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Evidence for Segregation of Heterologous GPI-anchored Proteins into Separate Lipid Rafts within the Plasma Membrane

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Abstract. Cholesterol and glycosphingolipid-rich membrane rafts, which are rich in GPI-anchored proteins and are distinct from caveolae, are believed to serve as platforms for signal transduction events and protein recycling. GPI-anchored proteins with diverse functions as well as caveolin may be recovered in a membrane fraction insoluble in cold non-ionic detergent. This study tests for possible heterogeneity in the protein composition of the lipid rafts and detergent-insoluble membrane complexes by examining the two GPI-anchored homologous human folate receptors (FR)-α and -β, the GPI-anchored human placental alkaline phosphatase (PLAP), and caveolin (control) in transfected CHO cells. Both FR and PLAP showed the equal distribution of cell-surface *vs.* sequestered (recycling) protein typical of GPI-proteins. Quantitative affinity purification of detergent-insoluble complexes using biotinylated folate or specific antibodies demonstrated a strong association of the homologous FR-α and FR-β in the same detergent-insoluble complex and separate complexes containing either PLAP or caveolin. Immunogold localization experiments using antibody crosslinking to produce larger aggregates of GPI-anchored proteins for visualization by electron microscopy also showed a clear separation between FR- and PLAP-rich membrane microdomains. Thus, even though functionally diverse and heterologous GPI-anchored proteins are known to share endocytic and recycling vesicles, they may be segregated in distinct lipid rafts on the basis of their ecto(protein) domains facilitating clustering, compartmentalization and homotypic protein interactions.

Key words: Folate receptor — Placental alkaline phosphatase — Glycosyl-phosphatidylinositol — Lipid raft — Phosphatidylinositol-specific phospholipase C

Introduction

There is a considerable amount of physical and biochemical evidence to indicate lateral asymmetry in the distribution of lipids in biological membranes (Edidin, 1997; Simons & Ikonen, 1997; Brown, 1998; Kurzchalia & Parton, 1999). Specific lipid self-associations in the fluid bilayer, particularly involving sphingolipids and cholesterol, are believed to result in the formation of membrane microdomains or "rafts" that mediate interorganelle transport of membrane proteins or function as platforms for signal transduction events (Simons & Ikonen, 1997; Kurzchalia & Parton, 1999). Such membrane microdomains may be recovered in a low-density fraction that is insoluble in Triton X-100 at 4°C and referred to as detergent-insoluble, glycolipid-enriched complexes (DIGs) (Simons & Ikonen, 1997). Proteins, to which GPI anchors are attached in the endoplasmic reticulum, first become associated with DIGs in the Golgi complex, the site of synthesis of sphingolipids (van Meer, 1989; Brown & Rose, 1992). Membrane microdomains that constitute cell surface invaginations called caveolae can also be recovered in the form of DIGs (Schnitzer et al., 1995; Parton, 1996). Caveolae are typically associated with a protein called caveolin, which coats their cytoplasmic surface and is also observed in post-Golgi vesicles (Rothberg et al., 1992; Murata et al., 1995; Parton, 1996). Based on detergent partitioning and electron microscopic studies, it was originally proposed that GPI-anchored proteins occur inside caveolae (Rothberg et al., 1990; Ying, Anderson & Rothberg, 1992). Subsequent studies (Schnitzer et al., 1995) showed that isolated

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caveolae did not contain GPI-anchored proteins. It has also been noted that DIGs of GPI-anchored proteins occur in cells (e.g., lymphocytes, neuroblastoma cells, and the apical membrane of enterocytes) that do not have caveolae (Fra et al., 1994; Gorodinsky & Harris, 1995; Simons & Ikonen, 1997).

DIGs of both GPI-anchored proteins and caveolin are known to contain proteins involved in signal transduction (Anderson, 1993; Lisanti et al., 1994; Parton & Simons, 1995; Kurzchalia & Parton, 1999). For example, the Src family kinases, Lck and Fyn co-immunoprecipitate with GPI-anchored proteins in cold Triton X-100 (Casey, 1995) and trimeric G proteins and Ras are similarly associated with caveolin (Li et al., 1995; Song et al., 1996).

