Introduction

In model membranes, sphingolipids and cholesterol separate from disordered glycerolipids to form tightly packed, ‘liquid-ordered’ domains or ‘sphingolipid rafts’ that resist solubilization in non-ionic detergents (Brown and London, 1998; Rietveld and Simons, 1998). A similar phase separation appears to occur in cell membranes, from which detergent-insoluble, glycolipid-enriched vesicles (DIGs) can be isolated (Brown and Rose, 1992). DIGs, and by biochemical cross-linking (Friedrichson and London, 1998). However, other studies using immunohistochemistry (Tiveron et al., 1994), FRET (Varma and Mayor, 1998), optical tracking (Sheets et al., 1997; Simson et al., 1998) and biochemical cross-linking (Friedrichson and Kurzchalia, 1998) have demonstrated GPI proteins to be restricted, at high density, to microdomains on the cell surface. These differing results on the density and distribution of GPI proteins on the surface may reflect diversity in the organization of different GPI proteins and/or in the size and composition of sphingolipid rafts.

To analyse the distribution of GPI proteins on the cell surface, and their relation to detergent-isolated vesicles, we have studied Thy-1 and prion protein (PrP), two functionally different GPI proteins. Thy-1, a small cell adhesion molecule (CAM) found in the nervous system exclusively on adult neurons (Morris, 1992), appears to act as a negative regulator of transmembrane signalling (Nosten-Bertrand et al., 1996; Huerber et al., 1997; Hollrigel et al., 1998). PrP is found predominantly on neurons (Kretzschmar et al., 1986), although there is some glial expression (Moser et al., 1995); unlike Thy-1 (Tiveron et al., 1994), PrP is rapidly endocytosed, possibly to provide a Cu²⁺/Zn²⁺ uptake system (Pauly and Harris, 1998). To gain a fuller understanding of the organization of GPI domains in brain, we also studied three other GPI CAMs: TAG-1 (Furley et al., 1990) and F3 (Olive et al., 1995) expressed on neurons and oligodendrocytes (Krämer et al., 1997), and the GPI isofrom of NCAM of apparent Mr, 120 kDa (NCAM-120) that is expressed on glia (Krämer et al., 1997).

Most previous studies of the membrane organization of GPI proteins have used cell lines, in which GPI protein expression can be readily manipulated but whose sphingolipid content may differ significantly from that of normal cells. We have therefore concentrated here upon neuronal

**Functionally different GPI proteins are organized in different domains on the neuronal surface**

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We have investigated the organization, on the plasma membrane and in detergent-insoluble membrane vesicles, of two neuronal glycosylphosphatidylinositol-anchored (GPI) proteins: Thy-1, a negative regulator of transmembrane signalling; and prion protein, whose rapid endocytosis and Cu²⁺ binding suggest that it functions in metal ion uptake. Prion protein occurred on the neuronal surface at high density in domains, located primarily at the cell body, which were relatively soluble in detergent. Thy-1, although much more abundantly expressed on neurons, occurred at lower density over much of the surface of neurites (and in lower abundance at the cell body) in domains that were highly resistant to detergent solubilization. Detergent-insoluble membrane vesicles contained Thy-1 at a density similar to that on the neuronal surface. Vesicles containing each protein could be separated by immuno-affinity isolation; lectin binding showed that they were enriched in different glycoproteins. Our results demonstrate a structural diversity of the domains occupied by functionally different GPI proteins.

Keywords: detergent-insoluble glycolipid/neuron/prion protein/sphingolipid/Thy-1
membranes, which contain high levels of sphingolipids (Ledeen, 1978), and have used transgenesis as a tool to modulate the level of neuronal Thy-1. We have included thymocytes, which express high levels of Thy-1, and a Thy-1-transfected neural cell line (Tiveron et al., 1994), to permit comparison with other normal cells and with a cell line.

**Results**

**Surface localization of neuronal GPI proteins**

Immunohistochemical localization of Thy-1 was examined at levels of resolution that increased progressively by ~10-fold: immunofluorescence using light microscopy (Figure 1) and immunogold labelling using scanning (SEM; Figure 2) and transmission (TEM; Figure 3) electron microscopy. Glutaraldehyde-containing fixatives were used throughout to prevent antibody-induced redistribution of the GPI proteins (Mayor et al., 1994); the same pattern of labelling was obtained with monovalent

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**Fig. 1.** Immunofluorescence labelling of Thy-1 on neurites of primary cultured neurons fixed with 0.5% glutaraldehyde/0.5% paraformaldehyde. (A) Rat neurite labelled with fluorescein isothiocyanate (FITC)-conjugated Fab OX7 anti-Thy-1 antibody, 0.2 µm Z-axis series deconvolved to show the upper surface of the neurite. (B) Mouse neurite (lacking the Thy-1.1 epitope detected by OX7) immunolabelled as in (A). (C) The same field as in (B), phase contrast. Scale bar, 5 µm.

