separation in Triton X-114

See the Materials and methods section for the experimental details.

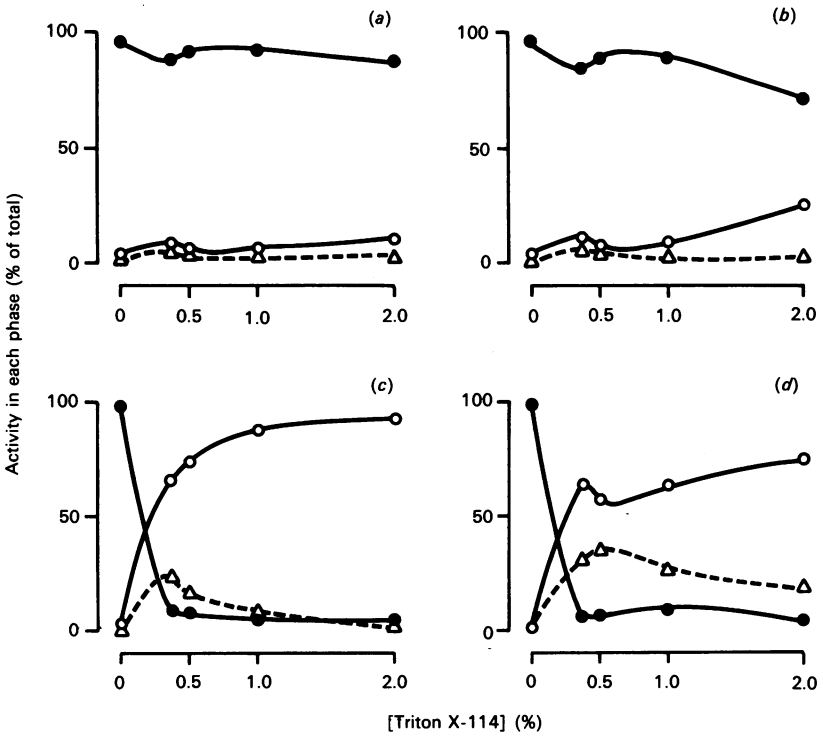

Fig. 2. Effect of Triton X-114 concentration on the solubilization and phase separation of kidney microvillar membranes

A pig kidney microvillar membrane fraction was subjected to differential solubilization and temperature-induced phase separation at the indicated concentrations of Triton X-114 as described in the Materials and methods section. The resultant phases were then assayed for enzyme activities: (*a*) alkaline phosphatase, (*b*) membrane dipeptidase; (*c*) dipeptidyl peptidase IV, (*d*) aminopeptidase N. The results are the means of duplicate determinations at each concentration of detergent, ●, detergent-insoluble pellet; ○, detergent-rich phase; △, aqueous phase.

concentration of 2% (v/v) and a total volume of 0.2 ml. Immediately the sample was vortex-mixed (1–2 s) and then placed on ice for 5 min. The sample was then centrifuged at 8800 *g* for 10 min at 4 °C in a fixed angle rotor. The supernatant was removed and layered on top of 0.3 ml of 6% (w/v) sucrose/10 mM-Tris/HCl/0.15 M-NaCl/0.06% (v/v) Triton X-114, pH 7.4, in a second microcentrifuge tube (see Fig. 1). After incubation at 30 °C for 3 min, followed by centrifugation at 3000 *g* for 3 min in a swing-out rotor, the upper aqueous phase (0.2 ml) was transferred to a clean tube and fresh Triton X-114 was added to a final concentration of 0.5% (v/v). After mixing and incubating on ice for 5 min, this phase was overlayed on the same sucrose cushion, incubated at 30 °C for 3 min and then centrifuged at 3000 *g* for 3 min in a swing-out rotor. The resulting upper aqueous phase (0.2 ml) was removed a second time to a clean tube and fresh Triton X-114 was added to a final concentration of 2% (v/v) (Fig. 1). The sample was mixed, incubated on ice for 5 min and then at 30 °C for 3 min, before centrifugation at 3000 *g* for 3 min in a swing-out rotor. The supernatant (0.18 ml) was removed to a clean tube; this is the final aqueous phase. The sucrose cushion was removed from above the detergent-rich phase, which was then made up to 0.18 ml with 10 mM-Hepes/NaOH, pH 7.4. The original detergent-insoluble pellet was washed (not resuspended) with 0.2 ml of 10 mM-Hepes/NaOH, pH 7.4, and centrifuged at 8800 *g* for 10 min at 4 °C. The pellet was finally resuspended in 0.18 ml of 10 mM-Hepes/NaOH, pH 7.4. It is essential that the detergent-insoluble pellet and detergent-rich phase are completely resuspended prior to assaying for activity. This can best be achieved by freeze–thawing the phases and mixing them thoroughly by drawing the solution up into a pipette several times. The three phases were then assayed for enzyme activities, and the activities recovered in each phase were expressed as percentages of the total activity in all three phases.

