The Membrane Domains Occupied by Glycosylphosphatidylinositol-anchored Prion Protein and Thy-1 Differ in Lipid Composition

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Glycosylphosphatidylinositol-anchored prion protein and Thy-1, found in adjacent microdomains or “rafts” on the neuronal surface, traffic very differently and show distinctive differences in their resistance to detergent solubilization. Monovalent immunogold labeling showed that the two proteins were largely separated in separate domains on the neuronal surface: 86% of prion protein was clustered in domains containing no Thy-1, although 40% of Thy-1 had a few molecules of prion protein associated with it. Only 1% of all clusters contained appreciable levels of both proteins (>3 immunogold label for both). In keeping with this distribution, immunoaffinity isolation of detergent-resistant membranes (DRMs) using the non-ionic detergent Brij 96 yielded prion protein DRMs with little Thy-1, whereas Thy-1 DRMs contained ~20% of prion protein. The lipid content of prion protein and Thy-1 DRMs was measured by quantitative nano-electrospray ionization tandem mass spectrometry. In four independent preparations, the lipid content was highly reproducible, with Thy-1 and prion protein DRMs differing markedly from each other and from the total DRM pool from which they were immunoprecipitated. Prion protein DRMs contained significantly more unsaturated, longer chain lipids than Thy-1 DRMs and had 5-fold higher levels of hexosylceramide. The different lipid compositions are in keeping with the different trafficking dynamics and solubility of the two proteins and show that, under the conditions used, DRMs can isolate individual membrane microenvironments. These results further identify unsaturation and glycosylation of lipids as major sources of diversity of raft structure.

The separation of membrane lipids into different phases creates diverse microenvironments within a biological membrane (1, 2). In particular, cholesterol is believed to condense with saturated phosphatidylcholine (PC) and sphingomyelin (SM) to form minute patches (40–100 nm wide) of lipids in a liquid-ordered phase (3–7), creating specialized lipid microenvironments called “rafts” within the disordered fluid phase formed by unsaturated lipids (8). These ordered microdomains control the access and egress of subsets of membrane proteins, regulating signaling systems at the cell surface (9). liquid-ordered domains resist solubilization in non-ionic detergents (6, 7, 10–13), enabling them to be isolated as detergent-resistant membranes (DRMs) that float at low density upon centrifugation (14). Lipid-anchored proteins partition into both leaflets of these domains, the glycosylphosphatidylinositol (GPI)-anchored proteins into the outer (surface) layer and the diacylated cytoplasmic proteins into the inner layer (9, 15, 16).

The membrane environment of GPI-anchored prion protein (PrP) is of particular interest since it is a candidate for choreonizing the conversion of PrP to the altered pathogenic conformation associated with prion disease (17–19). Immunolabeling shows PrP to be present on the neuronal surface in different, albeit often closely adjacent, domains to those occupied by Thy-1, the major GPI-anchored protein of mature neurons (20). These differences in surface localization are reflected in the different functions and trafficking of these proteins. Thy-1 inhibits the activity of Src family kinases attached to the inner leaflet of rafts (21), undergoes relatively slow internalization (22), and has a half-life of >100 h (23). PrP has a half-life of a few hours (24, 25); it is rapidly and constitutively endocytosed on neurons, leaving rafts (as defined by their insolubility in standard non-ionic detergents) to enter more soluble membrane domains on the cell surface, and thus coated pits and endosomes (22).

The rafts occupied by PrP are distinctly more soluble than those of Thy-1 (20), a result that could indicate differences in lipid composition in the membrane surrounding the two GPI-anchored proteins. The primary goal of this study is to characterize the lipid composition in immunooaffinity-isolated PrP and Thy-1 DRMs, to determine whether the lipid environment of these functionally different GPI-anchored proteins differs. We have examined in detail cholesterol, as well as three lipids that are found predominantly on the outer leaflet of the plasma membrane (26): PC as the major glycerolipid, SM as the major sphingolipid, and hexosylceramide (HexCer) as a glycosphingolipid.

The approach followed here, of analyzing lipid composition of immunoisolated DRMs, is valid only if the detergent fractionates the membrane into discrete lipid microenvironments that maintain their separate identity during solubilization and pugent-resistant membrane; ESI-MS/MS, electrospray ionization tandem mass spectrometry; GPI, glycosylphosphatidylinositol; HexCer, hexosylceramide; PrP, prion protein; SM, sphingomyelin.