Various GPI-anchored proteins such as FR, Thy-1, CD44, prion protein, and decay-accelerating factor (DAF) have been found to internalize and recycle to the cell surface although FR is the only known GPI-anchored protein with a transport function, i.e., internalization of folate (Kamen et al., 1988, 1989). Even though endocytosis and recycling of FR and other GPI-anchored proteins does not appear to involve clathrin (Rothberg et al., 1990; Bamezai, Goldmacher & Rock, 1992; Keller, Siegel & Caras, 1992; Danielsen & van Deurs, 1995; Ritter et al., 1995) internalized FR and DAF have been localized in known endocytic compartments in kidney sections (FR) (Hjelle et al., 1991; Birn, Selhub & Christensen, 1993) and in MA104 cells (FR, DAF) (Mayor, Sabharanjak & Maxfield, 1998). The recycling of FR and DAF in MA104 cells is characterized by a 3-fold slower rate of externalization compared with the transferrin receptor (Mayor et al., 1998). A roughly equal distribution between the cell surface and intracellular compartments at steady state appears to be characteristic of the recycling GPI-anchored proteins (Kamen et al., 1988; 1989; Mayor et al., 1998). The unique recycling kinetics of GPI-anchored proteins is critically dependent upon their cholesterol and sphingolipid-rich microenvironment (Chang et al., 1992; Ritter et al., 1995; Mayor et al., 1998, Chatterjee et al., 2001). The recycling of FR has been shown to be modulated by phorbol ester (Lewis et al., 1998b) and likely mediated by the actin cytoskeleton (Lewis, Smith & Kamen, 1998a). FR was the paradigm for an earlier model termed potocytosis for caveolar mediation of the recycling of GPI-anchored proteins (Anderson et al., 1992). The kinetic aspect of potocytosis elucidated by Kamen and coworkers is conceptually elegant and valid, but the association of FR with caveolae was initially contradicted by a report (Mayor, Rothberg & Maxfield, 1994) suggesting that the clustering and apparent caveolar localization of FR (and by inference, other GPI-anchored proteins) observed earlier was an artifact of cross-linking by the antibody probes; however, separate receptor clusters were not observed in that report. Subsequently, two electron microscopic studies (Danielsen & van Deurs, 1995; Wu et al., 1997) visualized rafts of GPI-anchored proteins using more appropriate probing and fixation conditions. In one (Danielsen & van Deurs, 1995), a GPI-anchored iron-binding protein was shown to occur in clusters in the apical surface of enterocytes in ultracryosections of mucosal tissues. We used a monovalent folate affinity analog to covalently label cell surface FR under transport-permissive conditions and showed that FR molecules are clustered in situ in epithelial cells and fibroblasts and that they are not associated with caveolin even during folate transport (Wu et al., 1997). FR rafts on the surface of cells have since been visualized by fluorescence resonance energy transfer microscopy (Varma & Mayor, 1998) and by chemical cross-linking (Friedrichson & Kurzchalia, 1998) and their diameter has been estimated to be < 70 nm.

GPI-anchored proteins, of which over 100 have been recognized to date, carry out a wide variety of physiologic functions including signal transduction and cell adhesion. In many instances, homotypic interactions, clustering or compartmentalization of one or a few proteins in lipid rafts may be expected to be crucial for their function. In view of the limited capacity of the minute lipid rafts, it is possible that individual GPI-anchored proteins may be packaged into distinct rafts or DIGs based on properties of their ecto (protein) domains. Even though the protein ectodomains of GPI-anchored proteins are known to target them to distinct organelles, it is of interest to test whether such a segregation of GPI-anchored proteins also occurs within the plasma membrane. In this report, the detergent-insoluble membrane fraction is quantitatively resolved into its constituent DIGs, in particular, those enriched in FR-α, FR-β, placental alkaline phosphatase (PLAP) or caveolin. Associations of these proteins on the plasma membrane are also examined by electron microscopy. FR-α and FR-β were chosen for this study as examples of homologous GPI-anchored proteins. The two proteins are single polypeptides with 257 (FR-α) or 255 (FR-β) amino acids, which are N-glycosylated at three (FR-α) or two (FR-β) sites, are functionally distinguishable (Wang et al., 1992; Shen et al., 1997; Maziarz et al., 1999) and share approximately 70 percent amino acid-sequence identity. FRs-α and -β bind folic acid with a relatively high affinity (K_D $< 10^{-9}$ M) and with a 1:1 stoichiometry (Antony, 1996). PLAP was chosen as an example of a heterologous GPI-anchored polypeptide with 530 residues (Kam et al., 1985). Caveolin was used as a control in these studies because it is known to be enriched in the detergent-insoluble membrane fraction, but is known to exist in a membrane microdomain distinct from that of FR and other GPI-anchored proteins.

Materials and Methods

Reagents and Antibodies

Biotin-SS-Folate was synthesized and purified for use as described previously (Fan, Vitols & Huennekens, 1991; Wu et al., 1997). Phosphatidylinositol-specific phospholipase C (PI-PLC) was from BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA. Lipofectamine, opti-MEM I reduced-serum medium and biotinylated goat anti-rabbit and goat anti-mouse antibodies were from Gibco BRL, Grand Island, NY. FuGENE 6 transfection reagent was from Roche Molecular Biochemicals, Indianapolis, IN. Folate-free RPMI (FFRPMI) medium and fetal bovine serum were from Irvine Scientific, Irvine, CA. Streptavidin-conjugated superparamagnetic iron oxide particles and monoclonal anti-human placental alkaline phosphatase antibody were from Sigma, St. Louis, MO. [^{3}H]Folic acid (20.2 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA. ^{125}I-Labeled sheep anti-mouse IgG (20 μCi/μg) and ^{125}I-labeled donkey anti-rabbit IgG antibody (11 μCi/μg) were purchased from Amersham Life Science, Arlington Heights, IL. Rabbit antiserum against FR-β was prepared as described previously (Ratnam et al., 1989). Affinity-purified rabbit anti-human caveolin 1 antibody was purchased from Transduction Laboratories, Lexington, KY. Monoclonal antibody Mov 19 against human FR-α was a kind gift from Centocor Pharmaceuticals, Malvern, PA. Monoclonal anti-human placental alkaline phosphatase, clone 8B6, was purchased from Sigma. Ten-nm colloidal gold-conjugated goat anti-rabbit IgG and 5-nm colloidal gold-conjugated goat anti-mouse IgG were purchased from Ted Pella, Redding, CA.