Differential solubilization in Triton X-114: two phase system

In a 1.5 ml microcentrifuge tube the membrane protein sample (final concentration 4 mg of protein/ml) was diluted with 10 mM-Hepes/NaOH, pH 7.4. Pre-condensed Triton X-114 was added to a final concentration of 1% (v/v) and a total volume of 0.2 ml. Immediately the sample was vortex-mixed (1–2 s) and then placed on ice for 5 min before centrifugation at 8800 *g* for 10 min at 4 °C in a fixed-angle rotor. The supernatant was removed to a clean tube, and the detergent-insoluble pellet was washed (not resuspended) with 0.2 ml of 10 mM-Hepes/NaOH, pH 7.4, followed by centrifugation at 8800 *g* for 10 min. The second supernatant was combined with the first to give the detergent-rich supernatant. The detergent-insoluble pellet was resuspended in 0.4 ml of 10 mM-Hepes/NaOH, pH 7.4.

Phospholipase and protease incubations

B. thuringiensis PI-PLC was used at a concentration of 10 units/ml and trypsin was used at a concentration of 4 mg/ml in 10 mM-Hepes/NaOH, pH 7.4. Papain was used at a concentration of 2.5 mg/ml in 0.1 M-Pipes/NaOH, pH 6.8. After incubation at 37 °C for 30 min, the membrane samples were made to 2% (v/v) Triton X-114 and subjected to differential solubilization and temperature-induced phase separation in Triton X-114 as described above.

Enzyme and protein assays

Alkaline phosphatase, acetylcholinesterase and 5′-nucleotidase were assayed spectrophotometrically with *p*-nitrophenyl phosphate, acetyl thiocholine iodide and 5′-AMP respectively as

