Glycolipid-anchored Proteins in Neuroblastoma Cells Form Detergent-resistant Complexes without Caveolin

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Abstract. It has been known for a number of years that glycosyl-phosphatidylinositol (GPI)-anchored proteins, in contrast to many transmembrane proteins, are insoluble at 4°C in nonionic detergents such as Triton X-100. Recently, it has been proposed that this behavior reflects the incorporation of GPI-linked proteins into large aggregates that are rich in sphingolipids and cholesterol, as well as in cytoplasmic signaling molecules such as heterotrimeric G proteins and src-family tyrosine kinases. It has been suggested that these lipid-protein complexes are derived from caveolae, non-clathrin-coated invaginations of the plasmalemma that are abundant in endothelial cells, smooth muscle, and lung. Caveolin, a proposed coat protein of caveolae, has been hypothesized to be essential for formation of the complexes. To further investigate the relationship between the detergent-resistant complexes and caveolae, we have characterized the behavior of GPI-anchored proteins in lysates of N2a neuroblastoma cells, which lack morphologically identifiable caveolae, and which do not express caveolin (Shyng, S.-L., J. E. Heuser, and D. A. Harris. 1994. *J. Cell Biol.* 125:1239–1250). We report here that the complexes prepared from N2a cells display the large size and low buoyant density characteristic of complexes isolated from sources that are rich in caveolae, and contain the same major constituents, including multiple GPI-anchored proteins, α and β subunits of heterotrimeric G proteins, and the tyrosine kinases fyn and yes. Our results argue strongly that detergent-resistant complexes are not equivalent to caveolae in all cell types, and that in neuronal cells caveolin is not essential for the integrity of these complexes.

A diverse group of proteins is attached to the plasma membrane by a glycosyl-phosphatidylinositol (GPI)[1] anchor (reviewed by Cross, 1990; Englund, 1993). This group includes lymphocyte and trypanosome surface antigens, adhesion molecules, exofacial enzymes, and receptors. The core structure of the anchor consists of a phosphoethanolamine residue amide-linked to the COOH-terminal amino acid of the protein, three mannose residues, an unacetylated glucosamine residue, and a phosphatidylinositol molecule that is embedded in the outer leaflet of the lipid bilayer. This core is transferred en bloc to the polypeptide chain in the endoplasmic reticulum, following cleavage of a hydrophobic COOH-terminal domain that serves as a signal for anchor addition.

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1. *Abbreviations used in this paper*: chPrP, chicken prion protein; DAF, decay-accelerating factor; FRT, Fisher rat thyroid; GPI, glycosyl-phosphatidylinositol; PIPLC, bacterial phosphatidylinositol-specific phospholipase C; PrP^{C}, cellular isoform of the prion protein; PrP^{Sc}, scrapie (infectious) isoform of the prion protein.

It has been known for a number of years that glycolipid-anchored proteins, in contrast to many transmembrane proteins, are relatively insoluble in nonionic detergents at 4°C (Vitetta et al., 1973; Letarte-Muirhead et al., 1974; Low, 1989). Recently, it has been proposed that this phenomenon results from incorporation of GPI-linked proteins into large complexes that contain sphingolipids and cholesterol, as well as cytoplasmic signaling molecules such as src-family tyrosine kinases and GTP-binding proteins (Stefanova et al., 1991; Brown and Rose, 1992; Sargiacomo et al., 1993; Zurzolo et al., 1994; Shenoy-Scaria et al., 1994). Although described in detergent extracts, these complexes are presumed to represent microdomains of the plasma membrane that exist in intact cells. The proposed functions of these domains include protein sorting in polarized cells (Simons and Wadinger-Ness, 1990) and transmembrane signaling in lymphocytes (Robinson, 1991; Brown, 1993).

Several recent studies have suggested that the detergent-resistant domains represent caveolae, which are non-clathrin-coated invaginations of the plasmalemma that have been described in some cell types, including epithelial cells, smooth muscle cells, and fibroblasts (Sargiacomo et al., 1993; Chang et al., 1994; Lisanti et al., 1994*a*,*b*). The basis for identifying the complexes with caveolae is, first, the observation that several GPI-anchored proteins that are enriched in

the complexes are clustered in caveolae after antibody labeling for electron microscopy (Rothberg et al., 1990; Ying et al., 1992; Mayor et al., 1994); caveolae may mediate endocytic uptake of some of these proteins (Parton et al., 1994). Second, electron microscopic examination of the isolated complexes reveals membranous vesicles, some of which are said to have the dimensions and appearance of caveolae (Chang et al., 1994; Lisanti et al., 1994*a*). Third, the complexes are enriched in caveolin, a 21-kD phosphoprotein that is thought to form part of the cytoplasmic coat of caveolae (Lisanti et al., 1993); cells that do not express caveolin appear to yield "incomplete" complexes (Sargiacomo et al., 1993; Zurzolo et al., 1994). Despite these correlations, however, the precise relationship between caveolae and the detergent-resistant complexes remains uncertain.

We have been studying the cell biological properties of a cellular isoform of the prion protein (PrP^c), a glycolipid-anchored surface protein that is expressed predominantly in neurons and glia in the CNS, and that is critically involved in the pathogenesis of an unusual group of transmissible neurodegenerative diseases called spongiform encephalopathies or prion diseases (reviewed by Prusiner and DeArmond, 1994). We found previously that chicken prion protein (chPrP), the chicken homologue of mammalian PrP^c, is endocytosed via clathrin-coated pits in neuroblastoma cells, as well as in embryonic neurons and glia (Shyng et al., 1993, 1994). Caveolae do not seem to be involved in internalization of chPrP in neuroblastoma cells, since we observed that these cells do not contain morphologically identifiable caveolae by thin-section or deep-etch electron microscopy, and since they do not express caveolin protein or mRNA (Shyng et al., 1994).

To help clarify the relationship between detergent-resistant complexes and caveolae, we have analyzed the properties of GPI-anchored proteins in lysates of neuroblastoma cells that lack caveolae. We report here that the detergent-resistant complexes isolated from these cells have all the major constituents of the complexes prepared from sources that are rich in caveolae and caveolin, such as epithelial cells, lung, and smooth muscle. The complexes are enriched in chPrP and other GPI-anchored proteins, as well as in several tyrosine kinases and α and β subunits of heterotrimeric G proteins. Our results demonstrate, first, that caveolin is not essential for the formation of these complexes. Second, they indicate that detergent-resistant complexes are not equivalent to caveolae in all cell types, and suggest that in neuronal cells additional molecular components might be required for the formation of caveolae. Finally, the association of chPrP with kinases and G proteins raises the possibility that PrP^c and other GPI-anchored proteins might be involved in signal transduction processes in neuronal cells.