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**Fig. 2.** Cold field emission SEM of immunogold labelling of Thy-1 (A–D, G) and PrP (E–G) on the surface of primary cultured neurons. (A) Thy-1 immunolabelling (white dots are 40 nm gold particles) on a rat neurite. Large arrows point to micrometre-scale unlabelled regions, the double-ended arrow indicates the extent of a Thy-1-labelled region, and small arrows indicate unlabelled gaps in the labelled region. (B) Fine structure of Thy-1 label (highlighted by backscattered electrons) within a single labelled region of a rat neurite; the arrow points to a cluster of high-density label. (C) Thy-1-labelled region of a bifurcating neurite from a Thy-1-overexpressing transgenic mouse; backscattered electrons. (D) Neuronal cell body (from a transgenic mouse) with extending neurites, backscattered electrons showing label around the rim of the cell body (small arrows; central area is above the plane of focus) and on neurites (larger arrows). (E) Rat neuronal cell body immunolabelled for PrP (main picture: secondary electrons; insert: backscattered electrons show arrowed cluster). (F) PrP immunolabelling (backscattered electrons) on a neurite extending from a normal rat neuron expressing exceptionally high levels of PrP [the parent cell body was labelled ~10 times more intensely than that shown in (E)]. (G) Double immunolabelling for PrP (40 nm gold) and Thy-1 (20 nm) on a rat neuron; backscattered electrons. The patch of clustered PrP immunolabelling on the cell body (higher power in the left inset) was the only PrP label found on this neuron or its proximal neurites; Thy-1 immunolabelling of one neurite is shown at higher power, right inset. Neurons were fixed with 0.5% paraformaldehyde plus 0.5% (Thy-1) or 2% (PrP) glutaraldehyde, at 37°C (A, E, F) or 4°C (B–D, G). Scale bars, 5 µm in (D) and (G), otherwise 1 µm.
Different GPI domains for Thy-1 and prion protein

Fig. 3. TEM of immunogold labelling of Thy-1 (A–F; 10 nm gold), PrP (G–J; 20 nm gold) or both (K, L; PrP 20 nm, Thy-1 5 nm gold). (A and B) Longitudinal sections of neurites of primary cultured neurons from normal rat (A) and transgenic mouse (B); small arrows point to 100–500 nm gaps in regions of Thy-1 labelling, the larger arrow (A) points to a high-density cluster of labelling. (C) Thy-1 labelling (arrow) clustered on filopodium of transfected NG115 cell line. (D) Intracellular Thy-1 labelling within vesicles (arrows) of growth cone, 24 h after cells were introduced into culture. (E and F) Thy-1 labelling of cell bodies of a neuron (E) and thymocyte (F). (G) Small clusters of PrP labelling on a neurite (arrows; the cluster on the left is in a coated pit). (H) Larger cluster of PrP labelling on a cross-section of neurite near the cell body and (J) on a neuronal cell body. (K and L) Thy-1 and PrP (arrows) labelling of transgenic mouse (K; the expansion is at a varicosity containing mitochondria) or normal rat (L) neurites; smaller arrows show PrP within the 100–500 nm gaps in Thy-1 labelling, the larger arrow in (K) shows PrP in the midst of Thy-1 labelling. Fixation as before at 4°C except for (A) (37°C). Scale bar, 100 nm.

Fab and divalent IgG fractions of antibodies. Cells were fixed at either 4 or 37°C; no temperature-dependent difference in the pattern of labelling for Thy-1 or PrP was observed (examples of labelling at both temperatures are shown in Figures 1–3).

Thy-1 on primary cultured neurons and a transfected cell line. Immunofluorescent labelling of Thy-1 on rat neurons, which appeared by conventional optics to be continuous on the surface of cell bodies and neurites, was resolved by image deconvolution into discrete clusters of surface staining bounded by regions with relatively little labelling (Figure 1A). SEM showed the labelled regions (Figure 2A and B) to be irregular in size (in the submicrometre range) and shape, bounded by micrometre-scale unlabelled regions. There was a fine structure to the labelled regions, visible in the SEM (Figure 2B) and especially the TEM (Figure 3A); unlabelled gaps (~100–500 nm wide) were inserted within clusters of Thy-1 label.

Within Thy-1-labelled regions, the immunogold was relatively dispersed—the label only approached saturating density in occasional clusters on the surface of normal neurites (Figures 2B and 3A). Higher density packing of Thy-1 immunolabel was seen in three situations. First, dense Thy-1 immunolabel was found within intracellular vesicles in the vigorously extending growth cones of normal neurons shortly after explantation into culture (Figure 3D), in sharp contrast to the low level of surface
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ing of the growth cone and its adjacent distal neurite at this stage. (It took approximately a week in culture for the labelling intensity of Thy-1 to plateau on the surface of primary neurons.) Secondly, when the Thy-1 level was increased 8-fold [by transgenic overexpression of mouse Thy-1.1 (Kollia

t et al., 1987)], there was a corresponding increase in the density of Thy-1 surface labelling (Figures 2C, D and 3B). The overall pattern of Thy-1 immunolabelling was conserved (Figure 2C), although the unlabelled gaps were reduced in size and frequency (Figure 2C compared with 2B; 3B compared with 3A). Thirdly, Thy-1 was clustered at high density into discrete patches, separated by very long (several micrometres) stretches of unlabelled membrane, on a Thy-1-transfected neural cell line (Figure 3C; Tiveron et al., 1994).