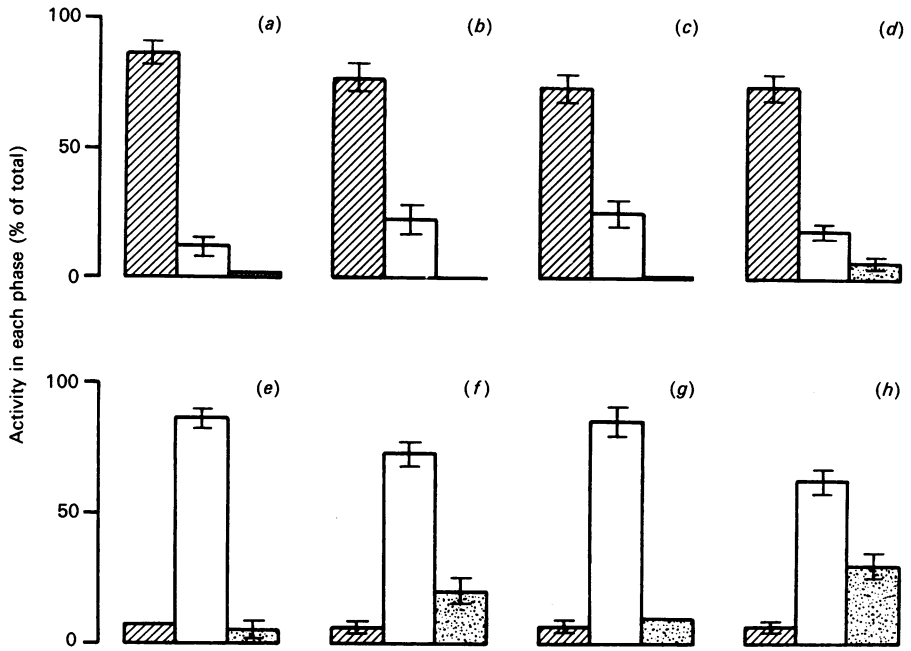

Fig. 3. Solubilization and phase separation of kidney microvillar membranes

A pig kidney microvillar membrane fraction was subjected to differential solubilization and temperature-induced phase separation in Triton X-114 as described in the Materials and methods section. The resultant phases were then assayed for enzyme activities: (*a*) alkaline phosphatase, (*b*) membrane dipeptidase, (*c*) aminopeptidase P, (*d*) 5′-nucleotidase, (*e*) dipeptidyl peptidase IV, (*f*) angiotensin-converting enzyme, (*g*) aminopeptidase N, (*h*) aminopeptidase A. The results are the means ± S.E.M. for four separate phase separations. ▨, Detergent-insoluble pellet; □, detergent-rich phase; ▩, aqueous phase.

substrates (Bessey *et al.*, 1946; Ellman *et al.*, 1961; Heinonen & Lahti, 1981). Aminopeptidases N and A and dipeptidyl peptidase IV were assayed fluorimetrically with Ala-4-methyl-7-coumarylamide, Glu-4-methyl-7-coumarylamide and Gly-Pro-4-methyl-7-coumarylamide respectively as substrates (Fulcher & Kenny, 1983). Membrane dipeptidase, angiotensin-converting enzyme and aminopeptidase P were assayed using Gly-D-Phe, benzoyl-Gly-His-Leu and Gly-Pro-Hyp respectively as substrates. The products were separated and quantified by reverse-phase h.p.l.c. (Hooper & Turner, 1987, 1988*b*; Littlewood *et al.*, 1989). Protein was determined using bicinchoninic acid in a microtitre plate assay with BSA as standard.

RESULTS

Effect of the Triton X-114 concentration on the differential solubilization and temperature-induced phase separation

The effect of the Triton X-114 concentration on the differential solubilization and temperature-induced phase separation of both G-PI- and transmembrane polypeptide-anchored ectoenzymes was examined (Fig. 2). In the absence of detergent the microvillar membranes sedimented on the first centrifugation. On increasing the concentration of Triton X-114, the polypeptide-anchored proteins dipeptidyl peptidase IV and aminopeptidase N were recovered predominantly in the detergent-rich phase, whereas those proteins with a G-PI anchor, i.e. alkaline phosphatase and membrane dipeptidase, were recovered predominantly in the detergent-insoluble pellet. As the concentration of Triton X-114 was increased from 0.37% to 1.0%, a substantial amount of aminopeptidase N was recovered in the aqueous phase. Only on increasing the concentration of Triton X-114 to 2% was the recovery of aminopeptidase N in the aqueous phase decreased to < 20%. However, on increasing the concentration of Triton X-114 from 1% to 2%, the amounts of alkaline phosphatase and membrane dipeptidase recovered in the detergent-rich phase increased slightly. Thus for the three phase system a concentration of 2% Triton X-114 was used, whereas a concentration of 1% was used in the two phase system (see the Materials and methods section).