The work presented here has been reported in abstract form (Gorodinsky, A., G. F. Wu, and D.A. Harris. 1994. GPI-anchored proteins in neuronal cells form detergent-resistant complexes without caveolin. *Mol. Biol. Cell.* 5:320a).

Materials and Methods

Antibodies and Reagents

A rabbit antiserum raised against a bacterial fusion protein encompassing amino acids 35–96 of chPrP has been described previously (Harris et al., 1993). A mouse monoclonal antibody (1H4) raised against human decay-accelerating factor (DAF) (Coyne et al., 1992) was a gift from Dr. D. M. Lublin (Washington University, St. Louis, MO). Rabbit antisera against α and β subunits of G proteins (P-960 [Casey et al., 1990] and U-49 [Mumby et al., 1986], respectively) were kindly provided by Dr. M. Linder (Washington University). A mouse monoclonal antibody (TD.1) against clathrin heavy chain was a gift of Dr. F. Brodsky (University of California, San Francisco). A rabbit antiserum against $p62^{yes}$ was a gift of Dr. M. Sudol (Rockefeller University, NY), and a mouse monoclonal antibody against $p59^{fyn}$ was purchased from Transduction Laboratories. An anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell culture reagents were from the Tissue Culture Support Center at Washington University. Biotin-X-NHS was obtained from Calbiochem (La Jolla, CA), [^{32}P]-γ-ATP from Amersham Corp. (Arlington Heights, IL), and all other reagents from Sigma Immunochemicals (St. Louis, MO).

Phosphatidylinositol-specific phospholipase C (PIPLC) was prepared by the procedure of Low (1992) from supernatants from cultures of *Bacillus subtilis* carrying a plasmid encoding PIPLC from *Bacillus thuringiensis*.

Cell Lines

N2a mouse neuroblastoma cells (CCL131, American Type Culture Collection, Rockville, MD) were grown in MEM containing 10% FCS, nonessential amino acids, and penicillin/streptomycin in an atmosphere of 5% CO_2/95% air. The transfected line of N2a cells expressing wild-type chPrP is the clone designated A26 that we have described previously (Harris et al., 1993).

To prepare the DAF-expressing line, N2a cells were transfected with Lipofectin (GIBCO BRL, Gaithersburg, MD) using a 1:10 mixture of pRSVneo (Ulrich and Ley, 1990) and a pBC12/CMV expression plasmid (Cullen, 1986) containing a human DAF cDNA (Lublin and Atkinson, 1989). Antibiotic-resistant clones were selected in 700 μg/ml geneticin (G418), expanded, and then maintained in 300 μg/ml geneticin.

The chPrP transmembrane chimera consists of amino acids 1-241 of chPrP fused to amino acids 2310–2364 of a mutant version of the bovine cation-independent mannose-6-phosphate receptor. A plasmid encoding this mutant (346), which is impaired in its ability to undergo coated pit-mediated internalization (Jadot et al., 1992), was kindly provided by Dr. S. Kornfeld (Washington University). A cDNA encoding the chimeric protein was constructed by recombinant PCR (Higuchi, 1989) using Vent polymerase (New England Biolabs, Beverly, MA), and cloned into the pBC12/CMV expression vector. Transfection was carried out as described above.

Preparation of Detergent Lysates

Cells grown to confluence in 100-mm dishes were solubilized at 4°C in a lysis buffer containing 1% Triton X-100, 20 mM PIPES (pH 6.5), 2 mM EDTA, 0.5 mM PMSF, 1 μg/ml pepstatin, and 1 μg/ml leupeptin. This buffer is similar to ones used by Brown and Rose (1992) and Sargiacomo et al. (1993); in agreement with the latter authors, we find that extraction at pH 6.5 produces a higher yield of detergent-resistant complexes than extraction at pH 7.5.

Gel Filtration of Triton-insoluble Complexes

A modification of the procedure of Cinek and Horejsi (1992) was used. Detergent lysates prepared as above were cleared by centrifugation (16,000 *g*, 10 min at 4°C), and 250 μl was applied to a 0.7 × 9-cm column of Sepharose 4B. The column was eluted at 4°C with lysis buffer, and 15 0.3-ml fractions were collected. Proteins in each fraction were precipitated with cold methanol and analyzed by SDS-PAGE followed by immunoblotting. The column was calibrated using intact and lysed erythrocytes as described by Shenoy-Scaria et al. (1994). Intact erythrocytes eluted in fractions 6 and 7 (the void volume of the column), while hemoglobin from the lysed erythrocytes eluted in fractions 10–14.

Isopycnic Centrifugation of Triton-insoluble Complexes

We employed either a continuous sucrose gradient, as described by Brown and Rose (1992) and Sargiacomo et al. (1993), or a step-gradient. For the continuous gradient, uncleared detergent lysates were mixed with an equal volume of 80% sucrose in lysis buffer without Triton, and then overlaid with

a linear 5–30% gradient of sucrose in lysis buffer without Triton. After 16–20 h of centrifugation in an SW-41 rotor (170,000 *g*) fractions were collected from the bottom of the tube using a no. 20 stainless steel syringe needle. Proteins in each fraction were precipitated with methanol and analyzed by SDS-PAGE followed by immunoblotting.

For the step gradient, uncleared detergent lysates were mixed with an equal volume of 80% in lysis buffer without Triton, and then overlaid with steps of 22 and 5% sucrose in the same buffer. Low-density complexes were collected from the 22%/5% sucrose interface using a syringe, diluted, and collected by centrifugation.

Detection of Surface-biotinylated, GPI-anchored Proteins

Cells were treated with biotin-X-NHS for 15 min at 4°C, and the reaction was then quenched with 50 mM glycine in TBS. Cells were lysed, and low-density complexes collected by fractionation on a sucrose step-gradient as described above. Complexes were resuspended in TBS containing 1% Triton X-114 plus protease inhibitors. (Triton X-114 was diluted from a 12% stock solution that was precondensed according to Bordier [1981]). After incubation at 37°C for 20 min, aqueous and detergent phases were separated by centrifugation. The detergent phase was diluted to the initial volume with TBS, and then incubated with PIPLC for 2 h at 4°C, and the phase separation repeated. Proteins in each phase were precipitated with cold methanol, separated by SDS-PAGE, and blotted. Blots were developed using HRP-conjugated streptavidin and enhanced chemiluminescence (ECL) (Amersham Corp.).

