# Amyloid precursor protein, although partially detergent-insoluble in mouse cerebral cortex, behaves as an atypical lipid raft protein

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Lipid rafts are regions of the plasma membrane that are enriched in cholesterol, glycosphingolipids and acylated proteins, and which have been proposed as sites for the proteolytic processing of the Alzheimer's amyloid precursor protein (APP). Lipid rafts can be isolated on the basis of their insolubility in Triton X-100 at 4 °C, with the resulting low-density, detergent-insoluble glycolipid-enriched fraction (DIG) being isolated by flotation through a sucrose density gradient. The detergent-insolubility of APP in mouse cerebral cortex relative to a variety of DIG marker proteins (alkaline phosphatase, flotillin, F3 protein and prion protein) and non-DIG proteins (alkaline phosphodiesterase I, aminopeptidase A and clathrin) has been examined. Alkaline phosphatase, flotillin, F3 protein and the prion protein were present exclusively in the DIG region of the sucrose gradient over a range of protein/detergent ratios used to solubilize the membranes and displayed a characteristic enrichment in the low-density fraction as the protein/detergent ratio was decreased. In contrast, most of the APP, alkaline phosphodiesterase I, aminopeptidase A and clathrin was effectively solubilized at all of the protein/detergent ratios examined. However, a minor proportion of these latter proteins was detected in DIGs at levels which remained constant irrespective of the protein/detergent ratio. When DIGs were isolated from the sucrose gradients and treated with excess Triton X-100, both the DIG marker proteins and APP, alkaline phosphodiesterase I and clathrin were predominantly resistant to detergent extraction at 37 °C. These results show that, although a minor proportion of APP is present in DIGs, where it is detergent-insoluble even at 37 °C, it behaves as an atypical lipid raft protein and raises questions as to whether lipid rafts are a site for its proteolytic processing.

Key words: alkaline phosphatase, Alzheimer's disease, flotillin, prion protein, Triton X-100.

## INTRODUCTION

The lipid raft model proposes that glycosphingolipids, cholesterol and acylated proteins are organized in domains within the membranes of mammalian cells [1,2]. A variety of studies with both model and cellular membranes have provided evidence for this clustered association of certain lipids and proteins in discrete domains of the lipid bilayer (reviewed in [3–7]). These domains are proposed to be involved in the apical sorting of membrane components in epithelial cells, in axonal sorting in neuronal cells, in signal transduction and endocytosis, and in the conversion of the cellular form of the prion protein into the scrapie isoform.

Insolubility in detergents, such as Triton X-100, at 4 °C has been adopted as the basis for isolating lipid rafts. This technique was first developed by Brown and Rose [8] on the basis of reports that both glycosphingolipids [9] and multiple glycosyl-phosphatidylinositol (GPI)-anchored proteins [10–13] were essentially insoluble in certain non-ionic detergents such as Triton X-100. Because of their high lipid content, the resulting detergent-insoluble glycolipid-enriched fraction (DIG) floats to a low density during sucrose-density-gradient centrifugation. By using this technique, DIGs have been shown to be enriched not only in cholesterol, glycosphingolipids and multiple GPI-anchored proteins [14–16], but also in certain transmembrane polypeptide-anchored proteins such as the haemagglutinin protein of influenza virus [17] and intestinal epithelial sucrase-isomaltase [18], and numerous acylated (myristoylated and/or palmitoylated) proteins, such as the Src family tyrosine kinases [16], nitric oxide synthase [19] and heterotrimeric G-proteins [15,16].

DIGs isolated from epithelial tissue possess a characteristically high content of the 21–24 kDa integral membrane protein caveolin-1/VIP21 [20–23]. Whilst caveolin is highly expressed in lung and muscle, it is undetectable in other tissues such as spleen, kidney, liver, brain and testis [16]. Despite the absence of caveolin, DIGs can be isolated from neuronal sources and are found to contain a number of GPI-anchored proteins, such as alkaline phosphatase, 5′-nucleotidase, F3 protein, the prion protein [24–27], tyrosine kinases, and $\alpha$- and $\beta$-subunits of heterotrimeric G-proteins [28,29]. Another protein, flotillin, has been found to be present exclusively in DIGs isolated from neuronal sources and can, therefore, be used in place of caveolin as a transmembrane polypeptide-anchored marker for these membrane domains [30–32].

Lipid rafts have been proposed as the site for the proteolytic processing of the Alzheimer's amyloid precursor protein (APP). Alzheimer's disease is a progressive neurodegenerative disorder characterized by the occurrence of senile plaques and neurofibrillary tangles throughout the brain cortex. The major component of the senile plaques is the amyloid $\beta$-peptide (A$\beta$), of $M_r \approx 4$ kDa, which is derived from the larger type I integral membrane APP, through sequential proteolytic cleavage by $\beta$- and $\gamma$-secretases [33,34]. APP can also be processed through a non-amyloidogenic pathway in which $\alpha$-secretase cleaves the protein within the A$\beta$ sequence, thereby precluding the formation

Abbreviations used: APP, amyloid precursor protein; A$\beta$, amyloid $\beta$-peptide; DIG, detergent-insoluble glycolipid-enriched fraction; GPI, glycosyl-phosphatidylinositol; MBS, Mes-buffered saline; TM, total membrane.

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of A$\beta$. Currently there are conflicting reports as to whether APP is present in lipid rafts, where its processing by the secretases may occur. Some studies have reported that a minor amount of APP is present in DIGs [31,35–37], while others have failed to detect it in such fractions [27,32,38].

