Molecular Biology of the Cell
Vol. 6, 929–944, July 1995

Insolubility and Redistribution of GPI-anchored Proteins at the Cell Surface after Detergent Treatment

Satyajit Mayor and Frederick R. Maxfield*

Department of Pathology, Columbia University, College of Physicians and Surgeons, New York, New York 10032

Submitted January 12, 1995; Accepted May 2, 1995
Monitoring Editor: Guido Guidotti

A diverse set of cell surface eukaryotic proteins including receptors, enzymes, and adhesion molecules have a glycosylphosphoinositol-lipid (GPI) modification at the carboxy-terminal end that serves as their sole means of membrane anchoring. These GPI-anchored proteins are poorly solubilized in nonionic detergent such as Triton X-100. In addition these detergent-insoluble complexes from plasma membranes are significantly enriched in several cytoplasmic proteins including nonreceptor-type tyrosine kinases and caveolin/VIP-21, a component of the striated coat of caveolae. These observations have suggested that the detergent-insoluble complexes represent purified caveolar membrane preparations. However, we have recently shown by immunofluorescence and electron microscopy that GPI-anchored proteins are diffusely distributed at the cell surface but may be enriched in caveolae only after cross-linking. Although caveolae occupy only a small fraction of the cell surface (< 4%), almost all of the GPI-anchored protein at the cell surface becomes incorporated into detergent-insoluble low-density complexes. In this paper we show that upon detergent treatment the GPI-anchored proteins are redistributed into a significantly more clustered distribution in the remaining membranous structures. These results show that GPI-anchored proteins are intrinsically detergent-insoluble in the milieu of the plasma membrane, and their co-purification with caveolin is not reflective of their native distribution. These results also indicate that the association of caveolae, GPI-anchored proteins, and signalling proteins must be critically re-examined.

INTRODUCTION

A diverse set of cell surface eukaryotic proteins including several receptors, enzymes, and adhesion molecules have a glycolipid modification at the carboxy-terminal end. This is a post-translational modification that serves as a membrane anchor and involves the replacement of the carboxy-terminal peptide sequence of the protein by a glycosyl-inositol phospholipid (GPI)[1] moiety (Ferguson and Williams, 1988; Low, 1989; Cross, 1990; Field and Menon, 1992; Englund, 1993). The structure and biosynthesis of the GPI moiety are now well understood, and it appears that all GPI-anchors have a common core glycan that bridges an ethanolamine residue in amide linkage with the protein and an inositol phospholipid (Mayor and Menon, 1990; Field and Menon, 1992; Englund, 1993; McConville and Ferguson, 1993). The main differences between GPI-anchors are due to numerous types of side chain modifications decorating the core glycan structure and the presence of various lipid backbones including glycerolipid and ceramide-based structures attached to a variety of mainly saturated

* Corresponding author: Department of Pathology, Rm. 15–420, College of Physicians and Surgeons, Columbia University, 630 West 168th St., New York, NY 10032.

[1] Abbreviations used: CHO, Chinese hamster ovary; DAF, decay-accelerating factor; FBS, fetal bovine serum; GPI, glycosylphosphoinositol-lipid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; HF, HEPES-buffered Hams F-12 medium; HRF$^-$, HEPES-buffered folate-deficient RPMI; mAb, mouse monoclonal antibodies; PI-PLC, phosphatidylinositol-specific phospholipase C; PLF, Pteroyl-lysyl-folate; TRVb-1, CHO cells expressing human transferrin receptor.

acyl and alkyl chains (McConville and Ferguson, 1993).

In spite of the extensive biochemical information on the GPI-moiety of GPI-anchored proteins, the functions of this ubiquitous protein modification are less understood, although it has been implicated in a variety of cell biological processes (Ferguson, 1994). The GPI-anchor has been proposed to act as an apical targeting signal for proteins in some epithelial cell types via its association with putative glycolipid rafts in the *trans*-Golgi network (Lisanti and Rodriguez-Boulan, 1990; Simons and Wandinger-Ness, 1990). GPI anchoring has also been shown to be important for the intracellular signalling capacity of several proteins especially in lymphocytes. In most cases the cross-linking of the protein is a prerequisite for their signalling function (Robinson, 1991; Brown, 1993).

In immunolocalization studies, GPI-anchored proteins have been found to be clustered at the cell surface, and a significant fraction of the clusters are localized to 50- to 60-nm caveolin/VIP-21–coated membrane invaginations called caveolae (Rothberg *et al.*, 1992; Dupree *et al.*, 1993). GPI-anchored proteins, a subset of membrane lipids (sphingomyelin, acidic and neutral glycolipids, and cholesterol), and caveolin/VIP-21 have been shown to be largely insoluble in nonionic detergents (mainly cold Triton X-100) (Hooper and Turner, 1988; Glenney and Zokas, 1989; Brown, 1992; Brown and Rose, 1992). Together these properties have been used by many investigators to purify caveolae or "caveolin/VIP-21–rich membranes" (reviewed in Brown, 1992; Lisanti *et al.*, 1993). Many cytoplasmically oriented signalling molecules, including heterotrimeric GTP-ases, small GTP-ases, and nonreceptor type protein-tyrosine kinases (PTKs) have been localized to caveolae, primarily in aggregates derived with Triton X-100 (reviewed in Brown, 1993). Based on this co-purification, GPI-anchored proteins have been proposed to mediate intracellular signalling in caveolae due to their association with PTKs in these structures (Anderson, 1993; Brown, 1993; Lisanti *et al.*, 1993).

However, we have recently shown, using fluorescently labeled monoclonal antibodies (mAbs) to different GPI-anchored proteins, that these proteins are not constitutively concentrated in caveolae; they are enriched in these structures only after cross-linking with polyclonal secondary antibodies (Mayor *et al.*, 1994). Analyses of the cell-surface distribution of GPI-anchored folate receptor and alkaline phosphatase by electron microscopy have also confirmed that these proteins are not constitutively enriched in caveolae (Mayor *et al.*, 1994; Parton *et al.*, 1994). Thus, multimerization of GPI-anchored proteins regulates their sequestration in caveolae, but in the absence of agents that promote clustering they are diffusely distributed over the plasma membrane (Mayor *et al.*, 1994).

The finding that GPI-anchored proteins are not normally clustered over caveolae raised questions about how these proteins become enriched in the caveolae preparations obtained from detergent-extracted cells. In this paper, we analyze the effect of Triton X-100 on the surface distribution of GPI-anchored proteins. We confirm that Triton X-100 treatment leads to an almost complete insolubility of GPI-anchored proteins at the plasma membrane whereas transmembrane proteins are effectively solubilized, and we show that the detergent causes a redistribution of GPI-anchored proteins at the cell surface. In view of these findings we critically discuss the connection between Triton X-100 insolubility, caveolae, and GPI-anchored proteins.