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Different Lipid Composition of Thy-1 and PrP DRMs

Phosphate determination was performed according to Rouser (33, 34) and described for sphingomyelin (33). Sphingosylphosphorylcholine, sphingosine, and psychosine were obtained from Matreya Inc. (Pleasant Gap, PA), standard synthesis of HexCer standards (18:1; 14:0/18:1; 17:0/18:1; 25:0) were performed as described previously (33). After solvent evaporation, samples were re-dissolved in dry ethanol immediately before homogenization, with Complete Mini protease inhibitor mixture (Roche Applied Science)). The postnuclear membrane pellet (18,000 × g, 40 min) was resuspended at 5 mg of protein/ml. This was diluted 1:1 in 1% Brij 96 (Fluka, Lot Number 402329/1) in buffer S, rotated gently for 30 min before a 1-ml aliquot was taken and frozen at −80 °C. Ten microliters of a synthetic lipid standard (18:1; 14:0/18:1; 19:0/18:1; 26:0) were added to 80 μl of a continuous 30–50% sucrose gradient in buffer S at 0.5% Brij 96 and centrifuged in a Beckman SW41 rotor (200,000 × g, 18 h). Sequential 1-ml fractions were removed, the position of the DRM fraction, identified by its opacity, was confirmed by immunoblotting for PrP and Thy-1, and an aliquot of the fraction (usually number 3) with the highest content of either protein was used directly for each DRM analysis. Lipid standards were quantitated using alkaline phosphatase-coupled secondary antibody, the enzymatic reaction was developed within its linear range, and blots were scanned on a Heidelberg 1200 flatbed scanner and analyzed with NIH Image.

Lipid Analysis—Lipid extractions in the presence of internal standards were performed according to the method of Bligh and Dyer (32) as described previously (33). After solvent evaporation, samples were re-suspended in methanol and further processed for mass spectrometry as described (33, 34). Nano-ESI-MS/MS analysis was performed on a Micromass QII triple-stage quadrupole tandem mass spectrometer equipped with a nano-ESI source (Z-spray) from Micromass. Argon was used as collision gas at a nominal pressure of 2.5 × 10−7 mbars. The cone voltage was set to 20 V. Resolution of Q1 and Q3 was set to achieve isotope resolution. Detection of PC and SM was performed by parent ion scanning for fragment ion 264 at a collision energy of 30 eV. SM detection in negative ion mode was done by applying a cone voltage of 100 V and a collision energy of 33 eV, selecting for a fragmentation of m/z 168. Cholesterol quantitation was performed as described (35) with a4-cholesterol (Cambridge Isotope Laboratories Inc., Andover, MA) in negative ion mode, selecting for fragment ions of m/z 80 at a cone voltage of 50 V and a collision energy of 130 eV. Synthetic lipid standards were obtained from Avanti Polar Lipids (Alabaster, AL), standard synthesis of HexCer standards (18:1; 14:0/18:1; 19:0/18:1; 26:0), and ceramide standards (18:1; 14:0/18:1; 17:0/18:1; 25:0) were performed as described for sphingomyelin (33). Sphingosylphosphorylcholine, sphingosine, and psychosine were obtained from Matreya Inc. (Pleasant Gap, PA), and fatty acids were from Merck (Darmstadt, Germany). Quantitative analyses were performed as described (33, 34). Phosphate determination was performed according to Rouser et al. (36). The significance of data was tested by analysis of variance with repeated measures; data that differed at p < 0.05 were then analyzed by paired, two-tailed t tests.

EXPERIMENTAL PROCEDURES

Preparation of DRMs (20)—For a source of rat brain, four 3-month-old virgin female Sprague-Dawley littersmates were used. All steps in the procedure were carried out at 4 °C independently for each brain with only the buffer/detergent solutions in common.