In Vitro Kinase Reactions

Triton-insoluble complexes were isolated on a continuous sucrose density gradient as described above, and were resuspended in kinase buffer containing 20 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.1 mM Na_3VO_4. For the detection of total kinase activity, 10 μCi of [^{32}P]-γ-ATP was added to 20-μl reactions, and incubation was carried out for 15 min at 25°C. Proteins were then analyzed by SDS-PAGE followed by autoradiography.

For the detection of tyrosine kinase activity, samples were incubated as above, except that 0.5 mM of unlabeled ATP was used. Proteins were then analyzed by SDS-PAGE followed by immunoblotting with an anti-phosphotyrosine antibody. The Triton-insoluble complexes used for these experiments were prepared in the presence of Na_3VO_4 to inhibit phosphatases.

Immunoblotting

Immunoblots on polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) were developed using HRP-conjugated secondary antibodies and visualized using ECL. Film images were digitized using an HP ScanJet IIp scanner, and band intensities quantitated using SigmaScan/Image (Jandel Scientific, Corte Madera, CA). Short exposure times were used to maintain linearity. To measure the total amount of protein in column or gradient fractions, blots were stained with Coomassie Blue, and the stain intensity in the corresponding lane measured from a scanned image of the blot. The concentration of specific immunoreactive proteins was calculated by dividing the digitized signal from the ECL film by the digitized signal from the Coomassie-stained blot.

Electron Microscopy

Triton-insoluble complexes isolated on a continuous sucrose density gradient were pelleted (150,000 *g*, 30 min) and fixed with 1% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4) for 1 h on ice. Samples were then postfixed in 1.25% OsO_4 in cacodylate buffer, incubated with 4% uranyl acetate, dehydrated with a graded series of ethanol, infiltrated with propylene oxide and embedded in PolyBed 812 (Polysciences Inc., Warrington, PA). Samples were then sectioned, post-stained with uranyl acetate and lead citrate, and examined using an electron microscope (10A; Carl Zeiss, Inc., Thornwood, NY).

Results

We have previously shown that N2a neuroblastoma cells do not contain morphologically identifiable caveolae, as demonstrated by both thin-section and deep-etch electron microscopy, nor do they express caveolin mRNA or protein (Shyng et al., 1994). In view of reports that caveolae can be isolated as detergent-resistant complexes containing multiple GPI-anchored proteins, it was of interest to characterize the biochemical properties of GPI-anchored proteins in lysates of N2a cells. In particular, our aim was to determine whether the complexes recovered from N2a cells were similar in composition and physical properties to those prepared from cell types that are rich in caveolae, such as epithelial and smooth muscle cells.

As model GPI-anchored proteins for our studies, we have used chPrP, the chicken homologue of mammalian PrP^c (Harris et al., 1991), and human DAF (Lublin and Atkinson, 1989). Both proteins were expressed by stable transfection of N2a cells, and recognized using specific antibodies.

ChPrP and DAF Expressed in N2a Cells Are Insoluble in Triton X-100

We first undertook to analyze the solubility of chPrP and DAF in 1% Triton X-100 at 4°C, conditions under which GPI-anchored proteins in many cell types are insoluble. Detergent lysates of N2a cells expressing each protein were centrifuged at 150,000 *g*, and the amount of chPrP or DAF in the supernatants and pellets determined by immunoblotting (Fig. 1). Both proteins were found to be almost completely insoluble in this assay, suggesting that in the presence of detergent they remained associated with molecular aggregates that were sufficiently large to pellet in an ultracentrifuge.

ChPrP and DAF Are Incorporated into Large Molecular Complexes with a Low Buoyant Density

To obtain information about the size of the detergent-resistant aggregates, we subjected lysates of N2a cells to chromatography on Sepharose 4B, as has been done for other cell types (Cinek and Horejsi, 1992). As shown in Fig. 2, both chPrP and DAF eluted in the void volume of the column, indicating these proteins were present in complexes that had a molecular mass greater than 2×10^7 D.

Previous studies have shown that complexes containing GPI-anchored proteins float at a low buoyant density after centrifugation in sucrose gradients, presumably because of their high content of sphingolipids and cholesterol (Brown and Rose, 1992). We therefore subjected detergent lysates of N2a cells to equilibrium centrifugation in 5–30% linear sucrose gradients, and assayed fractions for chPrP and DAF by immunoblotting (Fig. 3). Both proteins were recovered predominantly in fractions 5–7, which contained between 15 and 20% sucrose (Fig. 3 *D*), and had a density of 1.06–1.08 g/cc. Significantly, this region of the gradient contained less than 2% of the total cellular protein, most of which was found in denser fractions near the bottom of the tube (Fig. 3, *C* and *D*); these fractions presumably contained most of the soluble proteins in the cell. This result indicates that chPrP and DAF are enriched ~50-fold in the detergent-resistant complexes.

To test whether the GPI anchor was responsible for recruitment into the complexes, we constructed a transmembrane version of chPrP in which a portion of the COOH terminus of the protein containing the signal for GPI addition was replaced with the transmembrane and cytoplasmic domains of the cation-independent mannose-6-phosphate re-

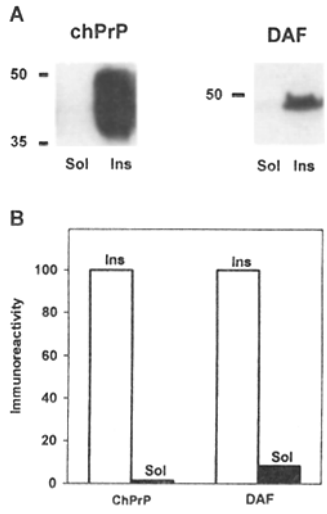

Figure 1. ChPrP and DAF are insoluble in Triton X-100 when expressed in N2a cells. Triton X-100 lysates of N2a cells expressing each protein were prepared at 4°C, and centrifuged at 150,000 *g* for 30 min at the same temperature. Molecular mass markers are given in kD. (*A*) The amounts of each protein in the supernatant (*Sol*) and pellet (*Ins*) were assayed by immunoblotting. (*B*) Densitometric quantitation of the film shown in *A*, with immunoreactivity expressed as a percentage of the maximum signal.