MATERIALS AND METHODS

Materials

Mouse mAb to the human folate receptor (MOv19; Coney *et al.*, 1991) were a gift from Dr. Richard Anderson and Centocor (Malvern, PA). Rabbit polyclonal antibody to caveolin/VIP-21 was obtained from Transduction Laboratories (Lexington, KY). Species-specific polyclonal IgGs to primary antibodies were obtained from Pierce Chemical (Rockford, IL). Mouse mAb to decay-accelerating factor (DAF), 1A10, (Davitz *et al.*, 1986) was a gift from M. Davitz (New York University, New York, NY). Labeling of mAbs and transferrin with the fluorophore Cy3 (Biological Detection Systems, Pittsburgh, PA) was carried out according to the manufacturer's instructions. Pteroyl-lysyl-folate (PLF) (McAlinden *et al.*, 1991) was obtained from Dr. J. Hynes (Medical University of South Carolina). Gold (10 nm)-conjugated antibodies were obtained from Amersham Life Science (Buckinghamshire, England). Poly-D-lysine–treated cover-slip bottom dishes were made and used for growing cells for all microscopy studies as previously described (Mayor *et al.*, 1993). All chemicals were obtained from Sigma Chemical (St. Louis, MO) and tissue culture supplies were obtained from Life Technologies (Gaithersburg, MD) unless otherwise specified.

Cells and Cell Culture

G3G2 cells (3T3-L1 cells transfected with cDNA of the human folate receptor; Lacey *et al.*, 1989) were grown on cover slip dishes in DMEM supplemented with 10% calf serum and 500 μg/ml Geneticin (DMEM-CS). All CHO cells lines were maintained in Hams F-12 medium supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. DAF-expressing CHO cells were obtained by the co-transfection of TRVb-1 cells (CHO cells expressing the human transferrin receptor; McGraw *et al.*, 1987) with a hygromycin-resistant plasmid pHEBO (Sugden *et al.*, 1985) and pCDM8-derived plasmid consisting of the DAF cDNA (Caras *et al.*, 1987; Medof *et al.*, 1987). Folate receptor–expressing CHO cells were derived from TRVb-1 cells by transfection with pMFRα, a plasmid constructed from the eukaryotic expression vector pMEP4 (Invitrogen, San Diego, CA) and the full length cDNA of the human folate receptor identical to that found in KB cells (Wang *et al.*, 1992), and provided by Dr. M. Ratnam (Medical University of Ohio, Toledo, OH). The folate receptor cDNA was placed in front of the metallothionein promoter in the multiple cloning site in the eukaryotic expression vector pMEP4. MA104 cells, a monkey kidney epithelial cell line, were maintained in folate-deficient DMEM-FBS and plated on coverslip dishes 5 days before the experiment.

Labeling of Cells and Triton X-100 Extractions

To label with antibodies recognizing cell-surface GPI-anchored proteins, the cells were pre-incubated in F-12 medium supplemented

with 0.2% bovine serum albumin, 1.2 g/l glucose, and 4.5 g/l HEPES, pH 7.4 (HF-BSA) for 10 min at 37°C. The cells were then incubated for 1 h at 0°C with Cy3-labeled mAb (20 μg/ml in the case of the anti-DAF monoclonal Cy3–1A10, and 4 μg/ml in the case of the anti-folate receptor monoclonal Cy3-MOv19). The cells were then rinsed (twice for 2 min) in medium 1 (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, pH 7.4) and then further incubated in medium 1 or with 1% Triton X-100 in medium 1 containing 2 μg/ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride at 0°C for 30 min. The cells were rinsed in ice-cold medium 1 and taken for fluorescence imaging, or fixed with 3% paraformaldehyde and 0.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 10 min at 0°C followed by 30 min at room temperature and subsequently processed for electron microscopy. Control incubation was performed with an irrelevant mouse mAb MOPC-21 (Sigma Chemical) at 20 μg/ml for the electron microscopic analyses, or Cy3-conjugated mAb to an irrelevant Ag (Thy 1.1 for CHO cells and DAF for 3T3-L1 cells) for fluorescence microscopy. No detectable fluorescence was observed in the control incubations with any of the irrelevant mAbs on the appropriate cells.

Before labeling with the fluorescent analogue of folic acid, PLF, (McAlinden *et al.*, 1991), the cells were first rinsed on ice with a low pH buffer (twice for 30 s with acid-saline [150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 3.0, adjusted with acetic acid] or twice for 2 min with a membrane-impermeant acid wash [50 mM sodium citrate, 280 mM sucrose, pH 4.6]; similar results were obtained with either condition) and then with medium 1 (twice for 2 min) and further incubated in HEPES-buffered folate-free RPMI 1640, pH 7.4 (HRF^-; Speciality Media, Lavallette, NJ), before the addition of PLF (15 nM) in HRF^-. PLF labeling of cells and detergent extraction was done in a manner similar to the labeling with antibodies described above except that HRF^- was used instead of HF-BSA as the labeling medium. The fluorescence due to labeling of human folate receptor-transfected CHO cells with PLF was completely abolished in the presence of 1.0 μM folic acid, and the staining of the DAF-transfected CHO cells with the fluorescent analogue was undetectable, confirming that the PLF was binding to the human-folate receptor, and that the folate receptor-transfected CHO cells expressed a bona fide high affinity folate receptor.

To show that the fluorescence labeling was specific for GPI-anchored forms of the proteins in question, phosphatidylinositol-specific phospholipase (PI-PLC; from *Bacillus thuringiensis*, a gift from Dr. Martin Low) digestion (10 U/ml, 2 h at 23°C or 0°C) was performed on cells stained with fluorescently labeled antibodies (1 h at 23°C or 0°C, respectively). Both at room temperature and on ice, PI-PLC released all the detectable fluorescence from the cells, confirming that the fluorescence was due to staining of GPI-anchored proteins in each case.