Differential solubilization and temperature-induced phase separation of kidney microvillar membranes

Pig kidney microvillar membranes were subjected to differential solubilization and temperature-induced phase separation in Triton X-114 as described in the Materials and methods section. The resulting three phases were assayed for the activities of eight different ectoenzymes (Fig. 3). The four ectoenzymes with G-PI anchors (alkaline phosphatase, membrane dipeptidase, aminopeptidase P and 5′-nucleotidase) were recovered predominantly (> 73%) in the detergent-insoluble pellet. Maximally, 26% and 7% of these four G-PI-anchored ectoenzymes were recovered in the detergent-rich and aqueous phases respectively. In contrast, those ectoenzymes anchored by a single membrane-spanning polypeptide (dipeptidyl peptidase IV, angiotensin-converting enzyme, aminopeptidases N and A) were recovered predominantly (> 62%) in the detergent-rich phase. Maximally, 8% and 31% of these four transmembrane-polypeptide-anchored ectoenzymes were recovered in the detergent-insoluble pellet and aqueous phase respectively.

Effect of phospholipase and proteases on the differential solubilization and temperature-induced phase separation of kidney microvillar membranes

Pig kidney microvillar membranes were incubated in the absence or presence of *B. thuringiensis* PI-PLC, trypsin or papain as described in the Materials and methods section, and then subjected to differential solubilization and temperature-induced phase separation in Triton X-114 (Fig. 4). The ectoenzyme

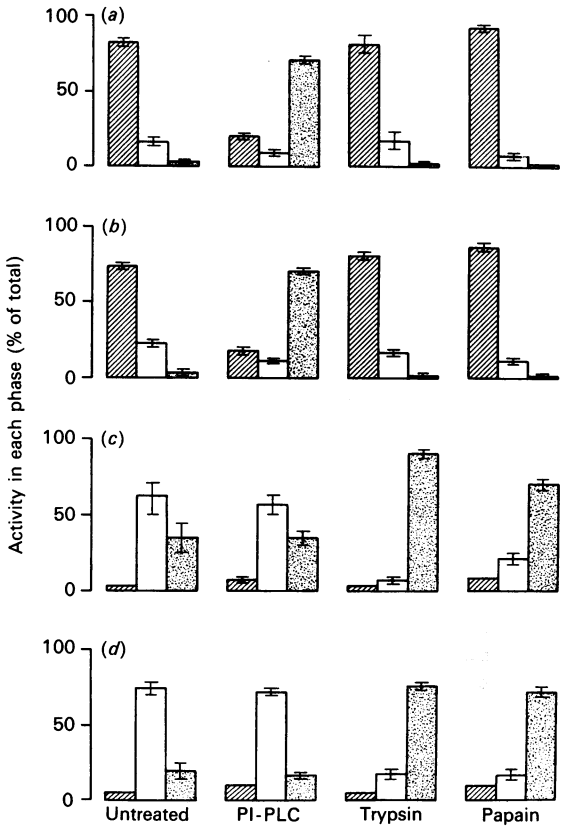

Fig. 4. Effect of phospholipase and proteases on the solubilization and phase separation of kidney microvillar membranes

A pig kidney microvillar membrane fraction was incubated in the absence (untreated) or presence of bacterial PI-PLC, trypsin or papain for 30 min at 37 °C prior to differential solubilization and temperature-induced phase separation in Triton X-114 as described in the Materials and methods section. The resultant phases were then assayed for enzyme activities: (*a*) alkaline phosphatase, (*b*) membrane dipeptidase, (*c*) angiotensin-converting enzyme, (*d*) aminopeptidase N. The results are the means ± S.E.M. for three separate incubations with each reagent. ▨, Detergent-insoluble pellet; □, detergent-rich phase; ▩, aqueous phase.

activities in the untreated membrane sample partitioned into the three phases as observed previously (Fig. 3). On treatment of the membranes with bacterial PI-PLC, the activities of alkaline phosphatase and membrane dipeptidase were recovered predominantly (> 70%) in the aqueous phase, consistent with the removal of the hydrophobic lipid anchor. In contrast, the partitioning of the two ectoenzymes anchored by a transmembrane polypeptide, angiotensin-converting enzyme and aminopeptidase N, was unaffected by treatment with PI-PLC. When the microvillar membranes were incubated first with either trypsin or papain, there was no observable difference in the partitioning of alkaline phosphatase and membrane dipeptidase (Fig. 4). However, both angiotensin-converting enzyme and aminopeptidase N were recovered predominantly (> 76% and > 70%) in the aqueous phase after treatment of the membranes with trypsin and papain respectively.