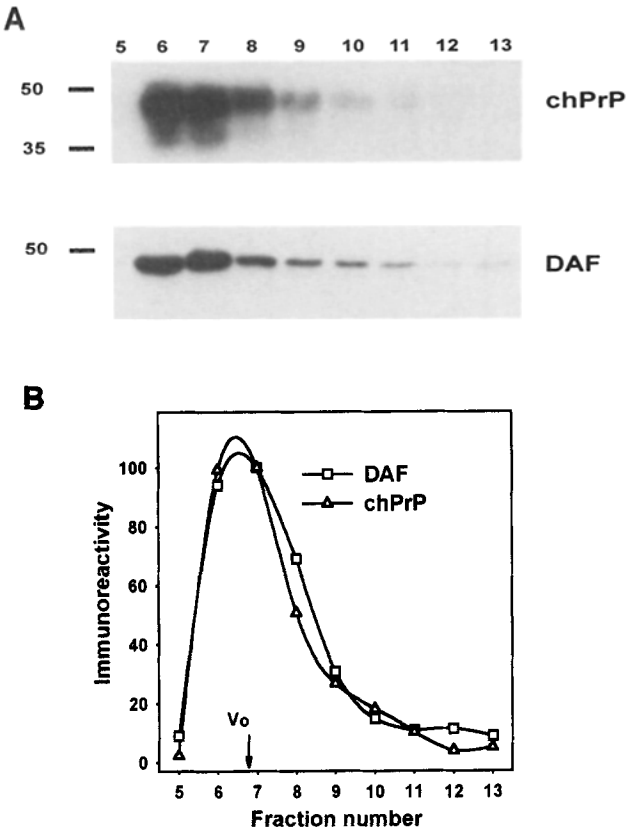

Figure 2. ChPrP and DAF in detergent lysates are present as high molecular mass complexes. N2a cells expressing each protein were lysed with Triton X-100 at 4°C, and the lysates fractionated on Sepharose 4B the same temperature. (*A*) Fractions were immunoblotted with anti-chPrP or anti-DAF antiserum. (*B*) Elution profile, based on densitometric quantitation of the immunoblots shown in *A*. Both proteins elute in the void volume of the Sepharose column, which is indicated by the arrow labeled V_o. The included volume of the column encompasses fractions 10–14 (see Materials and Methods).

ceptor. We confirmed by surface iodination that this chimeric protein was expressed on the plasma membrane of transfected N2a cells, and that it was not GPI-anchored because it was not released by digestion with bacterial PIPLC (data not shown). We found that the transmembrane form of chPrP, in contrast to the GPI-anchored version, fractionated near the bottom of the sucrose gradient (Fig. 3), indicating that the GPI anchor is essential for incorporation of the protein into low-density complexes.

Triton-resistant Complexes Contain Other GPI-anchored Surface Proteins

To determine whether endogenous GPI-anchored proteins are present in complexes from N2a cells, we surface biotinylated untransfected cells, and subjected detergent extracts to centrifugation on sucrose gradients as described above. Proteins in the region of the gradient between 15 and 20% sucrose were then phase separated in Triton X-114 before and after digestion with PIPLC (Fig. 4 *A*). A number of biotinylated proteins are present in the detergent phase in the absence of PIPLC treatment (lane *2*), consistent with the idea that the low-density complexes contain a variety of integral membrane proteins that are exposed on the cell surface. Most of these proteins are shifted into the aqueous phase by treatment with PIPLC (lane *5*), suggesting that they contain a GPI anchor. The same set of PIPLC-releasable proteins is seen when chPrP-expressing N2a cells are analyzed, but in addition a prominent 40–50-kD band is observed (Fig. 4 *B*, lane *5*); immunoprecipitation of this band confirms that it is chPrP (not shown), demonstrating that chPrP molecules on the cell surface are incorporated into detergent-resistant complexes.

Triton-resistant Complexes Contain Heterotrimeric G Proteins and Non-receptor Tyrosine Kinases

Several GTP-binding proteins and src-family tyrosine kinases have been reported to be associated with GPI-anchored proteins in detergent extracts from MDCK cells, smooth muscle, and lung (Sargiacomo et al., 1993; Arreaza et al., 1994; Lisanti et al., 1994*a*; Chang et al., 1994). We used specific antibodies to identify these signaling molecules in detergent lysates of N2a cells after centrifugation on sucrose density gradients (Fig. 5, *A* and *B*). To recognize heterotrimeric G proteins, we used a rabbit polyclonal antibody P-960, which has highest affinity for α_i and α_o, but also recognizes α_s, α_z, and α_t (Casey et al., 1990); and U-49, which is specific for the β_1 subunit (Mumby et al., 1986). Western blot analysis revealed a distinct peak of G_α immunoreactivity along the sucrose gradient, in the same region as the Triton-insoluble complexes containing chPrP and DAF (fractions 5–7). β subunit immunoreactivity was distributed more uniformly across the gradient, but when correction was made for the amount of protein in each fraction, the β subunit was highly enriched in fractions 5 and 6. Thus, both α and β subunits of heterotrimeric G proteins were enriched in low-density complexes from N2a cells.

Immunoblotting of gradient fractions with antibodies to two different src-family tyrosine kinases, fyn and yes, also showed peaks of immunoreactivity in the low-density region of the gradient (fractions 6 and 7) (Fig. 6, *A* and *B*). To test whether kinases in these fractions were catalytically active,