The pattern of staining of caveolin/VIP-21 in folate receptor–expressing cells was detected with a polyclonal antibody to caveolin/VIP-21. Cells were treated with or without Triton X-100 as described above and fixed with 3% paraformaldehyde for 10 min at 0°C followed by 20 min at room temperature. The fixed preparations were rinsed in medium 1 and the cells that had not been treated with Triton X-100 were permeabilized with medium 1 containing 100 μg/ml saponin for 10 min at room temperature. All the samples were incubated with medium 1 containing 25 mM NH_4Cl and then with medium 1 containing 10 mg/ml bovine serum albumin (RIA grade). The cells were then incubated with affinity-purified rabbit antibody to caveolin/VIP-21 antibody (5 μg/ml) or with an irrelevant affinity-purified rabbit immunoglobin (rabbit antibody to mouse immunoglobin) at the same concentration. The cells were rinsed, and the primary antibody was visualized with rhodamine-conjugated secondary antibodies. The distribution of the GPI-anchored folate receptor on the same cells was detected with the fluorescent analogue of folate, PLF, added at the same time as the secondary antibody incubation. All the incubations with antibodies were carried out in medium 1 containing 10 mg/ml bovine serum albumin for 45 min at room temperature.

Fluorescence Microscopy

Fluorescence microscopy and digital image collection were performed using a Leitz Diavert fluorescence microscope equipped with a Photometrics (Tuscon, Arizona)-cooled CCD camera and driven by software from Inovison (Durham, NC) on a SPARC station 4/330 computer system (Sun Microsystems, Mountainview, CA) as described previously (Mayor *et al.*, 1993). For output purposes the digital images were transferred to a Macintosh Power PC and printed via Adobe Photoshop software on a dye-sublimation printer (Phaser IIsdx, Tektronix, Wilsonville, Oregon).

Quantitation of fluorescence was made using the Inovison image analysis software package. Total fluorescence per field was obtained by summing the pixel intensity over the whole field after subtracting an integer corresponding to a background value obtained from a field that did not contain any cells. Background fluorescence values including autofluorescence and nonspecific fluorescence were obtained by imaging fields of cells under the same illumination and exposure conditions. Fluorescence quantitation was carried out with a 10 or 25× magnification objective to obtain a large number (30–100) of cells per field, whereas the images for visualization purposes were obtained at a higher magnification (63×, NA 1.4 objective).

Electron Microscopy

The mAb-labeled and fixed preparations were incubated in medium 1 containing 25 mM NH_4Cl for 20 min, and then blocked in medium 1 containing 10% FBS. All incubations on fixed cells were carried out at room temperature. The blocked preparations were then sequentially incubated in polyclonal rabbit anti-mouse antiserum (20 μg/ml specific Ig concentration; ICN Biomedicals, Costa Mesa, CA) followed by 1/30 dilution of 10 nm gold-conjugated goat anti-rabbit antibodies (RPN 421; Amersham Life Science). The cells were rinsed in medium 1 containing 10% FBS (3 × 5 min) between incubations, then in medium 1 without FBS (3 × 1 min), and finally in 0.1 M sodium cacodylate, pH 7.4. The cells were post-fixed in 0.1 M sodium cacodylate, containing 1% glutaraldehyde, 1% tannic acid (Electron Microscopy Sciences) and 3.4 mM $CaCl_2$, at pH 7.4, for 30 min, and rinsed in 0.1 M sodium cacodylate (3 × 1 min). The cells were then osmicated (1% OsO_4, 1.5% $KFe(CN_6)$ in 0.1 M sodium cacodylate, then rinsed (3 × 5 min) in cacodylate buffer and finally water. The preparations were then dehydrated in graded ethanol washes and embedded in epon (EM-bed 812; Electron Microscopy Sciences). The glass cover-slip along with the epon-embedded monolayer was removed from each cover-slip bottom dish and baked in an oven (24 h, 60°C). The glass was dissolved in aqueous HF (50%), and the epon-embedded monolayers were dried in an oven at 60°C and cut lengthwise into thin 5-mm wide strips. Three or four of these strips were re-embedded in blocks such that the flat surface of the strips was arranged parallel to the bottom of the block base. Sections (50- to 60-nm thick) were cut from the these blocks on a RMC MT-7000 ultramicrotome perpendicular to the plane of the cell monolayer. The sections were stained in 4% uranyl acetate for 15 min followed by 0.4% lead citrate for 4 min before observation on a JEOL JEM-1200 EXII electron microscope at 80 KV. All images for the statistical data were taken at a magnification of 20,000× and collected at random.

Biochemical Analyses of Detergent Insolubility of GPI-anchored Folate Receptor

The extent of GPI-anchored folate receptor insolubility was also analyzed by biochemical procedures. Folate receptor–expressing CHO cells were grown in 60-mm dishes and labeled with PLF (5 nM) for 30 min at 0°C. To determine the extent of solubility of the folate receptor in 1% Triton X-100, the cells were extracted with 1 ml of cold 1% Triton X-100 as described above. The Triton extract representing the detergent soluble fraction was removed and kept for fluorescence analyses. Ice-cold 1% Triton X-100 solution (0.5 ml)

was added to the plates and the cells were scraped using a rubber policeman. The plates were rinsed and scraped with an additional 0.5 ml of Triton X-100 solution and the scrapings were pooled and taken for fluorescence analyses. To determine total surface receptors on the cells, plates were scraped directly into 1 ml (2 × 0.5 ml) of the 1% Triton X-100 solution. Folate receptor was quantitated in each of the above fractions by measuring the specific fluorescence (λ^{ex} = 490; λ^{em} = 530) of each of the fractions using an SLM 8000 spectrofluorometer (SLM Instruments, Rochester, NY). Nonspecific fluorescence was determined by either leaving out the fluorescent analogue or including 200 nM folic acid in the labeling reaction; similar values for nonspecific fluorescence were obtained in either case.

To estimate the extent of association of the detergent-insoluble GPI-anchored folate receptor with the "low-density vesicles" (Brown and Rose, 1992; Fiedler *et al.*, 1993; Fra *et al.*, 1994; Lisanti *et al.*, 1994b), PLF-labeled cells (60-mm dishes) were first extracted, scraped, and homogenized in 2 ml (2 × 1 ml) of 1% Triton X-100 in a Tris-saline buffer (0.15 M NaCl, 5 mM EDTA, 20 mM Tris-HCl, pH 7.3) containing protease inhibitors (2 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml pepstatin A). The homogenate (1.75 ml) was mixed with an equal volume of 2.7 M sucrose solution in the Tris-saline buffer and placed at the bottom of an ultracentrifuge tube. 5.5 ml of 30% sucrose followed by 2.5 ml of 5% sucrose were then layered on top of the homogenate and centrifuged at 38,000 rpm in a SW40Ti rotor for 18 h at 4°C in a Beckman L7–55 ultracentrifuge (Beckman Instruments, Palo Alto, CA). The step gradient was fractionated by collecting 1-ml fractions from the top, and the fluorescence in each fraction was quantitated as described above. Nonspecific fluorescence was determined for each fraction of the gradient by measuring the fluorescence of unlabeled cells or cells labeled in the presence of 100-fold excess folic acid. The nonspecific values in either case were indistinguishable from each other, and were substracted from the values obtained from the corresponding fractions containing the PLF-labeled cells.