Differential solubilization and temperature-induced phase separation of other membranes

Pig lung microsomal membranes were subjected to differential solubilization and temperature-induced phase separation as described in the Materials and methods section. The resulting three phases were then assayed for ectoenzyme activities (Table 2). As with the kidney microvillar membranes, alkaline phosphatase and aminopeptidase P partitioned predominantly (> 63%) into the detergent-insoluble pellet, and angiotensin-converting enzyme and dipeptidyl peptidase IV partitioned predominantly (> 73%) into the detergent-rich phase.

Pig and human erythrocyte ghosts were also subjected to differential solubilization and temperature-induced phase separation in Triton X-114. In order to pellet the erythrocyte membranes the initial centrifugation step was increased to 48000 *g* for 30 min. The inclusion of 20 μl of 0.2 M-Pipes/NaOH, pH 6.8, in the initial incubation mixture was also found to aid the formation of the detergent-insoluble pellet (see legend to Table 3). The resulting three phases were then assayed for ectoenzyme activities (Table 3). Both pig and human erythrocyte acetylcholinesterase partitioned predominantly (> 69%) into the detergent-insoluble pellet. When the membranes were treated with *B. thuringiensis* PI-PLC (1 unit, 1 h at 37 °C) prior to solubilization and phase separation, the pig erythrocyte acetylcholinesterase was recovered predominantly (93.6%) in the aqueous phase, whereas there was no significant difference in the partitioning of human erythrocyte acetylcholinesterase (results not shown). Under identical centrifugation and buffer conditions pig kidney microvillar alkaline phosphatase and dipeptidyl peptidase IV partitioned predominantly (> 92% and > 60%) into the detergent-insoluble pellet and detergent-rich phase respectively (Table 3).

Separation of G-PI anchored from transmembrane-polypeptide-anchored proteins by differential solubilization in Triton X-114

Pig kidney microvillar membranes were subjected to differential solubilization in Triton X-114 as described in the Methods section to generate a detergent-insoluble pellet and a

Table 2. Solubilization and phase separation of lung microsomal membranes

The pig lung microsomal membrane fraction was subjected to differential solubilization and temperature-induced phase separation in Triton X-114 as described in the Materials and methods section. The resultant phases were then assayed for enzyme activities. The results are the means ± S.E.M. for four separate phase separations.

Enzyme	Activity in each phase (% of total)		
	Detergent-insoluble pellet	Detergent-rich phase	Aqueous phase
Alkaline phosphatase	73.3 ± 1.2	19.1 ± 1.9	7.6 ± 1.1
Aminopeptidase P	63.9 ± 2.2	30.6 ± 1.0	5.5 ± 2.5
Angiotensin-converting enzyme	9.8 ± 0.8	73.1 ± 2.0	17.1 ± 1.2
Dipeptidyl peptidase IV	9.1 ± 0.7	87.5 ± 1.1	3.4 ± 0.9

Table 3. Solubilization and phase separation of erythrocyte and kidney microvillar membranes

Membrane fractions were subjected to differential solubilization and temperature-induced phase separation in Triton X-114 as described in the Materials and methods section, except that the initial incubation mixture contained 20 μl of 0.2 M-Pipes/NaOH, pH 6.8, and the detergent-insoluble pellet was obtained by centrifugation at 48000 *g* for 30 min at 4 °C. The detergent-insoluble pellet was washed with 0.2 ml of 0.2 M-Pipes/NaOH, pH 6.8, centrifuged at 48000 *g* for 30 min at 4 °C and finally resuspended in 10 mM-Hepes/NaOH, pH 7.4. The resultant phases were then assayed for enzyme activities. The results are the means ± S.E.M. for four separate phase separations.