The density of immunolabelling on neuronal cell bodies was lower, but of a similar pattern to that seen on neurites (Figures 2D and 3E).

Thy-1 labelling of thymus cells. To assess Thy-1 labelling on a freshly isolated cell, a rat thymus was teased apart directly into fixative prior to Thy-1 immunolabelling; the density of label (Figure 3F) was intermediate between that found on neuronal cell bodies and neurites.

PrP labelling of primary cultured neurons. PrP immunolabelling differed from that of Thy-1 in four respects. First, individual neurons differed >10-fold in their levels of expression of PrP, whereas the level of Thy-1 labelling was relatively homogeneous on different neurons (not shown). Secondly, PrP label was usually clustered at high density in discrete patches, particularly on cell bodies where clusters of 10–20 gold particles were usually seen by SEM (Figure 2E and G). Thirdly, PrP label occurred within coated pits (Figure 3G). Fourthly, PrP label typically decorated cell bodies, and was relatively rare on neurites (Figures 2E–G and 3G–L). Where PrP labelling occurred on neurites, it was within the 100–500 nm gaps in Thy-1 labelling (Figure 3K and L), although on neurites from Thy-1-overexpressing mice single particles of PrP immuno
cell were occasionally seen within a Thy-1-labelled region (Figure 3K).

Differential solubilization of GPI proteins from isolated brain membranes by non-ionic detergents

When a non-ionic detergent extract of membrane proteins, prepared at 4°C, is centrifuged to equilibrium on a density gradient, fully solubilized membrane proteins remain in the 40% sucrose sample in which they were loaded, while insoluble, lipid-rich vesicles (the DIGs containing GPI proteins) float as an opaque band at low density (Figure 4A) separate from most membrane proteins (Figure 4C). Thy-1 DIGs were released from isolated rat brain membranes by remarkably low concentrations (0.01%) of the non-ionic detergents Brij 96 and Triton X-100. They banded at low density on a sucrose gradient, and were stable as the detergent concentration was raised to 1% (Figure 4A). Of the other components of DIGs isolated in non-ionic detergent, only ganglioside GM1 showed the same simple solubilization pattern as Thy-1 (Figure 5A).

The solubilization properties of the glial GPI protein NCAM-120 were very different. When membranes were solubilized in 0.1% Brij 96 or Triton X-100, most NCAM-120 floated above the 40% sucrose fractions (Figure 5B); however, most (Triton X-100) or all (Brij 96) NCAM-120 was fully solubilized in 0.5% detergent (fractions 10–12; Figure 5A), suggesting that 0.5% Brij 96 solubilized all the glial DIGs containing NCAM-120.

Alternative splicing generates two longer, transmembrane forms of NCAM (-140 and -180) that are expressed exclusively on neurons but not within DIGs (Olive et al., 1995). NCAM-140 was fully solubilized by both detergents. No NCAM-180 appeared in the DIG fraction in Brij 96, even in 0.1% detergent, whereas some did appear in the DIG fraction in Triton X-100 (especially at 0.1%)
(Figure 5A and B). In both detergents, some NCAM-180 pelleted at the bottom of the gradient (Figure 5A and B) along with polymerized cytoskeletal proteins (not shown).

The neuronal/glial CAMs F3 and TAG-1 divided into fractions that corresponded in solubility to both Thy-1 and NCAM-120; following solubilization in 0.5% Brij 96 or Triton X-100, the major part floated at low density, although a proportion remained within the fully solubilized fractions (Figure 5A). This solubilized proportion was reduced in 0.1% detergent (not shown) and so might include the glial component of these CAMs. The major proportion, insoluble in 0.5% Brij 96, co-purified with Thy-1 (below) and so is in neuronal DIGs.

PrP separated into three fractions after solubilization in either Brij 96 or Triton X-100 (Figure 5A): a DIG fraction (~80% of the total PrP), a fully solubilized fraction (~15%) either Brij 96 or Triton X-100 (Figure 5A); and a fraction (~<5%) that pelleted at the bottom of the gradient.

**Increased stringency of solubilization**

*Mixtures of Brij 96 with sodium deoxycholate.* Brij 96 (0.5%), equilibrated to form mixed micelles with the strong ionic detergent sodium deoxycholate, failed to solubilize significant amounts of Thy-1 until the deoxycholate contribution reached 2% (Figure 4B). Thy-1 from the brains of overexpressing transgenic mice showed a pattern of detergent insolubility identical to that of endogenous Thy-1 (Figure 4B), as did DIGs containing the neuronal component of the CAMs F3 (Figure 6A) and TAG-1 (not shown). PrP, however, was >50% solubilized when the proportion of sodium deoxycholate was 0.2%, and fully solubilized at 0.5% of the ionic detergent (Figure 6A).