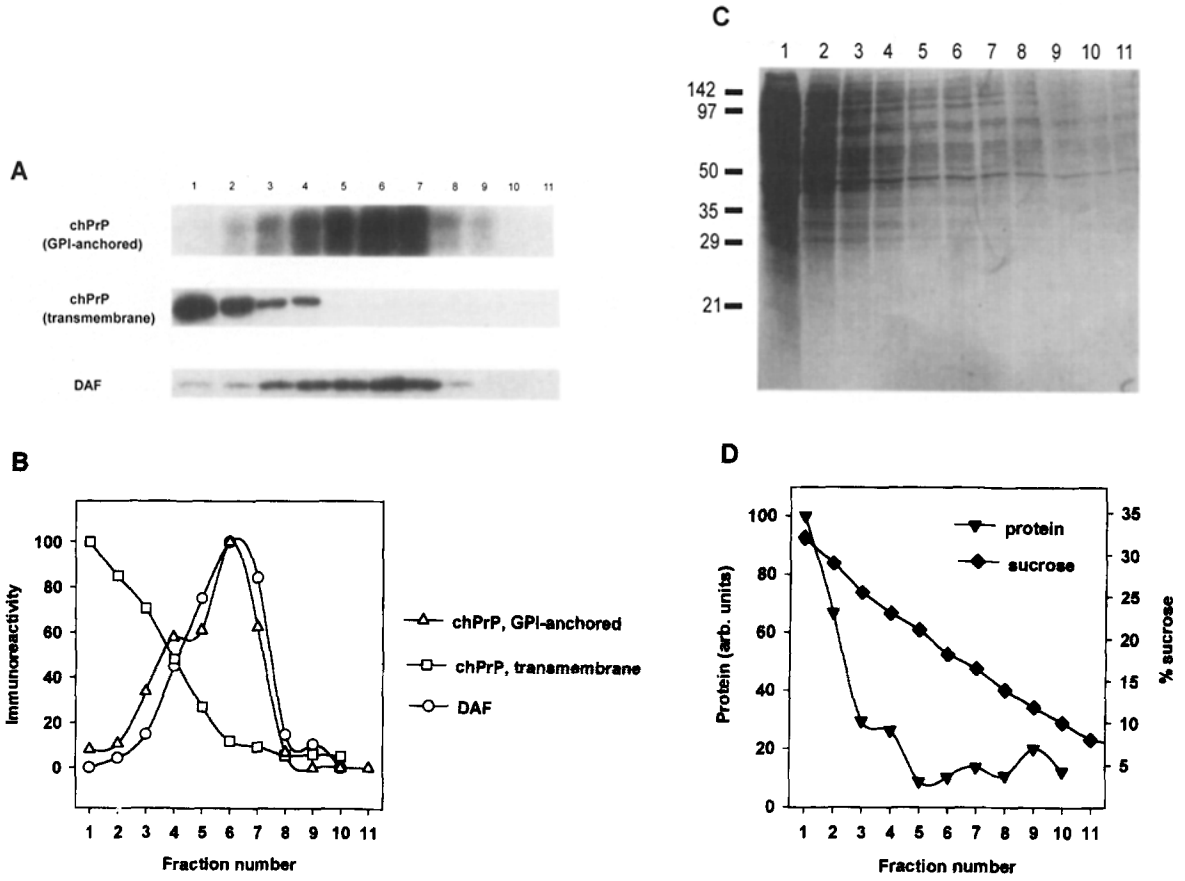

Figure 3. GPI-anchored forms of chPrP and DAF, but not a transmembrane form of chPrP, are present in low-density complexes after detergent extraction. N2a cells expressing either GPI-anchored chPrP, transmembrane chPrP (chPrP residues 1–241 fused to the transmembrane and cytoplasmic domains of the cation-independent mannose-6-phosphate receptor), or DAF were homogenized at 4°C in a buffer containing 1% Triton X-100. The homogenate was brought to 40% sucrose, overlaid with a continuous gradient of 30% to 5% sucrose, and centrifuged to equilibrium (170,000 *g* for 16 h). Gradient fractions were immunoblotted to detect the expressed proteins (*A* and *B*), and the blot stained with Coomassie to reveal the total amount of protein in each fraction (*C*). The triangles in *D* show a plot of the total protein in each fraction derived by densitometric analysis of the Coomassie stained blot, and the diamonds show a plot of the sucrose concentration in each fraction. Note that the two GPI-anchored proteins float at 15–20% sucrose (fractions 5–7), while the transmembrane form of chPrP is found near the bottom of the gradient (fractions 1–4) with the bulk of the cellular protein.

we carried out in vitro kinase assays using [^{32}P]-γ-ATP. We found that multiple proteins were phosphorylated, ranging in molecular mass from 40 to over 140 kD (Fig. 6 *C*, lane *2*). Several proteins were phosphorylated on tyrosine residues after incubation with unlabeled ATP, as shown by reactivity with anti-phosphotyrosine antibodies (Fig. 6 *C*, lane *4*). The prominent group of tyrosine-phosphorylated bands near 60 kD may represent phosphorylated forms of fyn, yes, or other src-family kinases. Of note, we have not observed any phosphorylated bands corresponding in size to caveolin (M_r = ~20 kD), in agreement with our previous report that caveolin protein and mRNA are absent in N2a cells (Shyng et al., 1994).

Triton-resistant Complexes Are Membranous Vesicles That Do Not Contain Clathrin

Examination of isolated Triton-resistant complexes by thin-section electron microscopy revealed a heterogeneous collection of vesicles having diameters of 200–800 nm (Fig. 7). We did not observe any structures resembling either caveolae or clathrin-coated vesicles.

Since we have recently shown that chPrP is endocytosed via clathrin-coated pits in N2a cells (Shyng et al., 1994), we were interested in determining where coated vesicles migrated in the sucrose density gradient under conditions used for isolation of detergent-resistant complexes. Immunoblotting of the gradient fractions with an antibody against the heavy chain of clathrin showed that this protein was localized primarily in the high-density fractions 1–4 (Fig. 8), with some recovered in the pellet that collects at the bottom of the tube (not shown). No clathrin heavy chain was detected in the low-density area of the gradient where GPI-anchored proteins were recovered. This distribution of clathrin is in accord with the reported resistance of clathrin-coated vesicles to Triton X-100 treatment, and with their high buoyant density, which is approximately equal to that of 52% sucrose (Woodward and Roth, 1978).

Discussion

It has been known for many years that, in contrast to many transmembrane proteins, GPI-anchored proteins from a va-

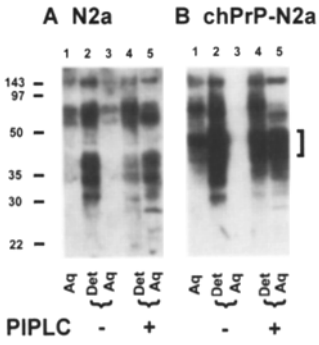

Figure 4. Triton-resistant complexes from N2a cells contain multiple GPI-anchored surface proteins. Untransfected N2a cells (*A*) or N2a cells expressing chPrP (*B*) were surface-biotinylated, lysed in Triton X-100 at 4°C, and the lysates fractionated on a sucrose step-gradient (see Materials and Methods). Complexes that collected at the 5%/22% sucrose interface were resuspended in 1% Triton X-114, and phase separation induced by warming to 37°C. The aqueous phase (lane *1*) was saved, and the diluted detergent phase was incubated at 4°C with (lanes *4* and *5*) or without (lanes *2* and *3*) PIPLC. The phase separation was then repeated to yield a second set of detergent phases (lanes *2* and *4*) and aqueous phases (lanes *3* and *5*). The proteins in each phase were methanol-precipitated, separated by SDS-PAGE using a Bio-Rad mini-gel apparatus, and blotted. Blots were then developed with HRP-streptavidin using ECL. Multiple biotinylated, integral membrane proteins are present in low-density complexes from both cell lines, and most of these are releasable by PIPLC (lane *5*). The bracket in *B* indicates a protein that is absent in untransfected N2a cells (*A*), and that can be shown by immunoprecipitation to be chPrP.