In order to clarify whether APP is a component of lipid rafts, we have examined the occurrence of APP in DIGs isolated from mouse cerebral cortex under a range of protein/detergent ratios and compared its distribution and enrichment in this fraction with both DIG marker proteins (flotillin, F3 protein, prion protein and alkaline phosphatase) and other non-DIG proteins (alkaline phosphodiesterase I, aminopeptidase A and clathrin). Our results show that, although a minor proportion of APP is present in DIGs, where it is insoluble in Triton X-100 even at 37 °C, it does not behave as a typical DIG protein.

## MATERIALS AND METHODS

### Materials

Anti-F3 antibody was a rabbit polyclonal antibody prepared against a fusion protein comprising the F3 protein Ig-like domains and was kindly given by Dr. G. Rougon (Institut de Biologie du Dévelopement de Marseille, Marseille, France). Anti-APP antibody 22C11 was a mouse monoclonal antibody from Boehringer-Mannheim (Lewes, East Sussex, U.K.) and recognizes amino acids 60–100 of APP. Anti-clathrin antibody was a mouse monoclonal antibody from Harlan Sera-Lab (Crawley Down, Crawley, Sussex, U.K.). Anti-flotillin antibody was a mouse monoclonal antibody from Transduction Laboratories (Lexington, KY, U.S.A.) and recognizes amino acids 312–428 of flotillin. Anti-prion antibody P45-66 was a rabbit polyclonal antibody and was kindly given by Dr. D. Harris (Washington School of Medicine, St. Louis, MO, U.S.A.). All other materials, unless otherwise stated, were from sources previously noted [27,39,40].

### Preparation of mouse cerebral-cortex total membranes (TMs)

One mouse cerebral cortex was homogenized initially by 30 passes of a Dounce homogenizer in 15 ml of 150 mM NaCl/20 mM $Na_2HPO_4$/2 mM $NaH_2PO_4$/20 % (v/v) glycerol, pH 7.4. Samples were further disrupted by sonication (30 % maximum power pulsed for 30 % of the time using a Branson Sonifier) for $10 \times 1$ min periods, with a 1 min cooling period between each round of sonication. The homogenized sample was then centrifuged at 5000 ***g*** for 20 min and the resulting supernatant at 100000 ***g*** for 90 min. The pelleted TM fraction was resuspended in 5 ml of Mes-buffered saline [MBS; 25 mM Mes (pH 6.5)/0.15 M NaCl].

### Isolation of DIGs

The cerebral cortex was dissected from four mouse brains and homogenized initially by 30 passes of a Dounce homogenizer in 7 ml of MBS containing 1 % (v/v) Triton X-100. The protein concentration of the homogenized sample was determined and solutions of 15, 10, 5 and 2 mg of protein/ml were prepared by diluting the original homogenate with MBS containing 1 % (v/v) Triton X-100. All the samples were then sonicated (30 % maximum power for $5 \times 20$ s with a 90 s cooling period between each round of sonication using a Branson Sonifier) and diluted with an equal volume of 80 % (w/v) sucrose in MBS lacking Triton X-100. The solubilized samples (4 ml) were then injected under a 10 ml 5–30 %-(w/v)-sucrose gradient in MBS lacking Triton X-100. Following centrifugation at 140000 ***g*** for 18 h at 4 °C in an SW-28 rotor (Beckman Instruments), 1 ml fractions were harvested from the bottom to the top of the gradient and the detergent-insoluble pellet at the base of the tube was resuspended by brief sonication in MBS.

### Preparation of pig kidney microvillar membranes

Pig kidney microvillar membranes were prepared as described previously [41]. The final membrane pellet was resuspended in MBS to a protein concentration of 8 mg/ml. DIGs were prepared from microvillar membranes as described for mouse cerebral cortex following the addition of an equal volume of 1 % (v/v) Triton X-100 in MBS.

### Incubation of DIGs with excess detergent

In order to reveal the location of the DIGs within the sucrose gradients, the $A_{620}$ of the harvested fractions was determined. Those fractions from the 5–30 %-(w/v)-sucrose region which exhibited high $A_{620}$ values were combined, diluted 10-fold with MBS and centrifuged at 100000 ***g*** for 90 min at 4 °C. The pelleted DIGs were resuspended in MBS and an equal volume of Triton X-100 in MBS was added, sufficient to give a final detergent concentration of 2 % (v/v). Following incubation for 2 h at either 4 °C or 37 °C, the remaining detergent-insoluble material was pelleted by centrifugation at 100000 ***g*** for 90 min at 4 °C. The insoluble pellets were resuspended in MBS to the same volume as the soluble supernatants.

### Enzyme and protein assays

Alkaline phosphatase (EC 3.1.3.1), alkaline phosphodiesterase I (EC 3.1.4.1) and aminopeptidase A (EC 3.4.11.7) were assayed with *p*-nitrophenyl phosphate, *p*-nitrophenylthymidine 5′-monophosphate and $\alpha$-glutamic acid *p*-nitroanilide respectively as substrate, and the products were quantified spectrophotometrically as described previously [11,42]. In the case of alkaline phosphodiesterase I, opaque membranes were removed from the reaction mixture by brief centrifugation (5 min, 11600 ***g***) prior to quantification of the reaction product. Protein was quantified using bicinchoninic acid [43] in a microtitre plate assay with BSA as standard following solubilization of membranes by the addition of an equal volume of 120 mM n-octyl $\beta$-D-glucopyranoside.