RESULTS

GPI-anchored proteins have been shown to be resistant to Triton X-100 detergent extraction from cell membranes (Hooper and Turner, 1988; Brown, 1992; Brown and Rose, 1992). To determine the distribution of the proteins in the insoluble membrane fraction after the detergent extraction procedure, we labeled DAF-transfected CHO cells with Cy3-conjugated mAb to DAF (Cy3–1A10) (Figure 1, A and C–E) or labeled folate receptor–transfected 3T3 cells with Cy3-conjugated mAb to the folate receptor (Cy3-MOv19) (Figure 1, B, F, and G). The cells were then treated with Triton X-100 at 0°C for 30 min (Figure 1, C–G) or incubated in buffer without Triton X-100 (Figure 1, A and B). In both the cell lines, the detergent extraction appears to create "holes" in the membrane, and an extremely granular appearance at the cell surface was rarely observed (Figure 1, E and G). In all cases the surface fluorescence was similar in brightness to the untreated controls, suggesting that a large fraction of the cell-surface GPI-anchored protein is insoluble in Triton X-100.

This cell surface redistribution was also observed when folate receptor–transfected CHO cells were examined with a fluorescein-labeled analogue of folic acid (PLF; McAlinden *et al.*, 1991); the uniformly diffuse surface fluorescence was transformed into a more mottled appearance after detergent extraction (Figure 2, A and C). This shows that the detergent-resistant property of the GPI-anchored proteins depicted in Figure 1 is not due to an artefactual situation induced by the binding of mAb to these proteins. To confirm that the detergent extraction procedure used in these studies (and the microscopic observation of the insoluble structures) was qualitatively similar to the procedures used by other investigators (Hooper and Turner, 1988; Brown and Rose, 1992; Sargiacomo *et al.*, 1993), we examined the detergent extractability of a transmembrane protein, the human transferrin receptor, which is also expressed in these cells (McGraw *et al.*, 1987). In the absence of detergent, the transferrin staining is present in mostly a clustered distribution (Figure 2B) due to the receptor being significantly enriched in coated pits at the cell surface at steady state (Jing *et al.*, 1990; McGraw and Maxfield, 1990). However, after detergent treatment all the detectable fluorescence due to Cy3-labeled transferrin binding to the transferrin receptor was extracted from the same cells in which the GPI-anchored folate receptor was almost totally insoluble (Figure 2, C and D).

To provide a comparable situation to the diffusely distributed GPI-anchored proteins, we examined the detergent extractability of a mutant transferrin receptor expressed in CHO cells lacking endogenous transferrin receptor (McGraw *et al.*, 1987). This receptor lacks the coated pit localization signal because it has the amino acids 3 through 59 deleted, but is still anchored in the membrane by a peptide transmembrane anchor and four cytoplasmic amino acids, and is able to bind transferrin with the same affinity as the wild-type receptor (Johnson *et al.*, 1993). This receptor (Δ3–59) is not concentrated in coated pits and is consequently endocytosed at bulk endocytic rates, similar to many GPI-anchored proteins (Widnell *et al.*, 1982; Keller *et al.*, 1991; Johnson *et al.*, 1993). Figure 3A shows that the Δ3–59 receptor has a diffuse distribution at the cell surface in live cells, and after detergent extraction there is no detectable transferrin fluorescence remaining in extracted membranous structures (Figure 3C). The results presented in Figures 1–3 are consistent with the previous observations of several investigators that GPI-anchored proteins are specifically retained in detergent-insoluble membranes whereas most other transmembrane proteins are extensively solubilized (Hooper and Turner, 1988; Hooper and Bashir, 1991; Sargiacomo *et al.*, 1993).

To determine the distribution of caveolae as characterized by caveolin/VIP-21 staining in folate receptor–expressing CHO cells before and after detergent treatment, these cells were stained with a polyclonal antibody to caveolin/VIP-21. Caveolin/VIP-21 staining in CHO cells was punctated without or with de-