Cell Culture and Transfections

The recombinant CHO cell line stably expressing FR-β (CHO-FR-β) has been described previously (Wu et al., 1997). CHO-FR-β cells were grown in FFRPMI medium supplemented with fetal bovine serum (10% v/v), penicillin (100 units/ml), streptomycin (100 g/ml), L-glutamine (2 mM), and L-proline 14.5 mg/l). The construction of the pcDNAI plasmids expressing FR-α and FR-β has been described previously (Yan & Ratnam, 1995). The cDNA for human PLAP was purchased from the American Type Culture Collection and was inserted into the expression plasmid pcDNAI at *Hind*III and *Xba*I sites. Transient transfections were carried out in 15-cm tissue culture plates (Corning) or in 6-well cell culture wells using lipofectamine or FuGENE according to the manufacturer's protocol.

PI-PLC Treatment

CHO cells (cultured in FFRPMI) were transiently transfected with the expression plasmids for FR-β or PLAP. 24 hr later, the culture medium was replaced with FFRPMI in the presence or in the absence of 15 mM NaN_3 and incubated at 37°C for 30 min. The cells ($\sim 1 \times 10^6$) were then washed with 1× HBSS (Hank's balanced salt solution) three times and incubated with 0.3 U of PI-PLC in 1× HBSS with or without NaN_3 (15 mM) at 37°C for 1 hr. After PI-PLC treatment, the cells were harvested and lysed in PBS containing 1% Triton X-100 at 37°C for 30 min. The cell lysates were finally subjected to quantitative western blot analysis as described below.

Membrane Preparations

All of the following procedures were carried out at 4°C or on ice. Cells from confluent cultures were washed with acid buffer (10 mM sodium acetate, pH 3.5/150 mM NaCl) to remove endogenous bound folates from the cell surface, followed by washing with PBS (10 mM sodium phosphate buffer, pH 7.5/150 mM NaCl). The cells were then suspended in lysis buffer (1 mM $NaHCO_3$, pH 7.2, containing 2 mM $CaCl_2$, 5 mM $MgCl_2$ and 1 mM phenylmethanesulfonyl fluoride). The cells were allowed to swell for 30 min then homogenized (50 strokes) in a dounce homogenizer. The homogenates were centrifuged for 10 min at 2,000 × g to sediment the nuclei and cell debris. The resulting supernatant was centrifuged for 45 min at 30,000 × g. The sedimented membranes were resuspended in acid buffer, sedimented again, resuspended, and washed twice with PBS. The membranes were finally resuspended in 1× HBSS containing a cocktail of protease inhibitors aprotinin, bestatin, chymostatin, EDTA, E-64, leupeptin, and pepstatin (Boehringer).

[^{3}H]Folic Acid Binding Assay

The binding of [^{3}H]folic acid to the membranes was quantitated as described (Wang et al., 1992). Membrane samples (10 μg protein) were incubated on a rotary shaker with 5 pmol of [^{3}H]folic acid (Moravek) in PBS for 30 min at 4°C. The membranes were then centrifuged at 12,000 × g for 15 min, rinsed twice with cold PBS, and dissolved in PBS containing 1% Triton X-100 at 37°C for 30 min. The samples were then subjected to liquid scintillation counting.

Affinity Purification of Detergent-insoluble Membrane Complexes

All the following procedures were carried out at 4°C or on ice. For purifying the FR-β-rich membrane fraction, membranes (100 μg total protein) were incubated with 50 pmol of Biotin-SS-Folate for 30 min. An equal volume of 1× HBSS containing 2% Triton X-100 was added to obtain a final concentration of 1 percent Triton X-100. After a further 30 min incubation, a prewashed suspension (0.5 ml in HBSS containing 1% Triton X-100) of streptavidin-coated magnetic particles (Sigma) was added and the mixture was incubated for 4 hr in a rotary shaker. The samples were then placed on a magnet stand to retain the magnetic particles. After washing the magnetic particles three times with 1× HBSS, the purified proteins on the magnetic particles were eluted in 100 μl standard of 2× sodium dodecylsulfate (SDS) sample loading buffer for western blot analysis. For purifying FR-α-rich and caveolin-rich membrane fractions, membranes (100 μg total protein) were incubated with 40 μg of Mov 19 or affinity-purified anti-caveolin antibodies, respectively, for 6 hr. At the same time, 0.5 ml of a prewashed suspension of streptavidin-coated magnetic particles was incubated with 200 μg of biotinylated goat anti-mouse or goat anti-rabbit antibody. Next, 1× HBSS containing 2% Triton X-100 was added to the membrane samples to obtain a final Triton X-100 concentration of 1 percent and they were incubated for 30 min. Unbound biotinylated goat anti-mouse or goat anti-rabbit antibodies were removed by washing the magnetic particles with 1× HBSS with the help of a magnetic stand to retain the particles. The antibody-coated magnetic particles (in 0.5 ml of HBSS containing 1% Triton X-100) were added to the membrane samples and incubated overnight on a rotary shaker. Finally, the magnetic particles were washed and the FR-α- or caveolin-enriched fractions recovered as described above for FR-β.