### SDS/PAGE and immunoelectrophoretic blot analysis

Samples were mixed with an equal volume of reducing electrophoresis sample buffer and boiled for 3 min. Proteins were resolved by SDS/PAGE using either a 5–15 %-polyacrylamide gradient gel or a 7–17 %-polyacrylamide gradient gel and transferred to Immobilon P (PVDF) membranes as described previously [44]. The membranes were blocked by incubation in PBS containing 0.1 % (v/v) Tween 20, 5 % (w/v) dried-milk powder, and 2 % (w/v) BSA overnight at 4 °C. All primary and secondary antibody incubations were performed in the same buffer as that used for blocking. The anti-clathrin antibody was used at a dilution of 1:1000, the 22C11 anti-APP antibody at 1:5000, the anti-prion antibody at 1:2000, the anti-F3 antibody at 1:2000, and the anti-flotillin antibody at 1:1000. Bound antibody was detected using peroxidase-conjugated secondary antibodies in conjunction with the enhanced-chemiluminescence (ECL®) detection method (Amersham Life Sciences, Little Chalfont, Bucks., U.K.).

## RESULTS

### The isolation of DIGs is unaffected by variations in the protein/detergent ratio

The effect of varying the protein/detergent ratio on the isolation of DIGs was examined. Mouse cerebral cortex was solubilized in 1% (v/v) Triton X-100 to give final protein concentrations of 15, 10, 5 and 2 mg/ml, and DIGs were isolated by flotation in a sucrose density gradient. At all the protein concentrations a single light-scattering band was observed within the 5–30%-(w/v)-sucrose region of the gradient (Figure 1A). Essentially all of the GPI-anchored alkaline phosphatase activity resided in the DIGs (fractions 6–10 of the sucrose gradient) irrespective of the protein/detergent ratio used during solubilization (Figure 1B). As caveolin-1 is absent from neuronal tissue, flotillin, which has been shown previously to be present in DIGs isolated from bovine cortical grey matter [30], rat cortical grey matter [31] and human neuroblastoma SH-SY5Y cells [32], was employed as a transmembrane protein marker for the detection of DIGs in the sucrose gradients. Flotillin was present exclusively in the DIGs region of the sucrose gradient (fractions 6–10), irrespective of the protein/detergent ratio used during solubilization (Figure 1C). Thus the distribution of two DIG marker proteins, alkaline phosphatase and flotillin, in DIGs isolated from mouse cerebral cortex was not affected by altering the protein/detergent ratio over a 7-fold range.

### The protein/detergent ratio affects the apparent distribution of other proteins in DIGs

The distribution of total protein was determined in the fractions harvested from the sucrose gradients. The majority of protein was solubilized at all of the protein concentrations studied and was subsequently detected in fractions 1–5 of the sucrose gradient (Figure 2A). As the protein/detergent ratio used during solubilization was increased the proportion of protein associated with the DIGs region of the gradient (fractions 6–10) increased. This situation was mirrored by the distribution of the transmembrane protein alkaline phosphodiesterase I (Figure 2B), which has been shown previously to be almost completely excluded from DIGs [27]. Only at the highest protein/detergent ratio (15 mg of protein/ml) was any of this protein detected in the DIGs region of the gradient (13% of the total activity). Similar results were obtained with another transmembrane protein, aminopeptidase A, which is completely excluded from DIGs isolated from porcine lung [39]. Aminopeptidase A was detected in the DIGs region of the sucrose gradient only at the two highest protein/detergent ratios (15 and 10 mg of protein/ml) (Figure 2C). The profile of APP distribution across the sucrose gradient was essentially identical with that of alkaline phosphodiesterase I and aminopeptidase A. APP was detected in DIGs only at the higher protein/detergent ratios used in the solubilization; at lower protein/detergent ratios APP appeared to be excluded completely from the DIGs (Figure 2D). However, it should be noted that, following concentration of the sucrose gradient fractions, APP could be detected in the DIGs fraction, even from the lowest protein/detergent ratio used to solubilize the membranes (see Figure 4 below). Thus, at high protein/detergent ratios, it would appear that proteins which are normally excluded from DIGs can be recovered in the DIGs region of the sucrose gradient, while at lower protein/detergent ratios the lack of detection of such proteins in DIGs may be due to limitations in assay sensitivity.

### DIG marker proteins are enriched as the protein/detergent ratio is decreased

DIGs isolated at the various protein/detergent ratios were concentrated by diluting fractions 6–10 of the sucrose gradients 10-fold in MBS followed by centrifugation at 100000 ***g*** for 90 min at 4 °C. The specific activities/signals of known DIG marker proteins within these concentrated samples were compared with a TM fraction before solubilization (Figure 3). As the protein/detergent ratio was reduced, the concentration of flotillin in DIGs increased (Figure 3A). At 15 mg of protein/ml the concentration of flotillin in DIGs was only 4-fold greater than in the TM fraction. However, at 5 and 2 mg of protein/ml, flotillin was 12-fold more concentrated in DIGs than in the TM fraction. The level of alkaline phosphatase activity in the TM fraction was so low relative to DIGs that no activity could be detected at the protein concentration used in the assays (Figure 3B). As was the case for flotillin, the specific activity of alkaline phosphatase was increased significantly at the lower protein/detergent ratios. At