The higher solubility of DIGs containing PrP compared with those containing neuronal CAMs implies that these two classes of protein exist in different lipid environments in the neuronal membrane; would these different environments be preserved in DIGs prepared in 0.5% Brij 96, or would they merge to produce a single environment of intermediate solubility? To test this, brain membranes, solubilized in 0.5% Brij 96, were applied to a sucrose gradient that contained 0.5% Brij 96/0.5% sodium deoxycholate throughout the gradient. Following centrifugation, all PrP remained in the 40% sucrose, whereas all the Thy-1 floated (Figure 6B), demonstrating that each protein retained its distinctive solubility characteristics in DIGs prepared in Brij 96.

Lectin labelling of immunoblots was used to detect a wider range of glycoproteins in the DIGs (Figure 6C). Many glycoproteins present in DIGs prepared in 0.5% Brij 96 were fully extracted by the combination of 0.5% Brij 96 plus 0.5% sodium deoxycholate (e.g. many bands detected by Maackia amurensis); other glycoproteins were present at markedly reduced levels in DIGs prepared at high stringency (e.g. the band at ~20 kDa detected by Arachis hypogaea). Overall, DIGs isolated in the mixed detergent, containing all the Thy-1 but no PrP, had a much simpler glycoprotein profile than those isolated in 0.5% Brij 96 alone.

**Increased temperature of solubilization.** In all of the work described thus far, DIGs were produced at 4°C. However, since phase partitioning by lipids shows a strong temperature dependence (Brown and London, 1998), we also solubilized membranes at 37°C in 0.5% Brij 96 and found that Thy-1 and PrP continued to float at low density on sucrose gradients (Figure 6D). To increase stringency further, rat brain membranes were solubilized at 37°C in a mixture of 0.5% each of Brij 96 and sodium deoxycholate; as an internal control, mouse brain membranes solubilized in the same detergent at 4°C were mixed with the rat extract and centrifuged together on the same gradient. Probing of the gradient fractions with species-specific Thy-1 antibodies showed that about half the rat Thy-1 remained insoluble in the mixed detergent even at 37°C (Figure 6E). The size of DIGs obtained from the mixture of rat and mouse membranes was analysed by both velocity sedimentation and gel filtration on a TSK G6000PWXL column, and found to span a wide range (10^6 kDa) that was not detectably altered with temperature of solubilization (not shown).

**Immunoaffinity purification of Thy-1 and PrP DIGs**

To determine whether the Thy-1-containing DIGs also contained PrP, we attempted to immunoaffinity purify Thy-1 from brain membrane extracted with 0.5% Brij 96. On standard immunoaffinity matrices (Sepharose 4B, Pierce Aminolinker) to which the anti-Thy-1 antibody was linked, whether before or after incubation with the DIGs, either directly, or indirectly via protein G, or via longer avidin bridges, very little (1–10%) of the Thy-1 DIGs bound to the beads, whether the beads were used in a column or batchwise, with incubation times ranging from 1 to 48 h (Figure 7A). This failure was not due to poor binding capacity of the antibody-coated beads because essentially all of the Thy-1 was retained if the DIGs were fully solubilized (Figure 7A). Equally, Thy-1 on the DIGs was accessible to antibody since liquid-phase immunoprecipitation brought down all of the Thy-1 (Figure 7A).

Reasoning that the problem was probably kinetic [slow...
Brij 96, also contained other neuronal GPI proteins effectively and specifically; economy favoured the routine use of Eupergit beads (Figure 7B), which could similarly be used to purify PrP DIGs (Figure 7D).

Immunoaffinity-purified Thy-1 DIGs, isolated in 0.5% Brij 96, also contained other neuronal GPI proteins (Figure 7C) and a broader range of glycoproteins detected with lectins (Figure 7E). The anti-Thy-1 beads removed almost all the glycoproteins from the DIG preparation (Figure 7E), suggesting that there were very few Thy-1-negative DIGs in 0.5% Brij 96. Thy-1 DIGs prepared in the more stringent mixed detergent (0.5% Brij 96/0.5% sodium deoxycholate) lacked many of the glycoproteins present after solubilization in 0.5% Brij 96 alone (Figure 7E), including the fully solubilized PrP (not shown). F3 and TAG-1, however, remained associated with Thy-1 DIGs in the mixed detergent (not shown).

Immunoaffinity purification of PrP in 0.5% Brij 96 copurified at most 10% of Thy-1 (Figure 7D) and comparably low proportions of F3 and TAG-1 (not shown). Lectin labelling showed that different glycoproteins were enriched in the PrP and Thy-1 DIGs (Phytolacca americana shown in Figure 7E). The anti-PrP beads reduced, but did not entirely remove, several glycoprotein bands present in the total DIG preparation (Figure 7E).