Freshly removed brain was homogenized in 0.32 μsucrose/buffer S (10 μM Tris-Cl, pH 8.0/0.02% Na2EDTA) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride (Sigma), added from 100× stock solution in dry ethanol immediately before homogenization, with Complete Mini protease inhibitor mixture (Roche Applied Science)). The postnuclear membrane pellet (18,000 × g, 40 min) was resuspended at 5 mg of protein/ml. This was diluted 1:1 in 1% Brij 96 (Fluka, Lot Number 402329/1) in buffer S, rotated gently for 30 min before a 1-ml aliquot was taken and frozen at −80 °C. Ten microliters of a synthetic lipid standard (18:1; 14:0/18:1; 19:0/18:1; 26:0) were added to 80 μl of a continuous 30–50% sucrose gradient in buffer S at 0.5% Brij 96 and centrifuged in a Beckman SW41 rotor (200,000 × g, 18 h). Sequential 1-ml fractions were removed, the position of the DRM fraction, identified by its opacity, was confirmed by immunoblotting for PrP and Thy-1, and an aliquot of the fraction (usually number 3) with the highest content of either protein was used directly for each DRM analysis. Lipid standards were quantitated using alkaline phosphatase-coupled secondary antibody, the enzymatic reaction was developed within its linear range, and blots were scanned on a Heidelberg 1200 flatbed scanner and analyzed with NIH Image.

Electron Microscopy—Adult mouse sensory neurons (37), maintained in culture for 3–5 days, were immunolabeled for 30 min at 10 °C with 5 nm (PrP) or 10 nm (Thy-1) of gold, to which the appropriate Fab was directly coupled in a limiting amount to achieve monovalent binding to surface antigen; the gold was titrated to a level at which it bound at >80% saturation of surface antigen (22). Cells were then fixed in 1% glutaraldehyde/1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and processed for viewing 80–100-nm-thick sections in a transmission electron microscope (Hitachi 7600 at 75 kV). Specimens were digitally photographed at ×10,000 (after viewing at ×100,000 if necessary to resolve the size/number of closely clustered grains). Label (>2,000 grains counted for both PrP and Thy-1) that was within 20 nm of another label on the same membrane was scored as within a single cluster. Control experiments using neurons taken from mice genetically null for Thy-1 (38) or PrP (39) showed label only for the expressed protein. With the 10 nm of gold used for Thy-1 labeling, smaller grains that would be scored as 5 nm (PrP) of label were occasionally seen, at a frequency of <1% of that seen for PrP label on wild type neurons.

RESULTS

Separation of PrP and Thy-1 on the Membrane and in DRMs—To determine the extent to which immunolabel for PrP and Thy-1 identify separate microdomains on the neuronal surface, primary cultured sensory neurons were labeled with Fab directly coupled to 5 nm (anti-PrP) or 10 nm (anti-Thy-1) of gold and viewed in the transmission electron microscope. Label for each GPI-anchored protein was present in clusters largely devoid of the other (Fig. 1, A–C). However, one or two immunogold labels for PrP or Thy-1 were frequently found at the border of Thy-1 clusters (Fig. 1, A, B, and E), and Thy-1 label was occasionally found beside a cluster of PrP (Fig. 1D).

To assess the extent of overlap between PrP and Thy-1, a cluster was defined operationally as any group whose individ-
Fig. 2. Extent of co-localization of PrP and Thy-1 label on sensory neurons. Each point represents a cluster of label for PrP (x axis) and Thy-1 (y axis). PrP-only clusters appear in the bottom right quadrant (the arrow indicates 3 points denoting clusters of 22 PrP immunogold labels), and Thy-1-only clusters appear in the top left quadrant (the arrow here indicates 2 points denoting clusters of 20 Thy-1 immunogold labels), and mixed clusters containing label for both GPI-anchored proteins are in the upper right quadrant (the arrow here indicates 2 points denoting 5 immunogold labels each for Thy-1 and PrP within the same cluster). For display purposes, a log/log plot has been used, with populous values (e.g. for clusters of 1 label) offset so as not to coincide.

Most gold grains were within 20 nm of another. Most PrP (86.1%) was found within clusters of 1–43 immunogold grains that contained no Thy-1; the remainder occurred primarily as 1–3 grains of PrP label associated with Thy-1 clusters of varying size (Fig. 2). On the other hand, a few grains of PrP label were often associated with Thy-1 clusters, so that 40% of the latter contained some PrP. Clusters containing label for both proteins appear in Fig. 2, in the upper right quadrant. Very few clusters (8/746, or 1.1%) contained >3 immunogold particles for both proteins (Fig. 2).