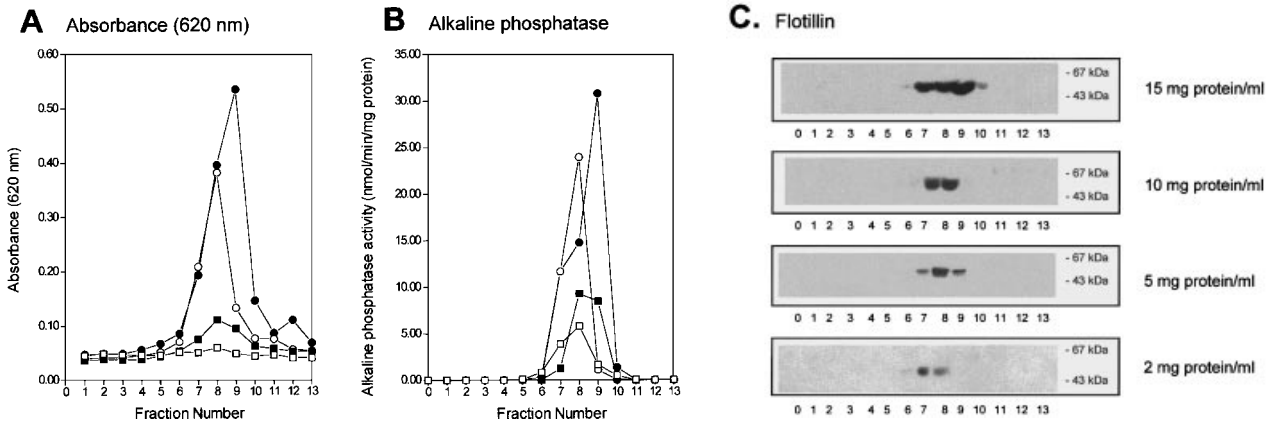


**Figure 1 Altering the protein/detergent ratio does not affect the isolation of DIGs**

Mouse cerebral cortex was solubilized in 1% (v/v) Triton X-100 at protein concentrations of 15 (●), 10 (○), 5 (■) and 2 (□) mg/ml and DIGs were prepared as described in the Materials and methods section. Sucrose-gradient fractions were harvested in 1 ml fractions (fraction 0, insoluble pellet; fraction 1, base of gradient; fraction 13, top of gradient). (**A**) Absorbance of fractions measured at 620 nm; (**B**) distribution of alkaline phosphatase activity in the sucrose gradient; (**C**) distribution of flotillin in the sucrose gradient as detected by Western blotting.