**Mixing of Thy-1 DIGs occurs in Triton X-100 but not Brij 96**

Membranes prepared from equal weights of co-homogenized rat and mouse brains were solubilized in 0.5% of either Brij 96 or Triton X-100 and DIGs prepared by gradient centrifugation. Rat Thy-1 was immunoaffinity purified on Eupergit beads before the total, unbound and eluted fractions were probed for rat and mouse Thy-1 (Figure 8). Following membrane solubilization in Brij 96 (or 0.5% Brij 96/0.5% deoxycholate; not shown), mouse Thy-1 DIGs were not retained on the anti-rat Thy-1 column. However, a significant proportion (10–50% in different experiments) of mouse Thy-1 was retained and eluted with the rat Thy-1 DIGs after solubilization in Triton X-100 (Figure 8).

**Ultrastructure of DIGs**

DIGs isolated in 0.5% Brij 96 (Figure 9A) or Triton X-100 (Figure 9B) were composed of small vesicles, many with diameters in the range of 0.2–1 μm, although smaller (~50 nm) and larger (several micrometres) vesicles were also present (Figure 9G and H). DIGs isolated in Triton X-100, but not in Brij 96, contained multi-lamellar myelin fragments (Figure 9B and C) and the transmembrane myelin-associated glycoprotein (detected by Western blotting; not shown). Immunogold labelling of Thy-1 on DIGs prepared from 0.5% Brij 96 without immunoaffinity purification revealed clusters of gold particles, at a density...
similar to that found on normal neurites (cf. Figure 3A and L), with intervening 100–500 nm stretches of unlabelled membrane (Figure 9D–H). Occasionally, vesicles had very low levels of Thy-1 (Figure 9H) and some were unlabelled (Figure 9G and H), from which we infer that a minor population of DIGs could have been Thy-1-negative, although cross-sections of vesicles containing very little Thy-1 must occasionally include unlabelled profiles.

Discussion

Most models of the organization of GPI proteins in sphingolipid rafts contain two general assumptions. First, rafts occupy small regions of the cell surface, coralling GPI proteins at very high density; consequently, evidence for dense packing of GPI proteins is taken as proof that rafts exist (e.g. Friedrichson and Kurzchalria, 1998; Varma and Mayor, 1998), whereas evidence for a dispersed distribution of GPI proteins questions their existence (e.g. Mayor et al., 1994; Jacobson and Dietrich, 1999). Secondly, rafts containing GPI proteins are homogeneous, so that all components within detergent-insoluble vesicles have access to each other.

Our findings argue that these assumptions should be modified. We propose that: (i) functionally different GPI domains differ structurally; (ii) different GPI proteins can cluster within sphingolipid rafts at different densities and predominantly on different parts of the same cell; and (iii) sphingolipid rafts cover relatively large areas of the cell surface.

We believe that our argument is strengthened by the convergence of results that have been derived from three complementary approaches: immunohistochemical labelling, detergent solubilization and immunoaffinity purification. We have used TEM and more especially SEM, rather than light microscopy, to provide the level of resolution required to visualize DIGs and the intervening membrane regions. In addition, we have based our interpretation on findings obtained using normal neurons and thymocytes, rather than transfected cell lines, on the grounds that GPI proteins are dependent for their localization in the membrane, and for their function, upon specific membrane lipids and transmembrane proteins that may not be present at physiological levels in cell lines.

Structural differences in domains enclosing functionally different GPI proteins

It is now recognized that at least two distinct types of DIG can co-exist on the cell surface: caveolae and the rafts enclosing GPI proteins (Schnitzer et al., 1995; Stan et al., 1997; Doyle et al., 1998). Very low levels of caveolin proteins and caveolae are present in brain, on non-neuronal cells (Ikezu et al., 1998), so we have largely avoided the need to separate caveolae from DIGs containing GPI proteins.

The problem of separating neuronal and glial DIGs was largely resolved by selective solubilization of most glial domains by 0.5% Brij 96. Thy-1 is exclusively neuronal in the adult nervous system (Morris, 1992), yet the anti-Thy-1 beads removed almost all glycoprotein (detected by lectins) from DIGs prepared in 0.5% Brij 96. This implies that most glial DIGs were solubilized; in fact the glial GPI protein NCAM-120 was fully solubilized by 0.5% Brij 96, as were significant proportions of the GPI proteins (F3, TAG-1 and PrP) that are expressed on both glia and neurons. A clue as to the cause of the relative insolubility of neuronal DIGs is given by ganglioside GM1, most of which remained in neuronal DIGs when glial DIGs were solubilized. Neuronal membranes are rich in gangliosides (Ledeen, 1978; Schwarz and Futerman, 1996) whose concentration in highly ordered lipid domains presumably contributes to the exceptional detergent resistance of neuronal DIGs.