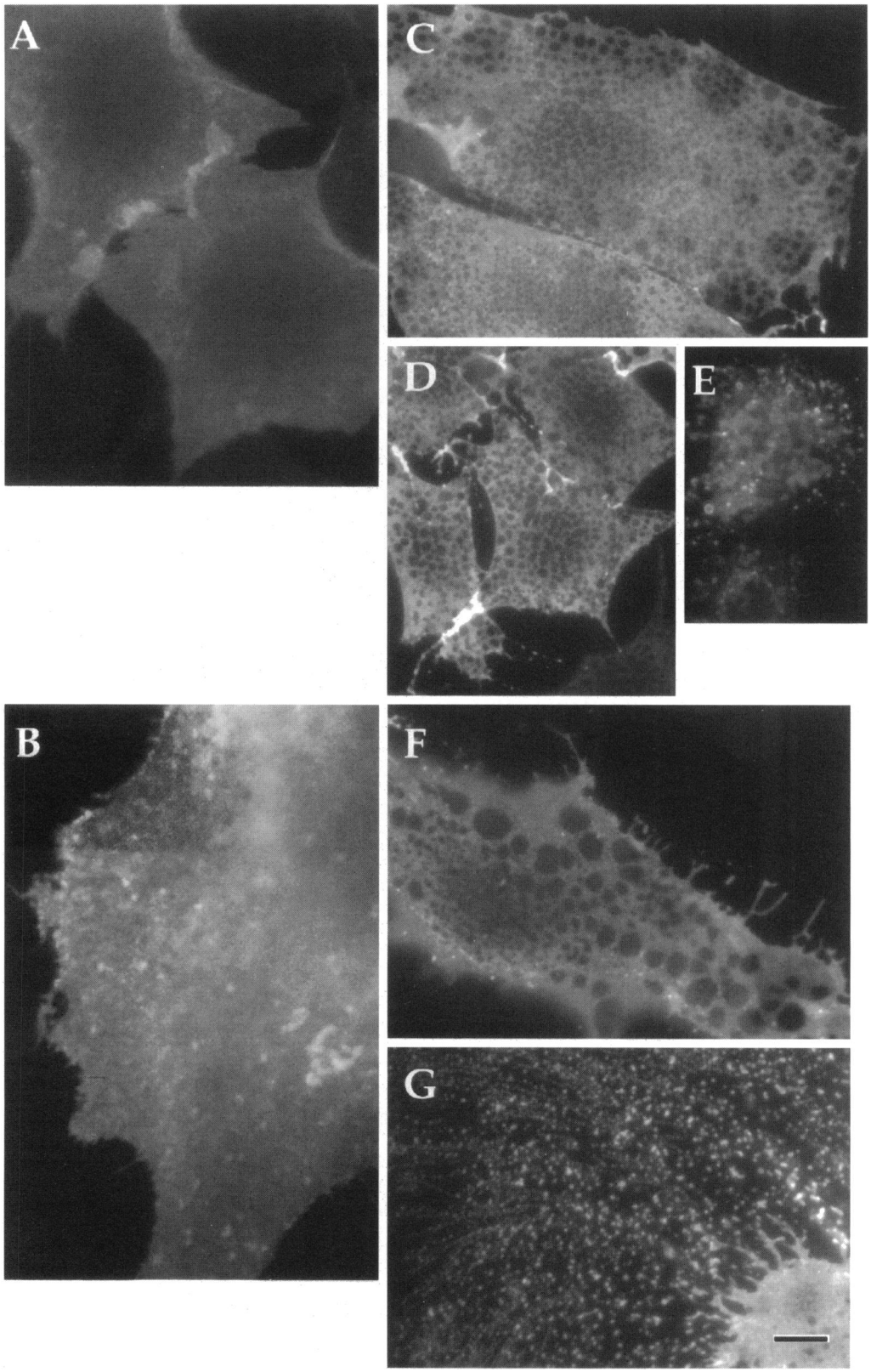

Figure 1. Surface distribution of mAb-labeled GPI-anchored proteins after Triton X-100 extraction. DAF-expressing CHO cells (A and C–E) and folate receptor–expressing 3T3-L1 cells (B, F, and G) were incubated with Cy3-labeled mAbs to DAF (Cy3–1A10) and the folate receptor (Cy3-MOv19), respectively. The cells were then treated with medium 1 (A and B) or medium 1 containing 1% Triton X-100 (C–G) for 20 min at 0°C. The cells were then imaged using an epi-fluorescence microscope equipped with a CCD camera as described in MATERIALS AND METHODS. Bar, 10 μm.

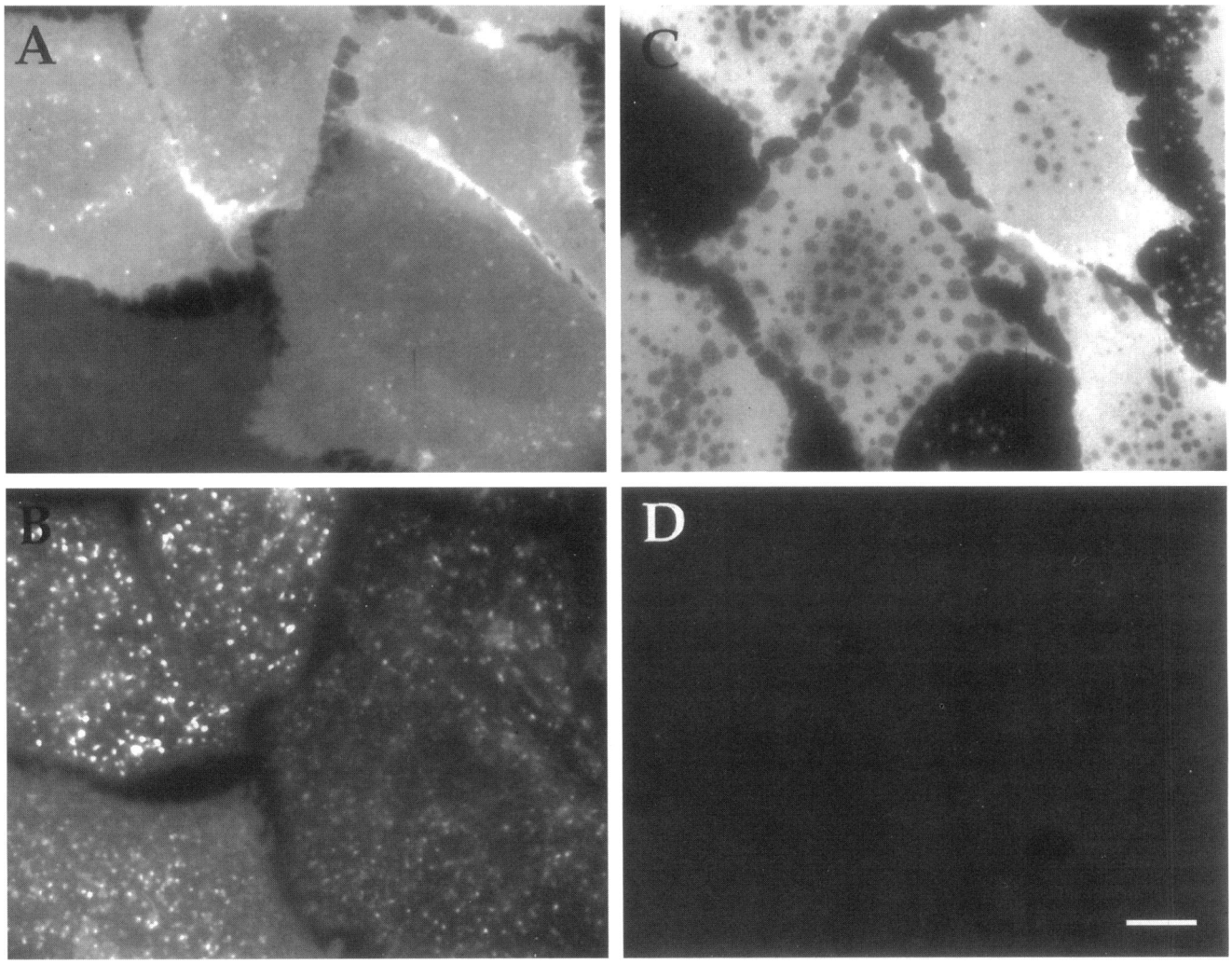

Figure 2. Effect of Triton X-100 extraction on the surface distribution of fluorescent analogues of folate and human transferrin bound to their cognate cell surface receptors. Folate receptor–expressing CHO cells were labeled with a fluorescent folate analogue, PLF (5 nM), (A and C) and Cy3-labeled transferrin (10 μg/ml) (B and D) for 30 min at 0°C, and after rinsing excess label the cells were either incubated in medium 1 at 0°C for 30 min in the absence of (A and B) or presence of 1% Triton X-100 (C and D). The same cells were then imaged for both fluorescein emission (A and C) and Cy3 emission (B and D) using appropriate filters for the two fluorescent species. Bar, 10 μm.

tergent treatment (Figure 4B, D) and resembled the staining pattern seen in several fibroblastic cell lines as described previously (Rothberg *et al.*, 1992; Dupree *et al.*, 1993). Similar to the data shown in Figure 2, the folate receptors at the cell surface in the same cells were not enriched in any punctate structure or distribution. Specifically, regions containing caveolin/VIP-21 were not detectably enriched in folate receptors.