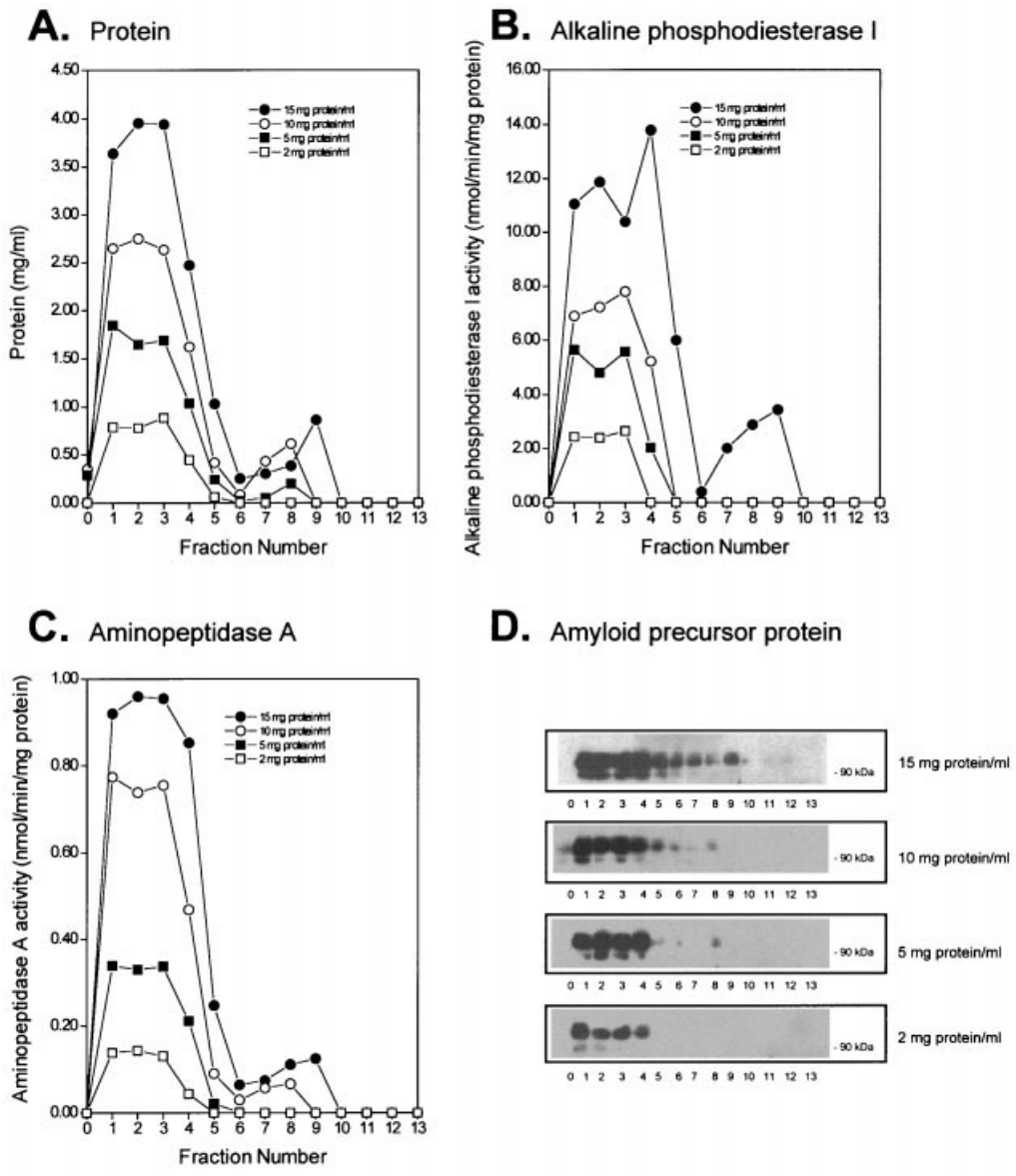


**Figure 2 Altering the protein/detergent ratio affects the association of proteins with DIGs**

Mouse cerebral cortex was solubilized in 1% (v/v) Triton X-100 at protein concentrations of 15, 10, 5 and 2 mg/ml and DIGs were prepared as described in the Materials and methods section. Sucrose gradient fractions were harvested in 1 ml fractions (fraction 0, insoluble pellet; fraction 1, base of gradient; fraction 13, top of gradient). (**A**) Distribution of total protein in the sucrose gradient. (**B**) Distribution of alkaline phosphodiesterase I activity in the sucrose gradient. (**C**) Distribution of aminopeptidase A activity in the sucrose gradient. (**D**) Distribution of APP in the sucrose gradient as detected by Western blotting.

2 mg of protein/ml the specific activity of alkaline phosphatase was approx. 3.5-fold greater than in DIGs isolated at a protein concentration of 15 mg/ml. Essentially the same pattern of enrichment was observed with two additional GPI-anchored proteins, the prion protein (Figure 3C) and the F3 protein (Figure 3D), both of which have been shown previously to fractionate into DIGs [24,26]. The specific signals for these two proteins were enhanced 11- and 4-fold respectively in DIGs isolated at 2 mg of protein/ml compared with those isolated at 15 mg protein/ml. Thus, although decreasing the protein/detergent ratio does not affect the distribution of DIG marker proteins across the sucrose gradient, presumably the removal of non-DIG proteins as the protein/detergent ratio is decreased results in an increased enrichment of the DIG marker proteins.

### APP is not enriched in DIGs as the protein/detergent ratio is decreased

DIGs isolated at the different protein/detergent ratios were also examined for any enrichment of APP, clathrin and alkaline phosphodiesterase I (Figure 4). In contrast with the DIG marker proteins, the amount of APP detected in DIGs remained constant irrespective of the protein/detergent ratio used during solubilization (Figure 4A), and the amount of this protein was on average 35% lower in DIGs than in the TM fraction. Like APP, the concentration of clathrin in DIGs remained constant irrespective of the protein/detergent ratio used during solubilization (approx. 40% of that observed in the TM fraction) (Figure 4B). Similar results were obtained for alkaline phospho-