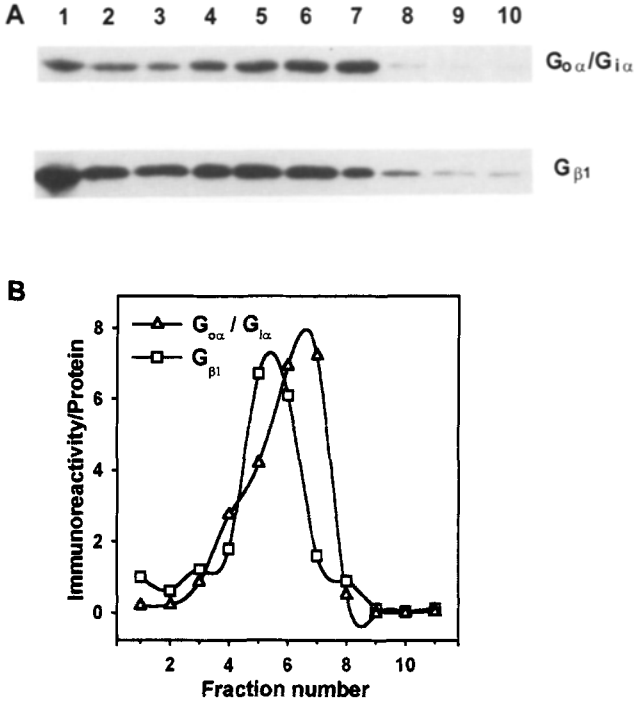

Figure 5. Heterotrimeric G proteins are enriched in Triton-resistant complexes. ChPrP-expressing N2a cells were extracted and fractionated on a continuous sucrose gradient as described in the legend to Fig. 3. (A) Fractions were immunoblotted with an antibody that recognizes $G_{o\alpha}$ and $G_{i\alpha}$ (40 kD), or with an antibody to $G_{\beta 1}$ (37 kD). (*B*) Films of the ECL-visualized immunoblots were quantitated by densitometry. The concentration of each G protein subunit was expressed as units of immunoreactivity divided by units of total protein in each fraction, the latter determined by scanning the corresponding lane of the blot after staining with Coomassie Blue (see Materials and Methods). The concentration on the ordinate is given in arbitrary units. Each of the G protein subunits is enriched in the same region of the gradient (fractions 5–7) that contains most of the GPI-anchored chPrP and DAF (compare with Fig. 3, *B*).

riety of cell types are relatively insoluble at 4°C in non-ionic detergents such as Triton X-100 (Vitetta et al., 1973; Letarte-Muirhead et al., 1974; Low, 1989). Presumably, this results from their incorporation into molecular aggregates or complexes that are resistant to disruption by the detergent. Recently, the composition and possible functions of these complexes have been subjects of intensive study.

Detergent-resistant complexes from several sources have been characterized, including those from a kidney epithelial cell line (MDCK) (Brown and Rose, 1992; Sargiacomo et al., 1993; Lisanti et al., 1993; Zurzolo et al., 1994), lymphoid cell lines (Stefanova et al., 1991; Cinek and Horejsi, 1992; Bohuslav et al., 1993), HeLa cells (Shenoy-Scaria et al., 1994; Rodgers et al., 1994), lung (Lisanti et al., 1994*a*), and gizzard (Chang et al., 1994). The complexes from all of these sources contain multiple GPI-anchored proteins. They also contain several kinds of cytoplasmic signaling molecules, including src-family tyrosine kinases, α and β subunits of heterotrimeric G proteins, and small GTP-binding proteins. Direct chemical analysis demonstrates that the complexes from MDCK cells are also enriched in neutral and acidic sphingolipids and cholesterol; these lipids are also likely to be major constituents of the complexes prepared from the other sources, which have a low buoyant density on sucrose density gradients. Finally, the complexes from MDCK cells, lung, and gizzard are enriched the proposed caveolar coat protein caveolin or VIP21 (Rothberg et al., 1992; Kurzchalia et al., 1992).

Whether the protein-lipid complexes that form in detergent extracts are identical to, or are derived from, structures that exist in intact cells has been a subject of considerable debate. One idea has been that the complexes represent non–clathrin-coated invaginations of the plasma membrane known as caveolae. Caveolae are abundant in endothelial cells, smooth muscle cells, and fibroblasts, and it has been proposed that they play a role in a number of apparently disparate processes, including transcytosis, uptake of extracellular nutrient molecules (potocytosis), calcium transport, and transmembrane signaling (reviewed by Anderson, 1993*a*,*b*; Lisanti et al., 1994*b*). The property of detergent insolubility forms the basis for two recent purification schemes for caveolae (Lisanti et al., 1994*a*; Chang et al., 1994).

The results reported here clearly demonstrate that, at least in some cell types, the insoluble protein-lipid complexes that form when cells are extracted with non-ionic detergents are not the same as caveolae. The detergent-resistant complexes we isolate from N2a neuroblastoma cells, which lack morphologically identifiable caveolae and do not express caveolin (Shyng et al., 1994), have all the essential properties of the complexes prepared from sources that are rich in caveolae, such as MDCK cells, lung, and gizzard. First, they are enriched in multiple glycolipid-anchored proteins, including chPrP and DAF, as well as in a number of unidentified PIPLC-releasable proteins that can be surface biotinylated. Second, the complexes from N2a cells contain several cytoplasmic signaling molecules, including the α_i, α_o, and β_1 subunits of heterotrimeric G proteins, and the src-family tyrosine kinases fyn and yes; moreover, protein kinases pres-