Quantitative analyses of the extent of insolubility of the folate receptor at the cell surface by two means showed that almost all of the surface PLF fluorescence was resistant to Triton X-100 extraction. As shown in Figure 5A, the fluorescence intensity directly measured by digital image analysis was not significantly reduced by Triton X-100 treatment. Furthermore, essentially all of the PLF-binding activity was in the detergent-insoluble pellet when cells were extracted with Triton X-100 before being scraped (Figure 5B). This is similar to previous analyses in which the data show that greater than 80% of the GPI-anchored proteins are resistant to Triton X-100 extraction (Hooper and Turner, 1988; Sargiacomo *et al.*, 1993). Furthermore, as shown in Figure 5C, most (>70%) of the detergent-insoluble GPI-anchored proteins fractionated at the 5%/30% sucrose interface similar to the low-density complexes characterized by several investigators (Brown and Rose, 1992; Fiedler *et al.*, 1993; Fra *et al.*, 1994; Lisanti *et al.*, 1994b).

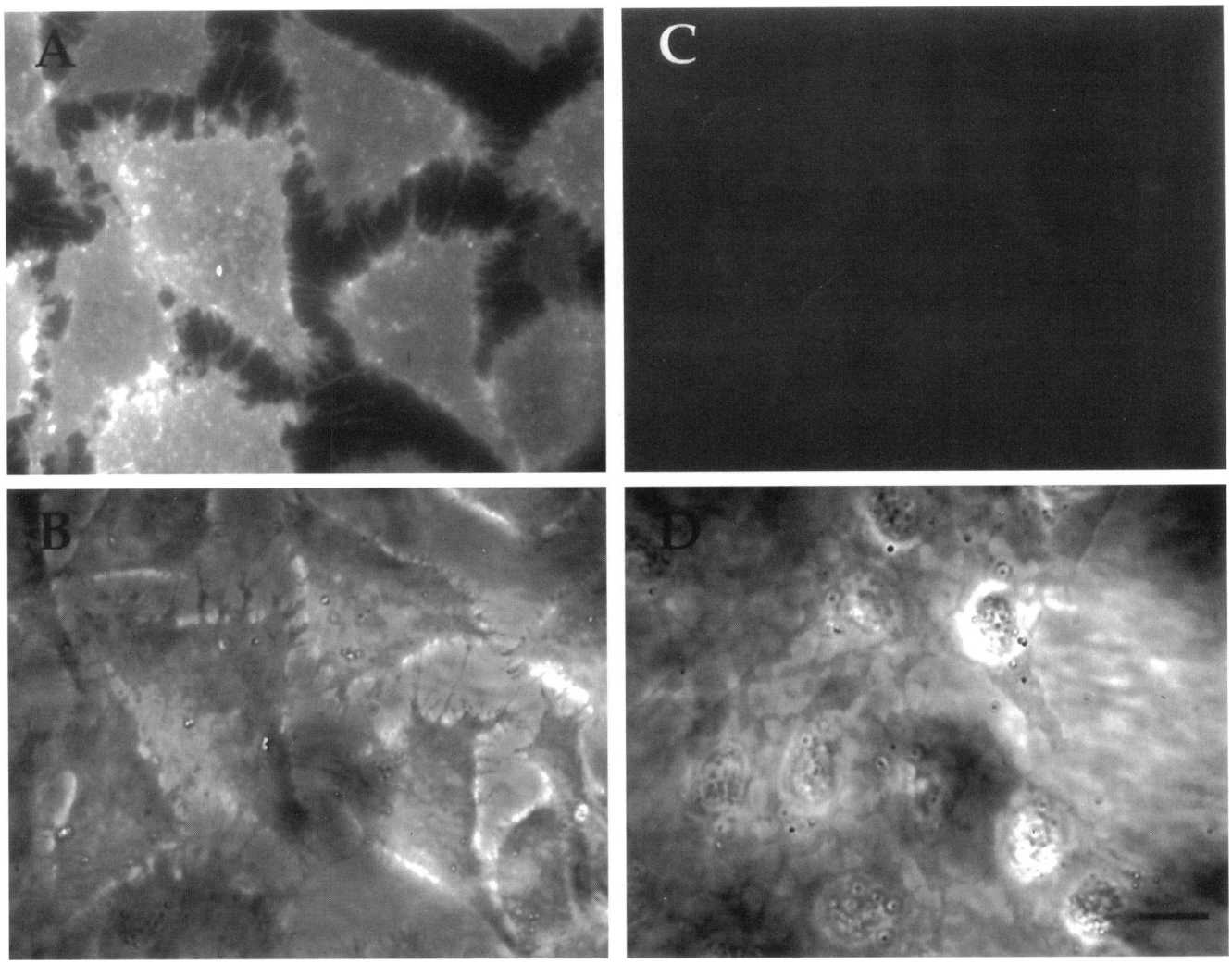

Figure 3. Detergent extractability of a diffusely distributed transmembrane protein from the plasma membrane of CHO cells. CHO cells expressing a mutant human transferrin receptor that lacks the coated pit localization domain, Δ3–59, (Johnson *et al.*, 1993) were incubated with Cy3-transferrin (10 μg/ml) and treated without (A and B) or with (C and D) Triton X-100 as described in Figure 2. Fluorescence (A and C) and phase images (B and D) were collected from the same cells. Bar, 10 μm.

Electron microscopic analyses of the surface distribution of the folate receptor on MA104 cells and DAF on CHO cells before and after Triton X-100 treatment are shown in Figure 6. The mAb-labeled GPI-anchored proteins were fixed under conditions that do not permit a redistribution of the GPI-anchored proteins during the immuno-gold (10 nm) labeling procedure (Mayor *et al.*, 1994). In the untreated condition, the GPI-anchored proteins appear to be randomly distributed at the cell surface, mainly in singlets. However, after the detergent treatment, there is an increased clustering of the GPI-anchored protein at the cell surface (compare images in Figure 6, A and C with Figure 6, B and D). To provide a quantitative estimate of the extent of clustering induced by Triton X-100 treatment, a large number of EM micrographs were randomly selected and examined, and a distribution of cluster sizes of gold particles was determined for each condition (Figure 7 and Table 1).