Standardized Western Blot Analysis

The samples were electrophoresed on 12% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose mem-

branes. The blots were probed with primary antibodies specific for FR-α, FR-β, PLAP or caveolin and the appropriate ^{125}I-labeled secondary antibodies. The band intensities were quantitated by phosphorimager (Storm 840) analysis using the program Image-Quant 1.2.

The western blots were standardized for quantitation as follows. Various amounts of membrane protein (0.5, 1, 2, 5, 10, 20, 40, and 80 μg) were resolved by SDS-polyacrylamide gel electrophoresis and electroblotted on nitrocellulose filters. The blots were probed with the primary antibodies specific for FR-α, FR-β, PLAP or caveolin and with the appropriate ^{125}I-labeled secondary antibody. The protein-band intensities were quantitated by phosphorimage analysis and plotted against the total membrane protein. A linear relationship with band intensity was obtained up to 20 μg of total membrane protein for FR-β and 40 μg of membrane protein for FR-α, caveolin and PLAP. In all of the quantitative western blot analysis in this study, the amount of each protein loaded was within this linear range.

Electron Microscopy

Transfected monolayer cells in 6-well standard cell culture plates were washed with Hank's balanced salt solution (HBSS) containing 1% bovine serum albumin and 30 mM NaN_3 (solution A) and incubated in this solution (1 ml/well) for 30 min at room temperature. The cells were treated at room temperature sequentially with either a rabbit antiserum against FR-β or with pre-immune serum (1:200 dilution) for 2 hr, 10-nm colloidal gold-conjugated goat anti-rabbit IgG (1:50 dilution) for 1.5 hr either mouse anti-PLAP monoclonal antibody or anti-FR-α (Mov 19) mouse monoclonal antibody (50 μg/ml) for 2 hr and 5-nm colloidal gold-conjugated goat anti-mouse IgG (1:50 dilution) for 1.5 hr. The cells were washed 4 times with buffer A in between antibody incubations and also after the final incubation described above. The cells were fixed in 3% glutaraldehyde and processed for electron microscopy by alcohol dehydration and embedding. Uranyl acetate and lead citrate post-staining was avoided in order to better visualize the gold particles. The sections were examined under a CM-10 Philips electron microscope. Negative controls included exclusion of individual primary antibodies and also transfection of cells with expression vector that did not contain a cDNA insert.

Results and Discussion

We have previously demonstrated the functionality of FR-β in recombinant CHO cells (CHO-FR-β) stably expressing the protein (Wu et al., 1997). For the purpose of the present studies, it was also desirable to establish that in CHO cells expressing FR and PLAP by transient transfection, the proteins retained the expected steady-state subcellular distribution of GPI-anchored proteins.

In CHO cells transiently expressing FR-β or PLAP, the relative distribution of each protein on the cell surface (PI-PLC-sensitive) and sequestered (PI-PLC-resistant) compartments in steady state was determined by first arresting endocytic and exocytic processes using sodium azide and then estimating the maximum fraction of each protein that could be released from the cell surface by PI-PLC. Approximately 50 percent of either FR-β or PLAP were

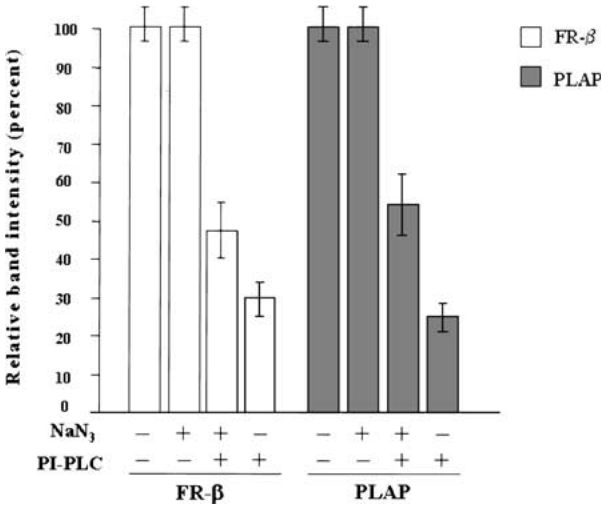

Fig. 1. Relative distribution of FR-β and PLAP between the cell surface and sequestered compartments. CHO cells transiently transfected with expression plasmids for FR-β or PLAP were incubated with or without 0.3 U of PI-PLC in 1× HBSS with or without 15 mM NaN_3 at 37°C for 1 hr. Then the cells were harvested and lysed. The cell lysates were subjected to standardized quantitative western blot analysis and the protein bands were quantitated on a phosphorimager as described under Materials and Methods.