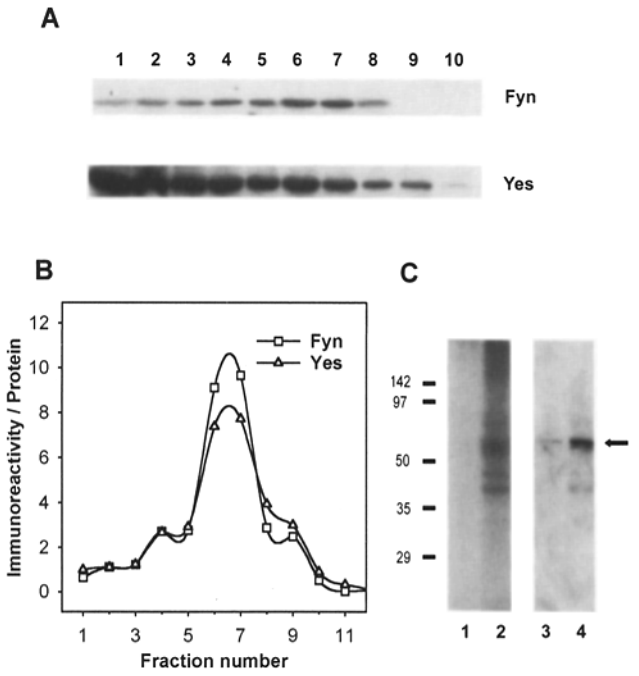

Figure 6. The src-family tyrosine kinases fyn and yes are enriched in Triton-resistant complexes, which display kinase activity in vitro. ChPrP-expressing N2a cells were extracted and fractionated on a continuous sucrose gradient as described in the legend to Fig. 3. (*A*) Fractions were immunoblotted with antibodies that recognize either fyn or yes (60 kD). (*B*) Films of the blots were quantitated by densitometry, and the concentration of each of the kinases was expressed per unit of total protein in each fraction, as described in the legend to Fig. 5 *B*. Each of the kinases is enriched in the same region of the gradient (fractions 6 and 7) that contains most of the GPI-anchored chPrP and DAF (compare with Fig. 3, *B*). (*C*) Triton-resistant complexes from chPrP-expressing N2a cells were collected at 15–20% sucrose after equilibrium centrifugation on sucrose gradients, as described in *A* and *B*. One set of samples was incubated with [^{32}P]-γ-ATP for 0 min (lane *1*) or 15 min (lane *2*), and then analyzed by SDS-PAGE and autoradiography. A second set of samples was incubated with non-radioactive ATP for 0 min (lane *3*) or 15 min (lane *4*), and then immunoblotted using an anti-phosphotyrosine antibody. Multiple proteins in the isolated complexes are phosphorylated in vitro, some of them on tyrosine. The arrow indicates the position of a prominent group of tyrosine-phosphorylated proteins near 55–60 kD that are likely to include fyn, yes, and perhaps other proteins of the src-family.

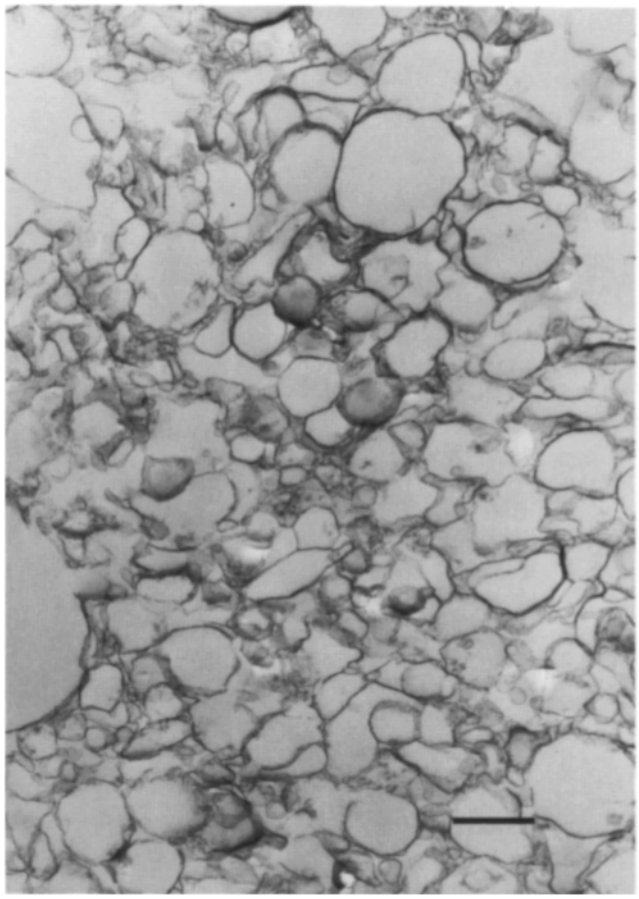

Figure 7. Triton-resistant complexes are membranous vesicles of heterogeneous size and shape. Complexes isolated from chPrP-expressing N2a cells by centrifugation on a continuous sucrose gradient were analyzed by thin-section electron microscopy. Bar, 500 nm.

ent in the complexes are catalytically active, as demonstrated by in vitro kinase assays. Third, in electron micrographs, the complexes from N2a cells are seen to consist of membranous vesicles of 200–800-nm-diam, similar in appearance to those from MDCK cells. Fourth, the physical properties of complexes are typical: they are large (>2 × 10^7 D), eluting in the void volume from a Sepharose 4B gel filtration column, and pelleting after centrifugation at 150,000 *g*; and they have a low buoyant density, floating at 15–20% sucrose after isopycnic centrifugation. Finally, preliminary thin-layer chromatographic analysis confirms the presence of glycosphingolipids in the complexes (unpublished data).

Our results do not rule out the possibility that, in other cell types, detergent-resistant complexes might be derived from caveolae. The complexes might represent microdomains of the plasma membrane that are present in both N2a cells and MDCK cells, but N2a cells might not express one or more components that allow these domains to assume the invaginated form of caveolae. This component might be caveolin, a hypothesis that could be tested by determining whether N2a cells transfected with a caveolin cDNA produce morphologically recognizable caveolae. On the other hand, recent work suggests that caveolin may not be an essential component of caveolae (Smart et al., 1994), thus arguing that other unidentified molecules may be important. In any case, the components we have detected in the complexes from N2a cells, including GPI-anchored proteins, kinases, G proteins, and probably lipids, are clearly insufficient on their own for the formation of caveolae.