Quantitative analyses of the distribution of DAF on CHO cells show that similar to our previous data describing folate receptors on MA104 cells (Mayor *et al.*, 1994), GPI-anchored DAF is randomly distributed at the cell surface; there is neither an enrichment nor depletion in either caveolae or clathrin-coated pits (Table 1). In the presence of Triton X-100 it is not possible to morphologically distinguish caveolae or clathrin-coated pits in the remaining membranous structure. However, there is a moderate increase in the surface density of gold particles of the folate receptor from 2.14 particles/μm to 4.61 particles/μm in MA104 cells and of DAF from 5.32 particles/μm to 6.34 par-

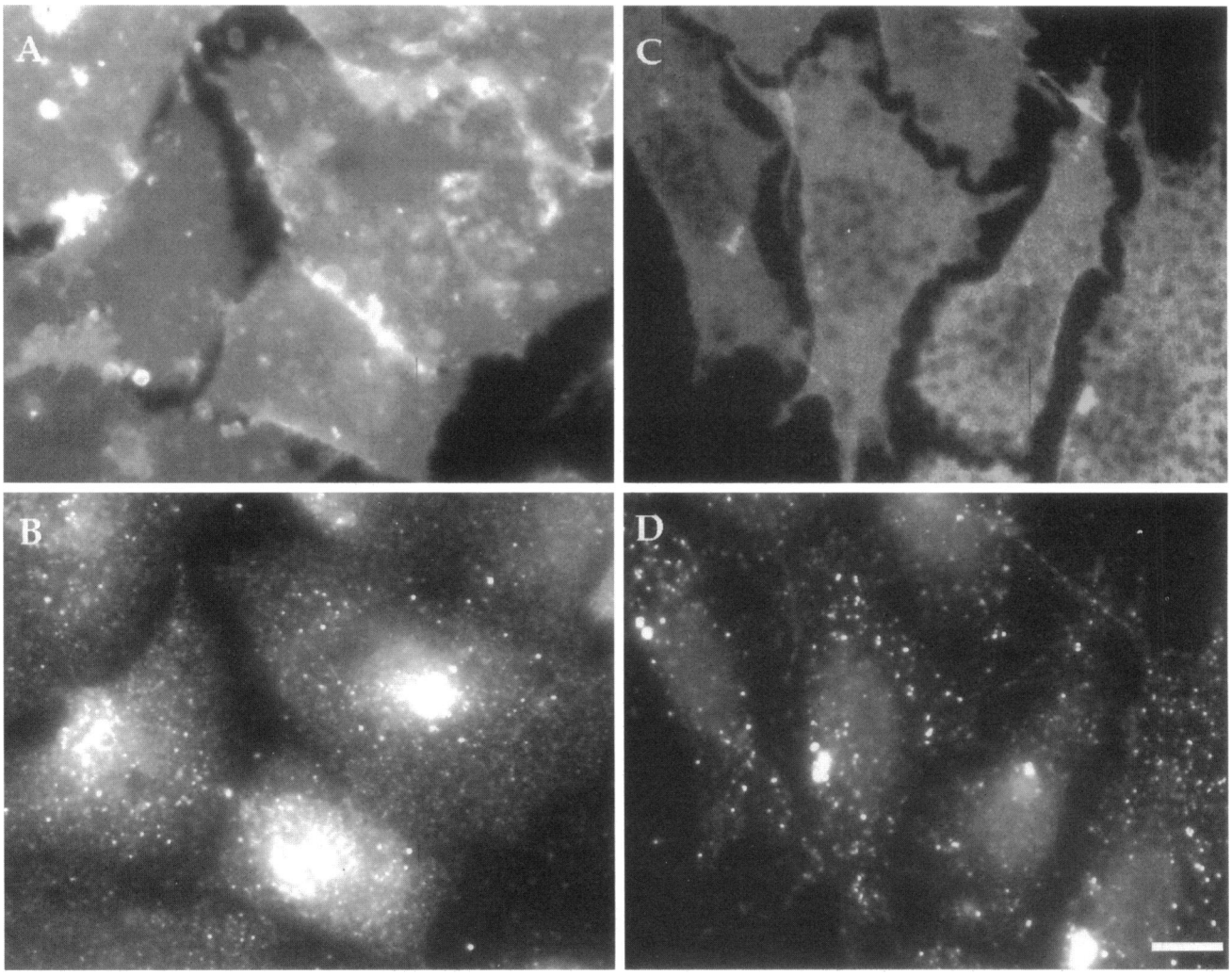

Figure 4. Effect of Triton X-100 extraction on the distribution of a fluorescent analogue of folate and caveolin. Folate receptor–expressing CHO cells were either incubated in medium 1 at 0°C for 30 min in the absence of (A and B) or presence of 1% Triton X-100 (C and D) and fixed. The cells were then stained for folate receptor (A and C) or caveolin (B and D) as described in MATERIALS AND METHODS and then imaged for both fluorescein emission (A and C) and rhodamine emission (B and D) using appropriate filters for the two fluorescent species. Bar, 10 μm.

ticles/μm in DAF-transfected CHO cells (Table 1). This is probably due to the creation of the holes that are observed in the detergent-treated cell membranes (Figures 1, 2, and 4) leading to a reduction in overall membrane surface area.

Using a definition of clustering consistent with previous analyses of the surface distribution of GPI-anchored proteins (greater than three gold particles; Rothberg *et al.*, 1990; Mayor *et al.*, 1994), the data show that after Triton X-100 treatment there is a 2.6-fold increase in clustering of DAF on CHO cells and an 8.6-fold increase in clustering of the folate receptors on MA104 cells (Table 1). Although these increases are significant, a much larger fraction of the GPI-anchored proteins were clustered after antibody-mediated cross-linking ($\geq$ 92%; Mayor *et al.*, 1994). These antibody-induced clusters were also much larger in size (Figure 8), and after antibody cross-linking the GPI-anchored proteins were specifically enriched in caveolae but not in coated pits (Mayor *et al.*, 1994).