Enzyme	Activity in each phase (% of total)		
	Detergent-insoluble pellet	Detergent-rich phase	Aqueous phase
Pig erythrocyte acetylcholinesterase	78.7±1.8	16.6±3.6	4.7±2.8
Human erythrocyte acetylcholinesterase	69.8±4.3	26.2±2.1	4.0±2.3
Pig kidney microvillar alkaline phosphatase	92.0±0.8	6.7±1.0	1.3±0.3
Pig kidney microvillar dipeptidyl peptidase IV	32.4±3.2	60.6±2.9	7.0±0.6

Table 4. Differential solubilization of kidney microvillar membranes

A pig kidney microvillar membrane fraction incubated in the absence or the presence of bacterial PI-PLC was subjected to differential solubilization in Triton X-114 as described in the Materials and methods section. The supernatant and pellet fractions were then assayed for ectoenzyme activities and the activities recovered in the detergent-insoluble pellet were expressed as percentages of the total activity in both fractions. The results for the untreated membranes are the means ± S.E.M. for four separate solubilizations. The results for the PI-PLC-treated membranes are the means of duplicate solubilizations which did not differ by more than 2.3%.

Ectoenzyme	Activity in detergent-insoluble pellet (% of total activity)	
	Untreated membranes	PI-PLC-treated membranes
Alkaline phosphatase	80.2±1.9	13.6
5′-Nucleotidase	63.5±2.3	13.0
Aminopeptidase P	67.8±1.0	28.6
Membrane dipeptidase	73.9±2.1	21.0
Aminopeptidase N	4.0±1.4	–
Aminopeptidase A	4.4±0.4	–
Dipeptidyl peptidase IV	4.5±1.0	–
Angiotensin-converting enzyme	3.1±0.6	–

detergent-soluble supernatant. The two fractions were then assayed for the various ectoenzyme activities (Table 4). Those ectoenzymes with a G-PI anchor were recovered predominantly (> 63%) in the detergent-insoluble pellet, whereas those ectoenzymes with a polypeptide anchor were recovered predominantly (> 95%) in the detergent-soluble supernatant. When the membranes were incubated with bacterial PI-PLC prior to detergent solubilization, the G-PI anchored ectoenzymes were then recovered predominantly (> 71%) in the detergent-soluble supernatant (Table 4).

DISCUSSION

The kidney microvillar membrane is enriched in a number of G-PI-anchored and transmembrane-polypeptide-anchored ectoenzymes (Table 1) (Hooper & Turner, 1988*a*; Turner & Hooper, 1990). This, together with the relative ease with which the membrane can be isolated in large quantities free from other membranes (Booth & Kenny, 1974), makes the microvillar membrane an ideal model system in which to study the structural and functional differences between these two types of membrane protein anchorage. The G-PI anchorage of alkaline phosphatase, 5′-nucleotidase, aminopeptidase P and membrane dipeptidase is well established. Aminopeptidases A and N and dipeptidyl peptidase IV are known to be anchored in the membrane by an uncleaved hydrophobic *N*-terminal signal sequence, and angiotensin-converting enzyme is anchored by a hydrophobic polypeptide residing near the *C*-terminus of the mature protein (see Turner & Hooper, 1990; Hooper, 1991*a*).

We have previously shown that detergents with low critical micellar concentrations (e.g. Triton X-100, Triton X-114 and Nonidet P-40) are relatively ineffective at solubilizing proteins anchored by a G-PI moiety, but that these detergents can solubilize substantial amounts of those proteins anchored by a single membrane-spanning polypeptide (Hooper & Turner, 1988*a*,*b*). In the present study we have exploited this differential solubilization in Triton X-114, followed by temperature-induced phase separation, to separate G-PI-anchored proteins from those proteins with a transmembrane polypeptide anchor. At 0 °C Triton X-114 effectively solubilized those kidney microvillar ectoenzymes with a polypeptide anchor, whereas the G-PI-anchored ectoenzymes were not solubilized and could be sedimented by low-speed centrifugation and recovered in the detergent-insoluble pellet (Fig. 3). The detergent-solubilized supernatant was then further fractionated by phase separation at 30 °C into a detergent-rich phase, which contains those ectoenzymes with a polypeptide anchor, and an aqueous phase. When the microvillar membranes were treated first with bacterial PI-PLC, the G-PI-anchored ectoenzymes (alkaline phosphatase and membrane dipeptidase) were recovered predominantly in the aqueous phase (Fig. 4), consistent with the removal of the hydrophobic lipid anchor by PI-PLC. Similarly, after treatment of the membranes with either trypsin or papain, which cleave the hydrophobic polypeptide anchors from angiotensin-converting enzyme and aminopeptidase N respectively, these two transmembrane-polypeptide-anchored ectoenzymes were also recovered predominantly in the aqueous phase. Thus this technique of differential solubilization and temperature-induced phase separation in Triton X-114 can distinguish between those proteins with a G-PI anchor, those with a transmembrane