released under these conditions relatively early (5 min, *results not shown*) or at the end of 1 hr (Fig. 1), indicating that approximately half of each protein was sequestered. In the absence of sodium azide, a significantly greater proportion of FR-β or PLAP was susceptible to PI-PLC, indicating that the sequestered proteins slowly re-emerged at the cell surface (Fig. 1). Thus, the sequestered proteins observed in the presence of sodium azide represent the populations of recycling protein molecules. These results suggest that FR and PLAP in the transiently transfected CHO cell system are present in functionally relevant membrane microdomains.

Detergent Insolubility of FR, PLAP, and Caveolin

Since the association of glycosphingolipids with GPI-anchored proteins in the Golgi is believed to confer the property of insolubility in cold non-ionic detergent (Schroeder, London & Brown, 1994), detergent insolubility may be viewed as a diagnostic feature of the occurrence of the proteins in lipid rafts. However, various GPI-anchored proteins appear to display variable degrees of detergent insolubility over a wide range (Brown & Rose, 1992; Solomon, Mallory & Finberg, 1998). To ensure that the majority of FR, PLAP, or caveolin could be recovered in a membrane fraction insoluble in cold Triton X-100, membranes from CHO cells expressing these proteins were trea-

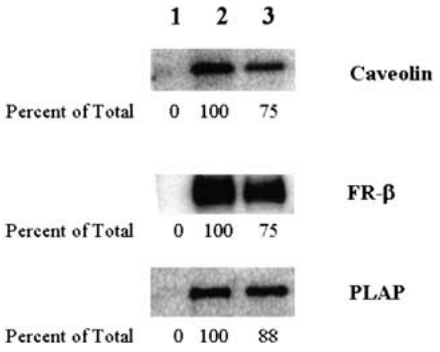

Fig. 2. Detergent-insolubility of FR, PLAP and caveolin. Membranes (20 μg total protein) were dissolved in a solution of 1 percent Triton X-100 in 1× HBSS at 4°C (lane *3*) or at 37°C (lane *1*) and incubated overnight. The samples were sedimented at 15,000 × g for 15 min at 4°C. The pellets were dissolved in 2× SDS sample loading buffer and subjected to standardized quantitative western blot analysis. Untreated membranes (20 μg total protein) were directly dissolved in SDS sample loading buffer and loaded in the control lane (lane *2*). The protein bands were quantitated by phosphorimager analysis as described under Materials and Methods.

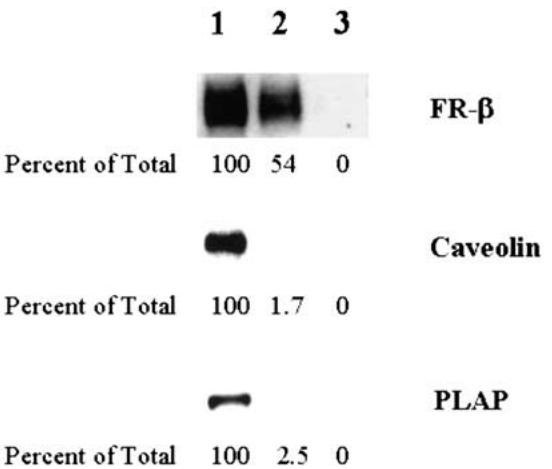

Fig. 3. Purification of detergent-insoluble complex of FR-β using biotin-SS-folate. CHO-FR-β cells were transiently transfected with an expression plasmid for PLAP. The plasma membranes from the transfected cells were prepared and treated with 1 percent Triton X-100 at 4°C and FR-β was purified from this sample using magnetic particles coated with biotin-SS-folate as described under Materials and Methods. The samples were subjected to standardized quantitative western blot analysis and the proteins quantitated using a phosphorimager as described under Materials and Methods. The purified sample was loaded in lane *2*. In a parallel negative control for this experiment (lane *3*), the same procedure was followed but with the exclusion of biotin-SS-folate. The corresponding amount of untreated membranes (10 μg total protein) was directly dissolved in SDS sample loading buffer and loaded in lane *1*.

ted with the detergent at 4°C or at 37°C and the insoluble proteins collected by sedimentation. The proteins were largely (75–88 percent) recovered in the detergent-insoluble sediment at 4°C but not at 37°C (Fig. 2). These results indicate that most, if not all, of