On the neuronal membrane, sphingolipid rafts containing PrP were more soluble than those containing Thy-1, yet pure Thy-1 is relatively hydrophilic (Kuchel et al., 1978) whereas PrP is relatively insoluble (Prusiner, 1998). The differing solubility of these GPI proteins in the membrane must therefore reflect differences in the content and degree of ordered packing of their surrounding lipids. We found that these differences exist at 37°C as well as 4°C, in contrast to studies with transfected cell lines where the solubility of GPI proteins at 37°C (Brown and Rose, 1992; Rijnboutt et al., 1996) presumably reflects lower levels and/or a different content of sphingolipids and cholesterol in the cell lines compared with neurons.

Our finding that DIGs are released from the fluid glycerolipid membrane by concentrations of non-ionic detergents as low as 0.01% suggests a sharp interface between the fluid and ordered lipid phases. Our data further imply that the highly ordered domains containing Thy-1, and less ordered domains containing PrP, are physically adjacent. All Thy-1 could be separated from PrP by high-stringency solubilization, confirming the immunohistochemical observation that the proteins exist in different environments. At lower stringency of solubilization (0.5% Brij 96) where neuronal PrP and Thy-1 domains remained intact, immunoaffinity purification of PrP co-purified a small fraction of Thy-1, while immuno-
Fig. 10. Model of how graded ordering of lipids induced by phase partitioning affects the partitioning of proteins in the intact surface membrane. (A) Diagram of surface membrane (extracellular surface uppermost). In bold are represented the sphingolipids (saturated, straight lipid chains, balloons representing glycosylation) forming a highly ordered domain in which Thy-1 is contained, bordered by PrP in a semi-ordered domain that contacts the fluid glycerolipid domain (‘kinked’, unsaturated lipid chains) in which the transmembrane proteins are embedded. Cytoplasmic protein tyrosine kinases (PYK) are shown partitioning into the semi- and fully-ordered lipid domains. (B) Proposed relative degree of lipid order (arbitrary scale) in the different regions of the membrane shown in (A).

affinity purification of the Thy-1 DIGs co-purified almost all of the PrP. This implies that no membrane soluble in 0.5% Brij 96 separates PrP and Thy-1 domains on the membrane, from which it follows that PrP domains must border Thy-1 domains with no intervening fluid phase membrane. This was confirmed immunohistochemically, since PrP label was adjacent to, but not within, the clusters of Thy-1 labelling, and the majority of Thy-1 domains lacked PrP.

The association of F3 and TAG-1 with Thy-1 DIGs in 0.5% Brij 96 was retained as the stringency of solubilization was increased, and through Thy-1 immunoaffinity purification. These three CAMs therefore appear to occupy the same neuronal domains.

The simplest model suggested by this data is that semi-ordered lipid domains containing PrP lie at the margins of the highly ordered Thy-1 domains (Figure 10A), presumably in a decreasing gradient of lipid order created by dynamic phase partitioning of sphingolipids and cholesterol, and the lipidated proteins contained within them (Figure 10B). Such a marginal zone of semi-ordered lipids would enhance interactions between GPI and transmembrane proteins that are essential for the function of both types of membrane protein. A large family of transmembrane receptors have GPI proteins as the ligand-binding subunit (the prototype being the receptor for ciliary neurotrophic factor; Davis and Yancopoulos, 1993). Other transmembrane receptors (e.g. FceR1 and T-cell antigen receptors; Field et al., 1995; Montixi et al., 1998), upon binding ligand, rapidly translocate from the fluid glycerolipid membrane into a region that is partially detergent resistant, similar to the semi-ordered PrP domain. Endocytosis, normally mediated by trafficking motifs within the cytoplasmic domain, poses an obvious problem for GPI proteins that the uPA receptor solves by binding to an endocytosing transmembrane receptor (Nykjaer et al., 1992). The location of PrP in a semi-ordered domain may be due to a similar requirement to bind to a transmembrane protein for endocytosis.

Different GPI proteins can cluster at different densities predominantly on different parts of the same cell

Thy-1 is quantitatively the dominant glycoprotein on the thymocyte surface: it is four times more abundant than the sum of the other major glycoproteins on this cell (Barclay et al., 1993). It is similarly abundant on the neuronal surface, yet on neither cell type, nor on DIGs isolated from brain, is Thy-1 uniformly clustered at high density. The variable, and overall relatively moderate, density of Thy-1 surface labelling that we find is unlikely to reflect destruction of antigen by the fixative since the latter retains >80% of Thy-1 antigenicity (Morris and Barber, 1983). Moreover, Thy-1 immunolabelling can be clustered at high density, as we show here on the surface of neurons of mice transgenically overexpressing Thy-1, on a Thy-1-transfected neural cell line (Tiveron et al., 1994) and on intracellular (presumably biosynthetic) vesicles in growth cones (cf. the high density of GPI-anchored 5’ nucleotidase as it arrives on the surface; Kenworthy and Eddin, 1998).