If the organization of PrP and Thy-1 on the neuronal membrane in vitro reflects that found with cultured neurons, it may be possible to immunoaffinity-purify a relatively pure population of PrP DRMs, whereas a substantial proportion of Thy-1 DRMs would have some PrP associated with them. In practice, immunoprecipitation would be unlikely to yield such clear separation since, for example, a few molecules of PrP within an otherwise Thy-1-containing DRM could cause the whole to be immunoprecipitated as a PrP DRM. Nonetheless, we previously observed purification broadly in keeping with the extent of separation of PrP and Thy-1 seen ultrastructurally; immunoprecipitation of DRMs with anti-PrP Immunebeads isolated 90% of PrP and 20% Thy-1, whereas anti-Thy-1 Immunebeads isolated >95% of the Thy-1 along with nearly 85% of PrP present in the Thy-1 DRMs (20).

In this study, to maximize the yield of DRMs in which either PrP or Thy-1 was dominant, the total DRM pool (Fig. 3A) was divided in two, and each was precleared twice with the reciprocal antibody before immunoprecipitating PrP or Thy-1 (Fig. 3B). The preclears were done rapidly with limiting amounts of antibody to favor removal, from the pool from which PrP would be immunoprecipitated, of DRMs expressing the highest levels of Thy-1, and vice versa. This strategy was successful. Preclearing with anti-Thy-1 enabled PrP DRMs that contained 40% of the PrP, with <2% of the Thy-1, to be isolated, and over 50% of Thy-1 DRMs were obtained that contained 20% of the PrP (Fig. 3B). Since Thy-1 is >10-fold more abundant than PrP (assessed by the degree of purification required from brain; (42–44)), the lipids associated with 20% of PrP present in the Thy-1 DRM would be a minor contaminant when compared with those associated with the more abundant Thy-1.

Significant Differences in Lipid Composition between PrP and Thy-1 DRMs—For each of the four independent preparations of PrP and Thy-1 DRMs, and the total pool of DRMs from which they were immunoprecipitated, the levels of cholesterol, ceramide,3 PC, SM, and HexCer, as well as the amount of fatty acid of different mass (i.e. chain length and saturation) attached to the latter three lipids, were measured. For each sample, duplicate lipid analyses were done, and the mean was used as the value for that sample.

The relative proportions of the lipids in Thy-1, PrP, and total DRMs is shown in Table I. The values are expressed as a percentage of the total lipid measured ([cholesterol] + [PC] + [SM] + [HexCer] + [ceramide]), as it is the relative proportions

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3 Only the major ceramide species, N-stearoylcereamide, was measured, as a control for the HexCer measurements.
of these components that is of interest. The results overall were remarkably consistent between the four preparations, giving standard deviations that were small when compared with the differences in mean values for the different DRMs. The proportion of most lipids differed significantly between the two immunoaffinity-purified DRMs and between each of these and the total DRM pool from which they were purified (Table I). The cholesterol level of PrP DRMs was slightly (1.12-fold), albeit significantly, higher than that of Thy-1 DRMs, although both were significantly lower than the cholesterol level of the total DRM. The same trend was evident with HexCer, although the level of this glycosphingolipid in Thy-1 DRMs was markedly lower than that of PrP DRMs. The contribution of the longer chain fatty acids attached to HexCer in Thy-1 and PrP DRMs was 74.3% of HexCer in Thy-1, and 76.0% in PrP. DRMs. The contribution of the longer chain fatty acids attached, which totaled (pooling hydroxylated plus non-hydroxylated) levels of PC than either PrP or total DRMs, and both Thy-1 and PrP had 2-fold higher levels of SM than the total DRMs. In addition, ceramide level was highest in Thy-1 DRMs (2- and 1.4-fold enriched over total and PrP DRMs, respectively).