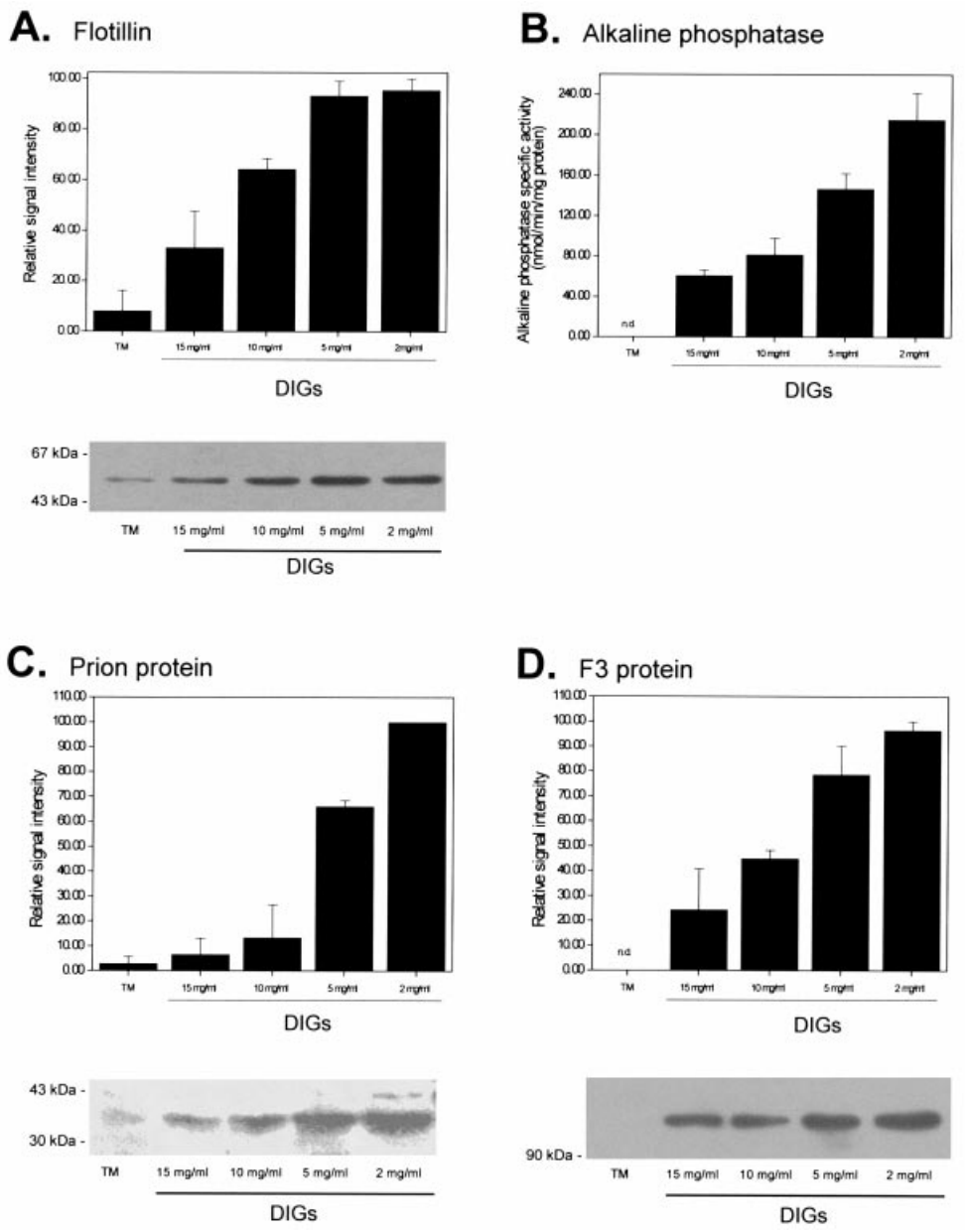


**Figure 3 Concentration of DIG marker proteins is increased at lower protein/detergent ratios**

DIGs were prepared as described in the Materials and methods section. Equal amounts of protein (20 $\mu$g) from a TM fraction and DIGs prepared from tissue solubilized in 1% (v/v) Triton X-100 at protein concentrations of 15, 10, 5 and 2 mg/ml were immunoblotted (**A**, **C** and **D**) or assayed (**B**) for the proteins indicated. Typical immunoblots are shown for flotillin, prion protein and F3 protein. The graphical quantifications shown are based on the mean relative densities (flotillin, prion protein and F3 protein) or the mean specific activity (alkaline phosphatase) from three separate sucrose gradients.

diesterase I, with the specific activity of this enzyme remaining relatively constant irrespective of the protein/detergent ratio used during solubilization of the tissue, and the amount of this enzyme in DIGs was approx. 35% of that in the TM fraction (Figure 4C). Although the specific activity of this enzyme was found occasionally to be enhanced at a protein concentration of 2 mg/ml, this difference was not statistically significant. Thus, although a minor amount of APP, alkaline phosphodiesterase I and clathrin are present in DIGs regardless of the protein/detergent ratio used during homogenization, there is no clear enrichment of these proteins as the protein/detergent ratio is decreased as observed for the DIG marker proteins.

### Proteins in DIGs isolated from mouse cerebral cortex are detergent-resistant at both 4 and 37 °C

Lipid raft proteins, although insoluble in Triton X-100 at 4 °C, are usually soluble at 37 °C [8]. This was confirmed under our experimental conditions by examining the detergent solubility of

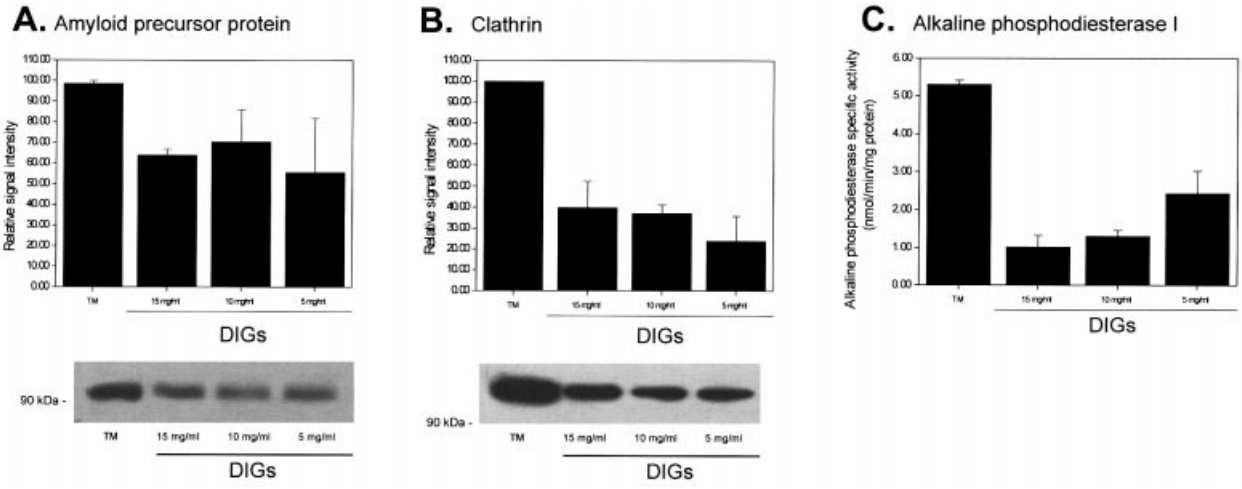


**Figure 4 Concentration of non-DIG proteins is unaffected by the protein/detergent ratio**

DIGs were prepared as described in the Materials and methods section. Equal amounts of protein (20 $\mu$g) from a TM fraction and DIGs prepared from tissue solubilized in 1% (v/v) Triton X-100 at protein concentrations of 15, 10, and 5 mg/ml were immunoblotted (**A** and **B**) or assayed (**C**) for the proteins indicated. Typical immunoblots are shown for APP and clathrin. The graphical quantifications shown are based on the mean relative densities of three immunoblots (APP and clathrin) or the mean specific activity (alkaline phosphodiesterase I) from three separate sucrose gradients.