The moderate density of Thy-1 labelling on normal thymocytes and neurons therefore implies that it is not a major component of sphingolipid rafts. Sphingolipids, more abundant than glycoproteins on neurons (Ledeen, 1978), are necessary and sufficient to form ordered lipid domains with cholesterol (Brown and London, 1988). On neurons and thymocytes these sphingolipid rafts must occupy large areas of the cell surface to accommodate, at subsaturating density, a protein as abundant as Thy-1. Since PrP occurred on neurites within the gaps in Thy-1-labelled areas, these small Thy-1-free areas were presumably semi-ordered lipid regions.

Although PrP was present at much lower levels than Thy-1 on the neuronal surface, it was clustered into discrete patches at relatively high density, especially at the cell body. Interestingly, the GPI-anchored folate receptor, which is endocytosed (Rijnboutt et al., 1996), is also clustered at high density (Varma and Mayor, 1998);
the clustering of both could be a function of their binding to transmembrane proteins responsible for leading them into endocytic trafficking. The high local density of normal cellular PrP would enhance the probability of protein-protein interactions thought to drive the pathological conversion of PrP in prion disease (Prusiner, 1998).

**Isolation of GPI domains as DIGs using non-ionic detergents**

Can the relative insolubility in non-ionic detergents of GPI proteins be used to isolate, intact and uncontaminated, the patches of surface membrane in which they are located?

The detergent most frequently used for such studies, Triton X-100, proved unsuitable for studying neuronal DIGs, for three reasons: (i) it promoted intermingling of GPI proteins derived from entirely different brains; (ii) it failed to segregate DIGs and myelin membranes; and (iii) it permitted association of DIGs with proteins not normally thought to be associated with them, such as transmembrane NCAM-180. The potential for artefactual intermingling of membrane components in detergent, seen here with Triton X-100, is particularly relevant to GPI proteins since mild detergents promote the exchange of GPI proteins between surface membranes of different cells (Medof et al., 1996).

We directed our attention to the detergent Brij 96, which has been found to be suitable for DIG isolation from lymphoid cells (Draberova et al., 1996). For solubilizing brain membrane proteins, Brij 96 suffered none of the disadvantages of Triton X-100, and had the unexpected advantage of solubilizing the glial domains. We doubt whether Brij 96 induced clustering or mixing of DIGs during solubilization of neuronal membranes, since the distribution of Thy-1 on isolated DIGs was similar to that seen on the surface of cultured neurons; Thy-1 DIGs could be cleanly isolated (by differential solubilization) from PrP DIGs, and the latter could be isolated (by immunoaffinity purification) from most of the Thy-1.

We therefore propose that DIGs provided by solubilization of brain membranes in Brij 96 yield vesicles that retain the molecular associations of the GPI proteins in the neuronal membrane. Brain provides material sufficient to allow non-Edman sequencing of the component proteins of isolated DIGs (work in progress), which should enable the identification of the full range of interacting proteins in different GPI domains.

**Materials and methods**

**Antibodies, cells and immunolabelling**

The specificity, purification, Fab production and coupling to biotin and fluorochromes of monoclonal anti-Thy-1 antibodies (OX7 for rat Thy-1; R194 for mouse Thy-1), and their use in light and TEM immunolabelling and immunoblotting, have been described (Tiveron et al., 1994). OX7 recognizes the Thy-1.1 epitope, present on all rat Thy-1 and a few mouse strains including the transgenic Thy-1.1-overexpressing strain used here (Kollias et al., 1987); most mouse strains (including the normal mice used here) have the alternative Thy-1.2 allele (Morris, 1992). Anti-PrP antibodies were made in this laboratory: their production, purification and specificity will be described in full elsewhere. Briefly, peptides corresponding to residues 23–42, 90–108, 98–116, 142–160 and 214–231 of the mouse PrP sequence were coupled to keyhole limpet haemocyanin by two methods: by introducing a C-terminal Cys residue whose -SH group was reacted with a bivalent cross-linker to induce antibodies used for immunoblotting; and by cross-linking -NH2 groups on the peptides and carrier using disuccinimidyl glutarate (Pierce), whose reaction closely mimics that of glutaraldehyde, to induce antibodies that react well with PrP in glutaraldehyde-fixed cells. Anti-peptide antibodies were immunoaffinity purified, and Fab fragments made and coupled to biotin as before. Specificity tests included lack of reaction with material from PrP knockout mice, identical reaction of antibodies detecting different epitopes, and absorption by relevant peptide. Rabbit antibodies to F3 (Olive et al., 1995) and TAG-1 (Furley et al., 1990), and the BDP-2 monoclonal anti-NCAM antibodies, were also used (Hirn et al., 1981). Antibodies were used in immunohistochemistry at saturating concentrations (20 μg/ml with 2–4 h incubations). Ganglioside GM1 was detected in a dot-blot with biotinylated cholera toxin A chain, and glycoproteins with biotinylated lecithins (Sigma).