The immunoaffinity-purified, and total, DRMs also differed significantly in the individual fatty acids attached to PC and SM, but not to HexCer. Values for the major lipid species are given in Table II. Thy-1 DRMs were significantly enriched in the major saturated fatty acids attached to PC (32:0 and 34:0) and SM (18:0). Conversely, PrP DRMs had a higher proportion of mono-unsaturated fatty acids attached to these lipids (for PC, 34:1, 36:1, 36:2, and 38:1; for SM, 16:1, 20:1, 22:2, and 24:1). Although the individual differences are not major, they add up to a pronounced shift toward unsaturation in the PrP DRMs. The extent of saturation for each lipid type is compared for Thy-1 and PrP DRMs in Fig. 4, showing the contrast between the significant differences in the extent of saturation of PC and SM, as well as the virtual identity of HexCer, in the two types of DRM. To give a view of the overall contribution of mono- and polyunsaturated fatty acids to the DRMs, the values for the individual lipid types were adjusted for the relative proportions of these lipids in the DRMs, and these values are given in Table III. Of the lipids investigated, 79.5% within Thy-1 DRMs were fully saturated when compared with just 68.5% in PrP DRMs.

Conversely, PrP DRMs had significantly more monounsaturated and polyunsaturated lipids than Thy-1 DRMs (Table III). Taken together, this higher level of lipid unsaturation might explain why PrP is more readily solubilized than Thy-1 from neuronal membrane (20, 22).

The most striking difference between Thy-1 and PrP DRMs, however, was in the uncharged glycosphingolipid hexosylceramide, which differed from both PC and SM in four respects. First, HexCer was over five times more abundant in PrP than Thy-1 DRMs (Table I). Second, the fatty acids attached were higher (1.4-fold) levels of PC than either PrP or total DRMs, and both Thy-1 and PrP had 2-fold

### DISCUSSION

The question investigated here is whether different rafts, enclosing functionally different proteins within the same mem-

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**Table I**

| Lipid | Thy-1 DRMs | PrP DRMs | Total DRM pool | PrP DRMs |
|-------|------------|----------|----------------|----------|
| PC    | 58.9 ± 6.0 | 52.7 ± 9 | 57.1 ± 1.1 | 57.1 ± 1.1 |
| SM    | 15.5 ± 1.0 | 17.4 ± 9 | 18.9 ± 2.0 | 18.9 ± 2.0 |

**Table II**

| Lipid          | Thy-1 DRMs | PrP DRMs | Total DRM pool | PrP DRMs |
|----------------|------------|----------|----------------|----------|
| PC             | 58.9 ± 6.0 | 52.7 ± 9 | 57.1 ± 1.1 | 57.1 ± 1.1 |
| SM             | 15.5 ± 1.0 | 17.4 ± 9 | 18.9 ± 2.0 | 18.9 ± 2.0 |

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*Levels refer to the major ceramide species, N-stearoylceramide, only.*

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*The full range of fatty acids detected, showing their proportion in the individual samples as well as mean values, is listed in Table I of the supplementary material.*
branes, differ also in their lipid composition. This is not a question specific to the organization of neuronal rafts. On mast cells, for instance, the FcR1 receptor and its downstream partner, LAT, are clustered in different, but adjacent, microdomains on the cell surface (45). These proteins also differ in their detergent solubility (46), suggesting, as with Thy-1 and PrP on neurons (20), that differences in lipid environment might accompany the different organization of surface membrane proteins within rafts.

How Separate Are PrP and Thy-1 on the Neuronal Surface?—As a point of reference, we determined quantitatively the extent of co-clustering of PrP and Thy-1 on the surface of primary cultured neurons. We have previously observed qualitatively the separate localization of these proteins using multivalent immunogold labels bound to cells that had first been fixed at either 4 or 37°C with glutaraldehyde (20). We opted here for a complementary procedure; monovalent immunogold was bound to living cells at 10°C before glutaraldehyde fixation. The same clustered, and largely separate, distribution of both proteins was observed. PrP occurred predominantly by itself; 84% of PrP had no Thy-1 label within 20 nm. However, 40% of Thy-1 occurred in clusters that had a few associated molecules of PrP, although only 1% of clusters were doubly labeled with >3 immunogold for both proteins.

Effectiveness of DRM Isolation of the Membrane Environment of PrP and Thy-1—If Brij 96 dissected Thy-1 and PrP rafts from the membrane with an accuracy of 20 nm, rendering each as an individual DRM, immunoaffinity purification, for example of PrP, would isolate not just the majority of the population of PrP-only DRMs, but also some DRMs containing large Thy-1 rafts with which a few molecules of PrP were associated. In practice, the stochastic process of detergent solubilization of membranes would be unlikely to excise rafts this cleanly, especially since clusters of both PrP and Thy-1 show considerable variation in size, shape, and proximity to each other on the neuronal surface (20). Some DRMs that included more than one raft could be produced. To characterize the lipid composition of individual Thy-1 and PrP rafts, rather than a mixture of both, it was important to reduce the contribution of DRMs containing high levels of the other protein in the purified Thy-1 or PrP DRMs.