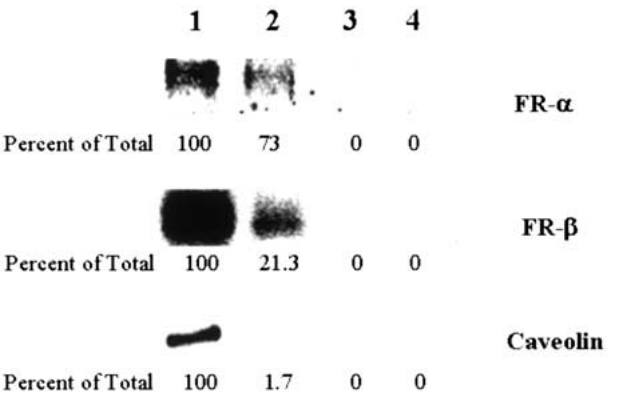

Fig. 4. Purification of detergent-insoluble complex of FR-α using monoclonal antibody Mov 19. CHO-FR-β cells were transiently transfected with the pcDNAI plasmid expressing the cDNA for FR-α. Plasma membranes were prepared from the transfected cells and treated with 1 percent Triton X-100 at 4°C and the FR-α purified, using the Mov 19 antibody as described under Materials and Methods. The samples were subjected to standardized quantitative western blot and phosphorimager analysis. The purified sample was loaded in lane *2*. In a parallel negative control experiment, the same procedure was followed but with the exclusion of Mov 19 (lane *3*). In a second parallel control experiment, the CHO-FR-β cells were transfected with the pcDNAI plasmid that did not contain the FR-α cDNA (lane *4*). The corresponding amount of untreated membranes (10 μg total protein) was directly dissolved in SDS sample loading buffer and loaded in lane *1*.

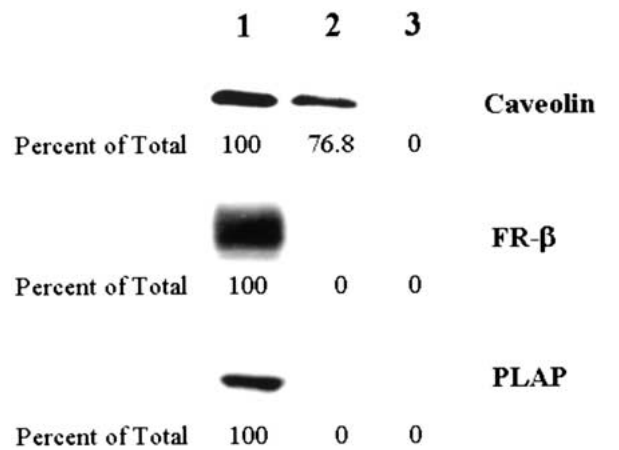

Fig. 5. Purification of detergent-insoluble complex of caveolin using anti-caveolin antibody. CHO-FR-β cells were transiently transfected with an expression plasmid for PLAP as in Fig. 3. Plasma membranes were prepared from the transfected cells and treated with 1 percent Triton X-100 at 4°C and the caveolin purified using anti-caveolin antibody as described under Materials and Methods. The samples were subjected to standardized quantitative western blot and phosphorimager analysis. The purified sample was loaded in lane *2*. The anti-caveolin antibody was excluded in a parallel negative control experiment (lane *3*). The corresponding amount of untreated membranes (10 μg total protein) were directly dissolved in SDS sample loading buffer and loaded in lane *1*.

the FR, PLAP or caveolin present in the detergent-treated membranes in the following experiments must