proteins in pig kidney microvillar membranes, where the differential detergent solubility of multiple proteins was first described [11]. Kidney microvillar membranes and DIGs isolated from this source were incubated at either 4 °C or 37 °C in the absence or presence of 2% (v/v) Triton X-100 (Figure 5A). Following detergent treatment the remaining insoluble protein was pelleted by centrifugation at 100000 ***g*** for 90 min, and the insoluble pellets were resuspended in MBS to the same volume as the solubilized supernatants. In the microvillar membranes, alkaline phosphatase was pelleted completely in the absence of detergent, and was mostly (70%) insoluble in the presence of Triton X-100 at 4 °C. However, at 37 °C this protein was effectively solubilized by the Triton X-100. In DIGs isolated from pig kidney microvillar membranes the detergent solubilization pattern for alkaline phosphatase was essentially identical with that for the intact microvillar membranes in that, although the enzyme was completely soluble in Triton X-100 at 37 °C, the same detergent concentration at 4 °C failed to solubilize the enzyme. As expected, 100% of the alkaline phosphodiesterase I was solubilized upon treatment of the kidney microvillar membranes with Triton X-100 at either 4 °C or 37 °C. The same procedure was applied to DIGs isolated from mouse cerebral cortex (Figure 5B). The DIG marker proteins flotillin and F3 protein were substantially detergent-insoluble, not only at 4 °C, but also at 37 °C, whereas the prion protein and alkaline phosphatase were almost entirely ($> 93\%$) detergent-insoluble at both temperatures. The minor amounts of APP and clathrin that are present in DIGs were found to be completely detergent-insoluble at both 4 °C and 37 °C, whereas alkaline phosphodiesterase I was substantially insoluble at both temperatures. Thus the minor fraction of APP and clathrin found in DIGs isolated from mouse cerebral cortex is completely resistant to detergent solubilization.

## DISCUSSION

In the present study we have compared the detergent-insolubility of APP from mouse cerebral cortex with that of typical DIG marker proteins in order to determine whether APP is a component of lipid rafts. Using the established method of flotation through a sucrose density gradient in the presence of Triton X-100 [8], we observed that in mouse cerebral-cortex multiple DIG marker proteins (alkaline phosphatase, flotillin, F3 protein and prion protein) were present exclusively in the low-density region of the gradient, regardless of the protein/detergent ratio used during solubilization of the tissue. In addition, these DIG marker proteins showed a characteristic enrichment in the low-density fraction as the protein/detergent ratio was decreased. The criterion of detergent-insolubility that is used to isolate lipid rafts was based on the observation that multiple GPI-anchored proteins were insoluble in certain detergents at 4 °C, but soluble at 37 °C. In kidney microvillar membranes the GPI-anchored alkaline phosphatase displayed this characteristic of insolubility in Triton X-100 at 4 °C, but complete solubility at 37 °C. In contrast, in the cerebral cortex, both the GPI-anchored prion protein and F3 protein and the transmembrane protein flotillin were significantly insoluble at 37 °C. The prion protein has been shown previously to remain associated with DIGs following detergent solubilization at 37 °C [26]. The difference in detergent-solubility of the DIG proteins at 37 °C between the cerebral cortex membranes and kidney microvillar membranes may well reflect differences in the lipid compositions of these two membranes. Whereas glycosphingolipids are major components of mouse cerebellar membranes [24], they are only minor components of epithelial cell membranes [8], and alkaline phosphatase has been shown to be more insoluble in cerebroside-containing synthetic vesicles than in vesicles of other lipid compositions [45].

Unlike the DIG marker proteins, most of the APP, alkaline phosphodiesterase I, aminopeptidase A and clathrin was effectively solubilized by the Triton X-100 at all of the protein/detergent ratios examined and were subsequently located in the high-density region of the sucrose gradient. However, as the protein/detergent ratio increased, an increasing proportion of these proteins was detected in the DIGs region of the sucrose gradient. The profile of detergent solubilization and pattern of enrichment of APP at various protein/detergent ratios was essentially identical with that of alkaline phosphodiesterase I and

**A.** Pig kidney microvillar membranes

| | Control (no detergent) | | Triton X-100 | | | |
|---|---|---|---|---|---|---|
| | 37°C | | 37°C | | 4°C | |
| | I | S | I | S | I | S |
| **Alkaline phosphatase** (microvillar membranes) | 100% | 0% | 0% | 100% | 70% | 30% |
| **Alkaline phosphatase** (isolated DIGs) | 100% | 0% | 0% | 100% | 100% | 0% |
| **Alkaline phospho-diesterase** (microvillar membranes) | 100% | 0% | 0% | 100% | 0% | 100% |