We therefore removed rafts containing the highest levels of Thy-1 from the DRM pool from which PrP rafts were immunoprecipitated, and vice versa for Thy-1 rafts, to enhance the selection of DRMs in which one or other, but not both, proteins were dominant. Three qualities of these immunopurified DRMs suggest that they substantially contain the different lipid environments surrounding PrP and Thy-1. First, the extent of co-purification of PrP or Thy-1 in the DRMs of the other proteins followed that expected from the immunolabeling (i.e., relatively pure PrP DRMs, but an appreciable PrP presence in Thy-1 DRMs). Second, major proportions of PrP (40%) and Thy-1 (>50%) DRMs were purified; the lipid analysis was not based on unrepresentative minor populations. Finally, the lipid content of each of these immunopurified DRMs was remarkably reproducible, indicating the robustness of the underlying

Fig. 4. Extent of lipid saturation in the Thy-1 and PrP DRMs. The percentages of PC (A), SM (B), or HexCer (C) that is saturated, or mono- or polyunsaturated, are shown (mean of four samples ± S.D.). The values have been summed for all chain lengths/level of saturation measured, including minor species not shown in Table II (see Table I in the supplementary material). Significant differences between values for Thy-1 and PrP DRMs are indicated using the * convention outlined in the legend for Table I.
processes. Taken together, these properties suggest that Brij
96 fractionated the neuronal membrane such that the me-
brane of many individual rafts remained relatively intact and
separate through all purification steps.

Although preclearing with the other antibody selected for
purer populations of Thy-1 and PrP DRMs, there was still an
appreciable presence of the other protein in each preparation;
true differences between PrP and Thy-1 DRMs are likely to
be somewhat greater than reported here. For instance, much of
the HexCer in Thy-1 DRMs might come from the 20% PrP
present in them. However, the immunoaffinity approach is a
marked improvement upon studying only the properties of the
total pool of DRMs. Not only do Thy-1 and PrP DRMs differ
from each other, they differ markedly from the total pool. Thus,
to understand how any particular protein interacts with its
lipid environment, it is mandatory to isolate that specific envi-
ronnement, rather than extrapolate from the properties of the
total pool.

Unsaturated Lipids as Important Structural Components of
Rafts—The common view that only saturated lipids contribute
to the formation of rafts (27) is challenged by the consistent
presence of 20–30% monounsaturated lipids in these DRMs.
Indeed, the proportion of these unsaturated lipids is one of the
key features distinguishing PrP from Thy-1 DRMs. Even
higher levels of unsaturated DRM lipids have been found by
others using a variety of methods from different cell types (see
Table II in the supplementary material).

It is improbable that such high and reproducible levels of
unsaturated lipids in DRMs are mere contaminants and not
integral components of membrane rafts. A direct structural
role for unsaturated lipids in rafts is possible since cholesterol
can form condensed, ordered phases with monounsaturated PC
(47–50). This effect is enhanced if the surrounding disordered
phase is rich in lipids unsaturated at both the sn-1 and sn-2
positions, as occurs in brain (50). Molecular dynamics simula-
tion of the interaction of cholesterol with PC shows the sterol
predominantly to associate with saturated fatty acid at the sn-1
position. Monounsaturated fatty acid is attached at the sn-2
position, where it makes very little contact with cholesterol
(51), which may explain why monounsaturated lipids can re-
side within an liquid-ordered phase.

There was also a small, but reproducible, presence of poly-
unsaturated lipids in the DRMs, particularly in PrP DRMs, in
which they were 3.4% of the total lipids analyzed. Much higher
levels of polyunsaturated lipids (13.3% of total) were found in
DRMs of RBL-2H3 cells expressing the FceR1 receptor, a pro-
portion that doubled when the receptor was activated (52) and
accessed its downstream signaling partners (53). The selective
enrichment of polyunsaturated lipids in PrP domains could
similarly reflect the requirement of this protein to switch be-
tween membrane subdomains during its rapid and constitutive
recycling from rafts through non-raft endosomal compartments
(22). The imperfect ordering of polyunsaturated lipids might be
important for accommodating transmembrane polypeptide hel-
ices that rafts would normally exclude (16).