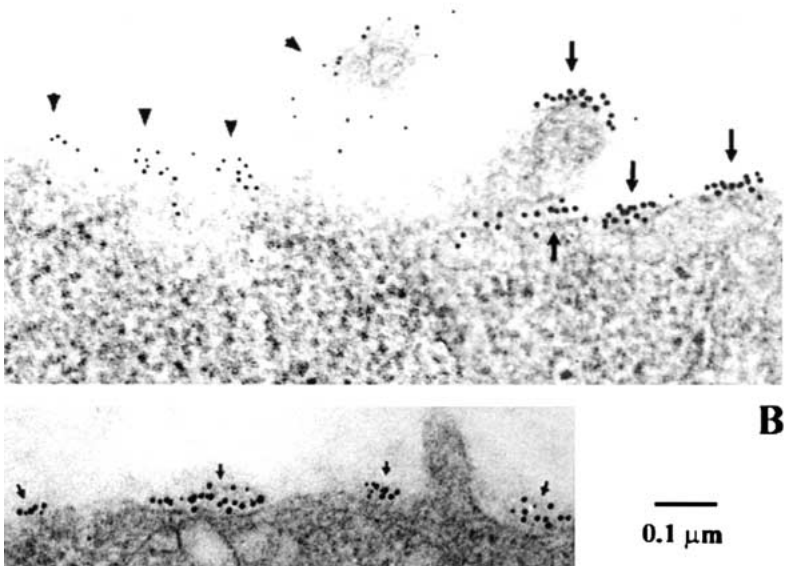

Fig. 6. Cell surface localization of FR-β, PLAP and FR-α by immunogold electron microscopy. CHO cells transfected with expression plasmids for FR-β and either PLAP (panel *A*) or FR-α (panel *B*) were probed in the presence of 30 mM NaN_3 with rabbit anti-FR-β antibody followed by 10-nm colloidal gold-conjugated goat anti-rabbit IgG and then with a mouse monoclonal antibody against either PLAP (panel *A*) or FR-α (panel *B*) followed by 5-nm colloidal gold-conjugated goat anti-mouse IgG. In panel *A*, arrows indicate clusters of 10-nm gold particles localizing FR-β, and the arrowheads represent clusters of 5-nm gold particles localizing PLAP. The arrows in panel *B* indicate clusters in which both 10-nm and 5-nm (FR-α) gold particles are colocalized. CHO cells transfected with vector that did not contain a cDNA insert did not show immunogold staining with any of the primary antibodies (*results not shown*). In addition, the rabbit pre-immune serum did not detect any protein in FR-β-expressing cells and 5-nm colloidal gold-labeling was virtually absent in PLAP or FR-α-expressing cells when the corresponding monoclonal antibody was excluded (*results not shown*).

represent the characteristic detergent-insoluble complexes of GPI-anchored proteins.

Fractionation of Detergent-insoluble Complexes

FR-β was affinity-purified using biotin-SS-folate-coated magnetic particles from the detergent-insoluble fraction of membranes from recombinant CHO-FR-β cells transfected with the cDNA for PLAP. The yield of FR-β by this method was 54 percent (Fig. 3). Appreciable amounts of caveolin or PLAP did not co-purify with the FR-β enriched fraction (Fig. 3), indicating a lack of significant association between FR-β and either PLAP or caveolin in the detergent-insoluble membrane fraction.

Magnetic particles coated with Mov 19, a mouse monoclonal antibody specific for FR-α, were used to purify FR-α from the detergent-insoluble fraction of CHO-FR-β cells transfected with the cDNA for FR-α. The yield of FR-α by this method was relatively high (73 percent) (Fig. 4). A significant fraction of the total FR-β (21.3 percent) co-purified with FR-α, whereas the co-purification of caveolin was poor (1.7 percent) (Fig. 4). It may be noted here that all of the CHO-FR-β cells used in this experiment stably expressed FR-β, whereas the fraction of the cells that expressed FR-α corresponded to the efficiency of transient transfection with the FR-α cDNA, which was determined to be ~30 percent (*results not shown*). Therefore, even though the proportion of FR-α purified in Fig. 4 appears to be about three times the proportion of FR-β that co-purified, the actual proportion of FR-β associated with FR-α in the transfected subpopulation of cells may be assumed to be almost quantitative. Figure 4 also shows that when untransfected CHO-FR-β cells were used, Mov 19 failed to purify a detectable amount of FR-β, indicating that the antibody did not directly bind to FR-β under the conditions of this experiment.

When anti-caveolin antibodies were used to purify caveolin from the detergent-insoluble membrane fraction of CHO-FR-β cells transfected with the cDNA for PLAP, caveolin was recovered with a high yield (76.8 percent) (Fig. 5). However, the caveolin-enriched fraction did not contain detectable amounts of either FR-β or PLAP (Fig. 5), indicating a lack of significant association of caveolin with these two proteins.

Together, the foregoing results indicate that the detergent-insoluble membrane fraction may be resolved into at least three distinct subfractions enriched in FR-α/FR-β, PLAP or caveolin. FR-α and FR-β appear to be physically associated in DIGs that are distinct from those of PLAP or caveolin. Despite the similarity in lipid composition between caveolae and rafts of GPI-anchored proteins, the caveolin-enriched subfraction did not contain detectable amounts of either FR or PLAP. This observation, together with the fact that FR and caveolin exist in separate membrane microdomains in situ supports the notion that DIGs are *bona fide* representatives of membrane microdomains. It may also be noted that a small amount of caveolin (1.7 percent, Fig. 3) appears

Table 1. Quantitative analysis of associations between FR-β and either FR-α or PLAP on the cell surface by electron microscopy

	Primary antibody detected	
	Anti-FR-β (10-nm gold) + Anti-FR-α (5-nm gold)	Anti-FR-β (10-nm gold) + Anti-PLAP (5-nm gold)
Total 10-nm gold	828	840
Total 5-nm gold	367	911
Number of 5-nm gold particles associated[1] with a 5-nm gold particle (percent of total 5-nm gold)	224 (61%)	535 (58.7%)
Number of 5-nm gold particles associated[1] with a 10-nm gold particle (percent of total 5-nm gold)	241 (65.6%)	60 (6.6%)

Data from randomly selected transfected CHO cells (two cells per column) expressing FR-β and either FR-α or PLAP and analyzed by immunogold electron microscopy as described under Methods. Negative control cells, i.e., cells that were transfected with vector alone, did not show significant immunogold staining for FR-β, FR-α or PLAP (< 1% of the positive signal).