Dorsal root ganglia were dissociated from 3- to 4-week-old Wistar rats. Thy-1.1 transgenic mice neurons, plated into laminin + mouse Ig-coated coverslips, were cultured for 7–9 days prior to assay in MEM-v with Na2 supplement (Gibco-BRL) plus 7.5 ng/ml nerve growth factor (NGF) (Serotec), 5 μg/ml brain-derived neurotrophic factor (BDNF) (Preprotech) and 1 μg/ml cholesterol as low-density lipoproteins (Sigma). Thy-1.2-transfected NG115 cells were as before (Tiveron et al., 1994). Cultured cells, washed once with phosphate-buffered saline (PBS), were fixed for 20 min, either at 4 or 37°C, with 0.5% paraformaldehyde containing glutaraldehyde (0.5% for Thy-1; 2% for PrP; Agar Scientific Ltd, Essex, UK; 50% vacuum distilled), blocked with 2% defatted powdered milk in PBS and immunolabelled. Immunofluorescence images, collected in a 0.2 μm Z-axis series, were deconvoluted using Openlab 2.0.4 Digital Confocal Module (Improvision, UK). For SEM, labelled cells were dehydrated in acetone and critical-point dried, coated with gold and viewed in an Hitachi S47000 or S35000N. Thymocytes were obtained by dissociating a rat thymus directly into 0.5% glutaraldehyde/0.5% paraformaldehyde and subsequently processed as for cultured cells.

**DIG isolation**

The membrane fraction pelleted by centrifuging a post-nuclear fraction of brain homogenate (30 ml at 25 000 g for 20 min) was resuspended at 5 mg protein/ml in 10 mM Tris–HCl pH 8.2/0.02% NaN3 (detergent buffer) and mixed with stirring in an equal volume of 2× detergent (for 30 min at 4°C or 37°C); the solution was chilled to 4°C, mixed with an equal volume of 80% sucrose and 1 ml of this sample overlaid with an 11 ml gradient of 5–30% sucrose in a Beckman SW41 ultracentrifuge tube (all sucrose solutions contained the same detergent, at the same final concentration, used for solubilization; all solutions were supplemented with 1 mM phenylmethylsulfonyl fluoride plus the Complete Mini protease inhibitor cocktail (Boehringer Mannheim, UK)). Following centrifugation (200 000 g for 18 h at 4°C), 12 × 1 ml samples of the gradient, plus the pellet resuspended in 1 ml of detergent, were taken for analysis. Protein was detected on blots using Protogold (British BioCell International).

Mixed detergent solutions were produced by dissolving both Brij 96 (Fluka) and sodium deoxycholate (BDH) to the desired proportion and specific activity puri flexted with an equal volume of 0.153 M ethanolamine–HCl pH 8.5 (15 min), collected and washed twice in 1% bovine serum albumin/PBS by centrifugation, and incubated in 20 μg/ml OX7 pre-bound to 20 μg of goat anti-mouse IgG10 nm colloidal gold (British BioCell International) for 1 h with mixing. The mixture was then centrifuged over a 20% sucrose cushion (14 000 g for 10 min in a microfuge), labelled DIGs floating at the interface were removed and pelleted by centrifugation (50 000 g for 30 min), and post-fixed in 2% glutaraldehyde/PBS (30 min) before processing for TEM.

**Immunofluorescence purification**

All procedures were carried out at 4°C. Antibody (5 mg/ml) was coupled to Eupergit CIZ beads as described (Grassel et al., 1989), or to MACS magnetic IgG microbeads (Miltenyi Biotech, UK) by antibody binding. DIG fractions (2 ml) from sucrose gradients were diluted with detergent solution and pelleted by centrifugation (1.5 ml at 50 000 g for 4 h). Pellets were resuspended in 0.5% glutaraldehyde/0.5% paraformaldehyde for 10 min, quenched with an equal volume of 0.153 M ethanolamine–HCl pH 8.5 (15 min), collected and washed twice in 1% bovine serum albumin/PBS by centrifugation, and incubated in 20 μg/ml OX7 pre-bound to 20 μg of goat anti-mouse IgG10 nm colloidal gold (British BioCell International) for 1 h with mixing. The mixture was then centrifuged over a 20% sucrose cushion (14 000 g for 10 min in a microfuge), labelled DIGs floating at the interface were removed and pelleted by centrifugation (50 000 g for 30 min), and post-fixed in 2% glutaraldehyde/PBS (30 min) before processing for TEM.
For liquid phase separation, DIG fractions were pre-cleared twice by incubation in normal mouse serum (10 µg/ml, 1 h) with sheep anti-mouse IgG (50 µg/ml), followed by centrifugation (3000 g for 10 min); Thy-1 was reacted with OX7 (50 µg/ml, 1 h) then immunoprecipitated by 250 µg/ml sheep anti-mouse IgG (1 h, then centrifugation at 3000 g for 10 min). Coupling of antibody to larger matrix beads followed the manufacturer’s instructions.

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References