**B.** Mouse cerebral cortex DIGs

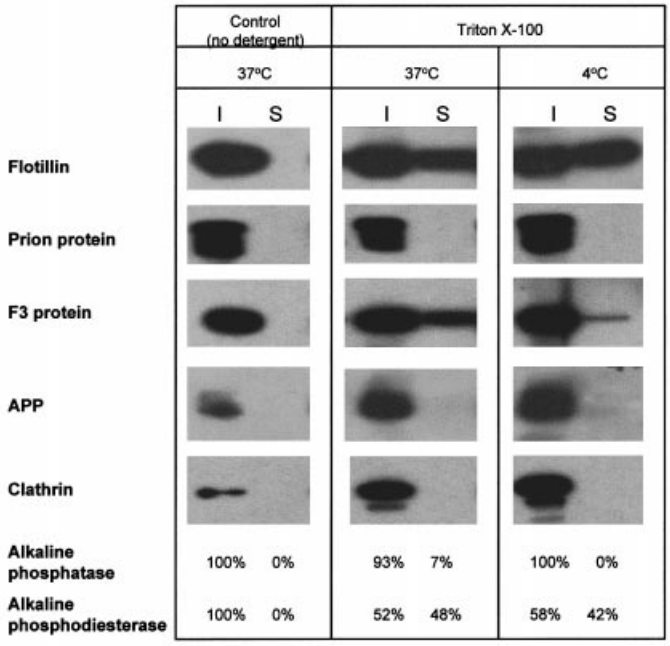


**Figure 5 All proteins in cerebral-cortex DIGs are resistant to solubilization by Triton X-100 at 37 °C**

Fractions were incubated at either 4 or 37 °C in the presence of 2% (v/v) Triton X-100. Control incubations in the absence of detergent were also performed at 37 °C. After detergent treatment the remaining insoluble protein was pelleted by centrifugation at 100 000 ***g*** for 90 min at 4 °C. The insoluble pellets were resuspended in MBS to the same volume as the solubilized supernatants. (**A**) Distribution of GPI-anchored (alkaline phosphatase) and transmembrane polypeptide-anchored (alkaline phosphodiesterase I) enzyme activities between detergent-insoluble (I) and detergent-soluble (S) fractions from kidney microvillar membranes and DIGs isolated from these membranes. (**B**) Distribution of DIG marker proteins (flotillin, alkaline phosphatase, prion protein and F3 protein) and non-DIG proteins (APP, clathrin and alkaline phosphodiesterase I) between detergent-insoluble (I) and detergent-soluble (S) fractions from DIGs isolated from mouse brain cerebral cortex.

clathrin, but significantly different from that observed for the DIG marker proteins. Thus, although APP, alkaline phosphodiesterase I, aminopeptidase A and clathrin are present in DIGs isolated from mouse cerebral cortex, they do not behave as typical DIG proteins. The observation that altering the protein/detergent ratio used during solubilization of the membranes can alter the apparent distribution of APP and other atypical DIGs proteins in the sucrose density gradients may account for the apparently conflicting reports on the presence of APP in DIGs [27,31,32,35–38]. One possible explanation to reconcile these conflicting data is that APP, alkaline phosphodiesterase I and clathrin are ineffectively solubilized contaminants of the DIGs fraction. However, this appears to be ruled out by the observations (i) that the concentration of these proteins in DIGs remained unchanged as the protein/detergent ratio was decreased, and (ii) that they remained insoluble following incubation of DIGs with excess Triton X-100 at both 4 and 37 °C. Collectively these results show that the protein/detergent ratio used during solubilization of membranes can affect the apparent distribution of certain proteins in DIGs, and that DIGs isolated from mouse brain cerebral cortex do not entirely exclude APP, alkaline phosphodiesterase I, aminopeptidase A and clathrin.

DIGs isolated from COS-7, HEK293 and Madin–Darby canine kidney ('MDCK') cells have been shown to retain the entire cell complement of APP [36]. In this instance APP was shown to interact directly with caveolin, and depletion of the latter protein using antisense oligonucleotides prevented $\alpha$-secretase cleavage, suggesting that caveolin may have a role in the $\alpha$-secretase-mediated processing of APP in non-neuronal cells. However, we have detected only a very small proportion of APP in DIGs isolated from COS-7 cells using the same methodology (E. T. Parkin, A. J. Turner and N. M. Hooper, unpublished work), and, as caveolin is absent from neuronal cells [16,27,28], the relevance of this observation to the processing of APP in the brain remains unclear.

Bouillot and co-workers [35] originally reported that 1–5% of the total neuronal APP was associated with DIGs isolated from rat embryonic cortical neurons. More recent studies have reported that a minor proportion of the total cellular APP is located in DIGs isolated either from APP-transfected rat hippocampal neurons [37] or from rat brain cortical grey matter [31]. It has been reported also that a large proportion of A$\beta$, along with various levels of presenilin-1 and presenilin-2, are present in DIGs isolated from either brain tissue or neuronal cell cultures [31,32,40]. The presence of these Alzheimer's-disease-related proteins in DIGs has led to the hypothesis that lipid rafts may be a site for the proteolytic processing of APP [31,36]. However, our observation that APP is present in DIGs from brain tissue at levels no higher than several other non-DIGs proteins brings into question whether lipid rafts are focal points for APP proteolytic processing. It is possible that APP in DIGs is rapidly processed such that the steady-state levels of this protein are lower than would otherwise be expected. Another possibility is that, as the A$\beta$ peptide has a high affinity for cholesterol [46] and ganglioside $G_{M1}$ [47–49], both of which are enriched in DIGs [8,25], this peptide rapidly translocates to DIGs after it is cleaved from APP. Finally, although depleting cells of cholesterol with methyl-$\beta$-cyclodextrin has been shown to decrease the production of A$\beta$ [37], this effect may not be entirely due to disruption of lipid rafts, as such treatment has also been shown to disrupt clathrin-coated pits [50] in which APP has been localized [51].

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