[1] "Association" between two gold particles is defined for the purpose of this analysis as the occurrence of the two particles within a distance of 40 nm in the electron microscopic image.

to specifically co-purify with FR-β. It has been reported that an undetermined fraction of caveolae exhibit a peripheral association with GPI-anchored proteins (Schnitzer et al., 1995) and that infrequently FR and caveolin clusters occur within a distance of 50 nm (Wu et al., 1997). Such a loose association between caveolae and FR clusters may have resulted in the marginal association of caveolin with FR-P in Fig. 3.

Cell Surface Localization

Lipid rafts are believed to be too minute to visualize individually using standard electron microscopy techniques, but antibody probes will produce large clusters of GPI-anchored molecules at the cell surface by cross-linking (Mayor et al., 1994). If an antibody against a specific GPI-anchored protein should generate an aggregate of lipid rafts containing the protein, it is reasonable to expect that such an aggregate should also contain other proteins present in those rafts. Based on this premise, CHO cells transfected with expression plasmids for FR-β and either PLAP or FR-α were probed with a rabbit antibody against FR-β and with a mouse monoclonal antibody specific for either PLAP or FR-α. Immunogold localization showed a clear separation of FR-β (10-nm gold) and PLAP (5-nm gold) molecules (Fig. 6*A*) in contrast to the colocalization of 10-nm and 5-nm gold particles labeling FR-β and FR-α (Fig. 6*B*). Quantitative analysis of the relative degree of self-association of the 5-nm gold particles *vs.* their association with 10-nm gold particles (Table 1), clearly demonstrates a strong tendency for FR-β and PLAP to display homotypic rather than heterotypic association. The results of the electron microscopic analysis thus complement the biochemical studies described above.

Physiologic Significance of Specialized Rafts

Based on the detergent solubility characteristics and targeting of chimeric proteins in which transmembrane and GPI anchors were interchanged, it is generally well-established that the association of GPI-anchored proteins with cholesterol and sphingolipids, and the subsequent targeting of most of them to the plasma membrane, occurs by virtue of their GPI anchor and is unrelated to their protein ectodomains (Brown, Crise & Rose, 1989; Lisanti et al., 1989; Simons and Ikonen, 1997). The present study illustrates three examples of GPI-anchored proteins (FR-α, FR-β and PLAP) that are sorted into microdomains of the plasma membrane based on the degree of similarity of their protein ectodomains and supports functional diversity of lipid rafts bearing GPI-anchored proteins on the cell surface. It has also been reported that by digital fluorescence microscopy, different GPI-anchored proteins (FR and DAF) may be observed in the same endocytic/recycling vesicles (Mayor et al., 1998). The estimated average diameter of membrane rafts of GPI-anchored proteins range from <70 nm (Varma & Mayor, 1998) to 200–300 nm (Jacobson & Dietrich, 1999); by these estimates, the surface area of an endocytic vesicle should be able to accommodate many membrane rafts. Therefore, it is possible that the distinct complexes of GPI-anchored proteins observed in this study recycle via the same endocytic pathway.

The simplest mechanism for the clustering of homotypic GPI-anchored polypeptides and a limited number of other proteins would be direct association of their ecto(protein) domains with a moderate affinity in the ER membrane. Such GPI clusters may be predisposed to associate with cholesterol and sphingolipids to form minute rafts in the Golgi, stabilizing the protein clusters. The solubility of certain GPI-

anchored proteins (Brown & Rose, 1992; Solomon et al., 1998) in cold non-ionic detergent could conceivably be due to the lack of such protein associations. Indeed, GPI-anchored proteins with different detergent solubility characteristics are known to be targeted to anatomically distinct parts of the neuronal cell surface (Madore et al., 1999). Segregation of GPI-anchored proteins is also evident from a recent report that GPI-anchored green fluorescent protein (GFP) and CD58 recycle between the plasma membrane and the Golgi apparatus by a pathway that is distinct from the FR recycling pathway, which occurs between the plasma membrane and endosomes (Nichols et al., 2001). The present study extends such a segregation to heterologous GPI-anchored proteins that reside in the same membrane organelle. Future studies should reveal more about the mechanism of the initial site of isotypic sorting of GPI proteins in the cell.

The requirement for clustering of GPI-anchored proteins at the cell surface for their optimal physiologic functioning is indicated by the inhibition of FR-mediated folate transport and GPI-protein-mediated signaling events by depletion of cholesterol (Chang et al., 1992; Stulnig et al., 1997). Ligation-induced homotypic interactions of raft-associated proteins may be required to initiate cell-signaling pathways (Brown & London, 2000). Given the rather small average size of a lipid raft estimated in situ and the large number of GPI-anchored proteins in the cell, it would appear to be necessary for the proteins to be segregated in order to accommodate an adequate number of protein molecules of any one type in a single raft and to ensure that proteins expressed at low levels are not too disperse on the cell surface. Segregation of GPI-anchored proteins, including receptors and co-receptors involved in signal transduction events, may also allow their compartmentalization. Furthermore, segregation and packaging of individual GPI-anchored proteins together with a few associated proteins into minute lipid rafts may allow close functional interactions among those molecules without spatial and steric constraints imposed by the presence of functionally unrelated proteins.

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