The results presented here are similar to those of Fra et al. (1994), who recently reported that several lymphoid cell lines lack caveolae by thin-section electron microscopy, and do not express caveolin protein or mRNA. When detergent extracts of these cells were analyzed by sucrose gradient centrifugation, thy-1, a GPI-anchored protein, as well as the ganglioside G_{M1} were found in the low-density region of the gradient. Presumably, this result reflects the existence of detergent-resistant complexes in lymphoid cell lysates, al-

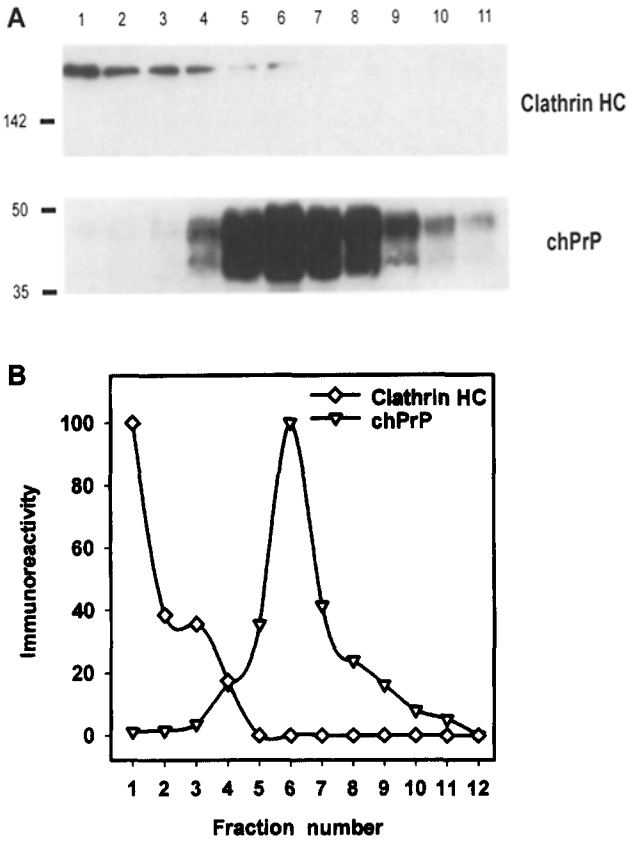

Figure 8. Triton-resistant complexes do not contain clathrin. ChPrP-expressing N2a cells were extracted and fractionated on a continuous sucrose gradient as described in the legend to Fig. 3. Fractions were immunoblotted with antibodies to either clathrin heavy chain or chPrP (*A*). The film of the immunoblot was quantitated by densitometry (*B*).

though the distribution of other components such as G-protein subunits and tyrosine kinases was not examined.

Our results differ significantly from those of two recent studies of detergent-resistant complexes from Fisher rat thyroid (FRT) cells, a line of epithelial cells which, like N2a cells, lack caveolin protein and mRNA (Sargiacomo et al., 1993; Zurzolo et al., 1994). Both of these studies conclude that complexes from FRT cells are incomplete. Although they are rich in glycosphingolipids, they are reported to exclude a model GPI-anchored protein (gDI-DAF), to be largely devoid of surface-labeled and metabolically labeled proteins, and to contain little in vitro phosphorylation activity. Both studies also suggest that the deficiencies in the FRT complexes are due to the absence of caveolin, which is proposed to play a role in recruiting GPI-anchored proteins and other elements to the complex, and which is enriched in complexes from MDCK cells, lung, and gizzard. In contrast, we have found that the complexes from N2a cells possess all of the components found in complexes from MDCK cells. A reasonable explanation for this discrepancy is that caveolin is not essential for the integrity of the complexes, and that FRT cells, but not N2a cells, lack other molecules essential for organizing the complexes.

We have previously reported that chPrP is endocytosed via clathrin-coated pits in N2a cells (Shyng et al., 1994). In the present study, we found that, after isopycnic centrifugation, the majority of clathrin heavy chain was found near the bottom of the gradient and in the pellet, well separated from the majority of the chPrP, which was present in the low-density fractions (Fig. 8). This result, along with our electron microscopic analysis of the low-density fractions (Fig. 7), indicates that most of the chPrP in N2a cells is found in detergent-resistant complexes that are distinct from clathrin-coated vesicles, which would remain intact in 1% Triton X-100 and migrate to the bottom of the gradient because of their high density (Woodward and Roth, 1978). This observation is consistent with our previous EM morphometric analysis, which indicated that, although gold-labeled chPrP molecules were three- to fivetime more concentrated over clathrin-coated pits than over undifferentiated areas of plasma membrane, at steady state only 10–15% of the total number of labeled molecules were localized over coated pits (Table I of Shyng et al., 1994). This percentage might be accounted for by the small amount of chPrP that was always found in the pellet at the bottom of the tube after centrifugation of sucrose gradients (data not shown). The chPrP molecules that not associated with coated pits, and that can often be observed in clusters over undifferentiated areas of membrane (Shyng et al., 1994), presumably correspond to those that are found in low-density complexes after extraction of cells. Whether the clusters observed by microscopy are related to the detergent-resistant complexes, and whether chPrP remains associated with the complexes after recruitment into coated pits are questions that require further investigation.

Although the study reported here has focused on N2a neuroblastoma cells, the results may also be applicable to other neuronal cell lines, and to neurons, which also lack morphologically identifiable caveolae (J. Heuser, personal communication), and which express only low levels of caveolin mRNA and protein (Glenney, 1989, 1992). Indeed, we have been able to detect low-density, chPrP-containing complexes in Triton extracts of chicken brain synaptosomal membranes after centrifugation on sucrose density gradients (Gorodinsky et al., 1994).

Although detergent-resistant complexes isolated from neuronal cells may not derive from caveolae, they may nevertheless correspond to microdomains of surface or internal membranes that are present in intact cells, and that serve a physiological function. One possibility is that these domains play a role in polarized sorting of proteins in neurons, analogous to the role that has been proposed for them in epithelial cells (Simons and Wadinger-Ness, 1990). Another intriguing possibility is that these domains operate in transmembrane signaling. Antibody-induced cross-linking of a variety of GPI-anchored proteins on lymphocytes stimulates tyrosine phosphorylation, elevation of cytoplasmic calcium, and mitogenesis; the tyrosine kinases and G proteins associated with the detergent-resistant complexes have been proposed to mediate these responses (Robinson, 1991; Brown, 1993). Whether similar effects occur in neurons, and whether PrP^{C} is involved, are interesting questions for future study. Finally, it will be important to investigate whether detergent-resistant domains play a role in the posttranslational conversion of PrP^{C} into the scrapie isoform of the prion protein (PrP^{Sc}), a process that is thought to occur either on the cell surface or in an endocytic compartment (Caughey and Raymond, 1991; Borchelt et al., 1992; Taraboulos et al., 1994).

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