polypeptide anchor, and those which either do not have or have lost the hydrophobic anchoring domain, i.e. are hydrophilic.

Pig lung microsomal membranes and pig and human erythrocyte ghosts were also subjected to differential solubilization and temperature-induced phase separation in Triton X-114 in order to assess whether the results obtained were due to a property of the kidney microvillar membrane rather than the type of anchorage of the protein. In agreement with the results obtained for the kidney membranes, the G-PI-anchored ectoenzymes in the pig lung membranes partitioned predominantly into the detergent-insoluble pellet, and those ectoenzymes with a polypeptide anchor partitioned predominantly into the detergent-rich phase (Table 2). Similar results have been obtained for other membrane samples including those from human placenta (S. J. Broomfield & N. M. Hooper, unpublished work), pig intestine and rat kidney and intestine (R. Koelsch, N. M. Hooper & A. J. Turner, unpublished work). With both pig and human erythrocyte membranes, the G-PI-anchored acetylcholinesterase partitioned predominantly into the detergent-insoluble pellet (Table 3). Pig erythrocyte acetylcholinesterase is susceptible to release from the membrane by bacterial PI-PLC (Low & Finean, 1977), whereas the human erythrocyte enzyme is resistant to release due to additional acylation of the inositol (Futerman *et al.*, 1985; Roberts *et al.*, 1988). Thus this technique of differential solubilization and phase separation in Triton X-114 would appear to be able to distinguish between G-PI-anchored and polypeptide-anchored proteins regardless of the membrane preparation, and also to be able to identify a protein possessing a G-PI anchor that is resistant to degradation by PI-PLC. As the technique requires the proteins to be associated with the lipid bilayer in order to sediment during the first centrifugation, it will not be applicable to purified protein samples. Repeated freeze–thawing of the membrane samples prior to solubilization and phase separation appears to result in a higher proportion of the G-PI-anchored proteins being recovered in the detergent-rich phase (results not shown), possibly due to disruption of the interactions within the lipid bilayer.

As it is essentially the first step, i.e. solubilization with Triton X-114 at 0 °C, which separates G-PI-anchored proteins from transmembrane-polypeptide-anchored proteins, pig kidney microvillar membranes were subjected only to the initial differential solubilization. The G-PI-anchored ectoenzymes were recovered predominantly in the detergent-insoluble pellet, whereas the transmembrane-polypeptide-anchored ectoenzymes were recovered predominantly in the detergent-soluble supernatant (Table 4). When the microvillar membranes were treated with PI-PLC prior to detergent solubilization, the now hydrophilic and soluble G-PI-anchored ectoenzymes were recovered predominantly in the detergent-soluble supernatant along with the polypeptide-anchored ectoenzymes. Thus this rapid and simple technique of differential solubilization in Triton X-114, which could be easily scaled up for preparative studies, separates effectively G-PI-anchored from polypeptide-anchored proteins without having to remove the lipid anchor.

In conclusion, differential solubilization and temperature-induced phase separation in the non-ionic detergent Triton X-114 can distinguish between cell-surface membrane proteins anchored by either a covalently attached G-PI moiety or a membrane-spanning polypeptide. As this technique does not rely on enzymic cleavage by phospholipases, recognition by antibodies or metabolic labelling of cells, it may prove to be more widely applicable and reliable as a means of identifying a G-PI anchor on an unpurified membrane protein.

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