 Unsaturated lipids in mammals are largely taken up in the
diet, and their contribution to membranes can vary consid-
erably during the life of an individual (54); if they are integral
components of rafts, then raft composition could also vary. Our
choice of closely matched brains as starting material followed
pilot studies that showed marked variation in the degree of
unsaturation of total, and immunoaffinity-purified, DRMs. For
instance, DRMs isolated from the brain of a female retired
breeder had 20% more unsaturated lipids than those from the
younger rats, yet the relative difference in the degree of satu-
ration between Thy-1 and PrP DRMs was maintained (55). Rela-
tive differences in lipid environments are presumably sufficient
to direct the selective partitioning of proteins into different
membrane microdomains.

HexCer Differences between Thy-1 and PrP DRMs—The
analysis of this glycosphingolipid (on neurons, effectively glu-
cosylceramid) showed a number of very interesting features.
The predominant long chain (C22–24) hydroxylated fatty acids
attached to HexCer were markedly different from the non-
hydroxylated, C18–20 fatty acids attached to sphingomyelin.
These two classes of sphingolipids, although present within the
same rafts, must be in quite separate metabolic pools, either
originating from different biosynthetic pathways (56) or modi-
fied by different lipases. Sphingolipids differ from glycerolipids
in being able to form a direct hydrogen-bonded network at the
cell surface (57, 58); this capacity is enhanced by the hydroxy-
lolation of the fatty acid (58), producing a more cohesive mem-
brane surface. In addition, the extent of interdigitation of fatty
acid into the opposite bilayer, thought to contribute to coupling
the inner and outer leaflets of the bilayer within rafts (16, 27),

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5 B. Brugger, C. Graham, I. Leibrecht, E. Mombelli, A. Jen, F.
Wieland, and R. Morris, unpublished results.

6 The overall level of saturation of lipids in this pilot study was, for
Thy-1 and PrP DRMs, respectively: saturated, 57.0 and 49.2%; monoun-
saturated, 40.6 and 47.6%; and polyunsaturated, 1.6 and 2.5% (cf. Table
III). As in the main study, this difference was due to PC and SM, but not
HexCer. The higher cholesterol level in PrP DRMs (56.5%, as compared
with 50.2% in Thy-1 DRMs) was also maintained despite the individual
levels being 3% higher than in the main study.

7 Mass spectrometric analysis does not distinguish between glucosyl-
and galactosylceramide. However, galactosylceramide is expressed in
brain only by oligodendrocytes (55), which express neither PrP (29) nor
Thy-1 (28). The HexCer measured in the PrP and Thy-1 DRMs must be
at least predominantly glucosylceramide.
should be considerably greater with C22–24 fatty acids attached to HexCer when compared with the dominant C18–20 fatty acids attached to SM. This may be particularly important in the cells that express very high levels of glycosylated sphingolipids that are suggested to protect the plasma membrane by forming a hydrated glycocalx over the surface (26). Further strengthening of the membrane may be afforded by interdigitation of the long fatty acid chains across the bilayer.

The high level of HexCer maintained within the PrP DRMs is particularly interesting. Hydrogen bonding between their carbohydrate headgroups markedly affects the membrane properties of glycosylated lipids and their interaction with GPI-anchored proteins (59, 60), an effect that could protect PrP against the hydrophobic conformational change that underlies its pathogenic action. It is notable that raft-associated PrP is relatively resistant to such conformational conversion (61, 62); its pathogenic action. It is presumably as PrP traverses the non-raft, non-glycosylated DRMs on its way to coated pits that it is more susceptible to such conformational conversion (61, 62); its pathogenic action. It is presumably as PrP traverses the non-raft, non-glycosylated DRMs on its way to coated pits that it is more susceptible to such conformational conversion (61, 62).

Glucosylceramide is the precursor to gangliosides, the dominant class of neuronal glycosphingolipid (63). It will be interesting, in further analysis, to determine the relative content of these anionic glycosphingolipids in PrP and Thy-1 DRMs, not only because of their possible interaction with GPI-anchored proteins, but to further our understanding of the contribution of the multiple layers of lipid diversity to the structure of membrane rafts (16).

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