DISCUSSION

Using fluorescently labeled mAbs to several GPI-anchored proteins, we have previously shown that they are diffusely distributed at the cell surface (Mayor *et al.*, 1994). Electron microscopic examination of the distribution of several GPI-anchored proteins, folate receptor on MA104 cells (Mayor *et al.*, 1994), DAF on CHO cells (this report), and alkaline phosphatase on

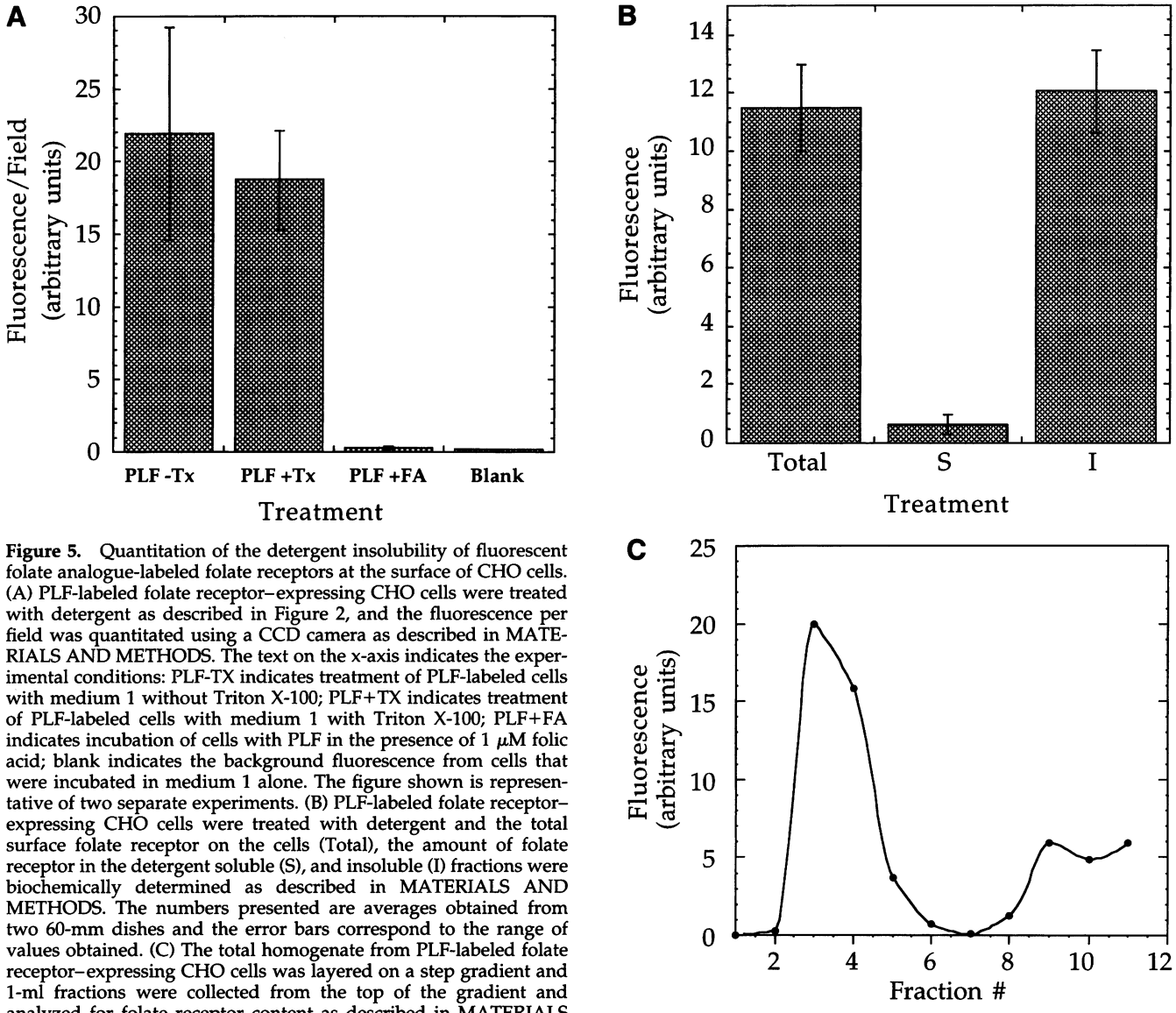

Figure 5. Quantitation of the detergent insolubility of fluorescent folate analogue-labeled folate receptors at the surface of CHO cells. (A) PLF-labeled folate receptor–expressing CHO cells were treated with detergent as described in Figure 2, and the fluorescence per field was quantitated using a CCD camera as described in MATERIALS AND METHODS. The text on the x-axis indicates the experimental conditions: PLF-TX indicates treatment of PLF-labeled cells with medium 1 without Triton X-100; PLF+TX indicates treatment of PLF-labeled cells with medium 1 with Triton X-100; PLF+FA indicates incubation of cells with PLF in the presence of 1 μM folic acid; blank indicates the background fluorescence from cells that were incubated in medium 1 alone. The figure shown is representative of two separate experiments. (B) PLF-labeled folate receptor–expressing CHO cells were treated with detergent and the total surface folate receptor on the cells (Total), the amount of folate receptor in the detergent soluble (S), and insoluble (I) fractions were biochemically determined as described in MATERIALS AND METHODS. The numbers presented are averages obtained from two 60-mm dishes and the error bars correspond to the range of values obtained. (C) The total homogenate from PLF-labeled folate receptor–expressing CHO cells was layered on a step gradient and 1-ml fractions were collected from the top of the gradient and analyzed for folate receptor content as described in MATERIALS AND METHODS. The 5%/30% sucrose interface was collected in fraction 3 and the 30%/40% interface was collected in fraction 9. ≥85% of the total fluorescence present in the homogenate was recovered in all the fractions. The data shown are the averages obtained from two 60-mm dishes and the error bars correspond to the range of values obtained.

A431 cells (Parton *et al.*, 1994), confirmed that these proteins are neither enriched in caveolae nor excluded from coated pits. The data presented here demonstrate that the diffuse nature of GPI-anchored proteins at the cell surface is not an artefactual consequence of the binding of mAbs to these proteins: a fluorescent analogue of folate, PLF, bound to the GPI-anchored human folate receptor at the cell surface also showed a diffuse distribution similar to that obtained with fluorescently labeled mAbs.

The data also show that GPI-anchored proteins at the cell surface are almost completely insoluble in Triton X-100 and become incorporated into low density complexes. Under these conditions transmembrane proteins such as the transferrin receptor and most other polypeptide-based membrane-anchored proteins are readily solubilized (Hooper and Turner, 1988; Hooper and Bashir, 1991; Sargiacomo *et al.*, 1993; this report).

The data presented here are contrary to the widespread interpretation that the low density Triton X-100 insoluble membranes represent a purified caveolae preparation (or a caveolin/VIP-21-rich preparation: 50- to 100-fold enriched) thereby accounting for the 100- to 200-fold enrichment of the GPI-anchored proteins contained in them (Anderson, 1993; Lisanti *et al.*,

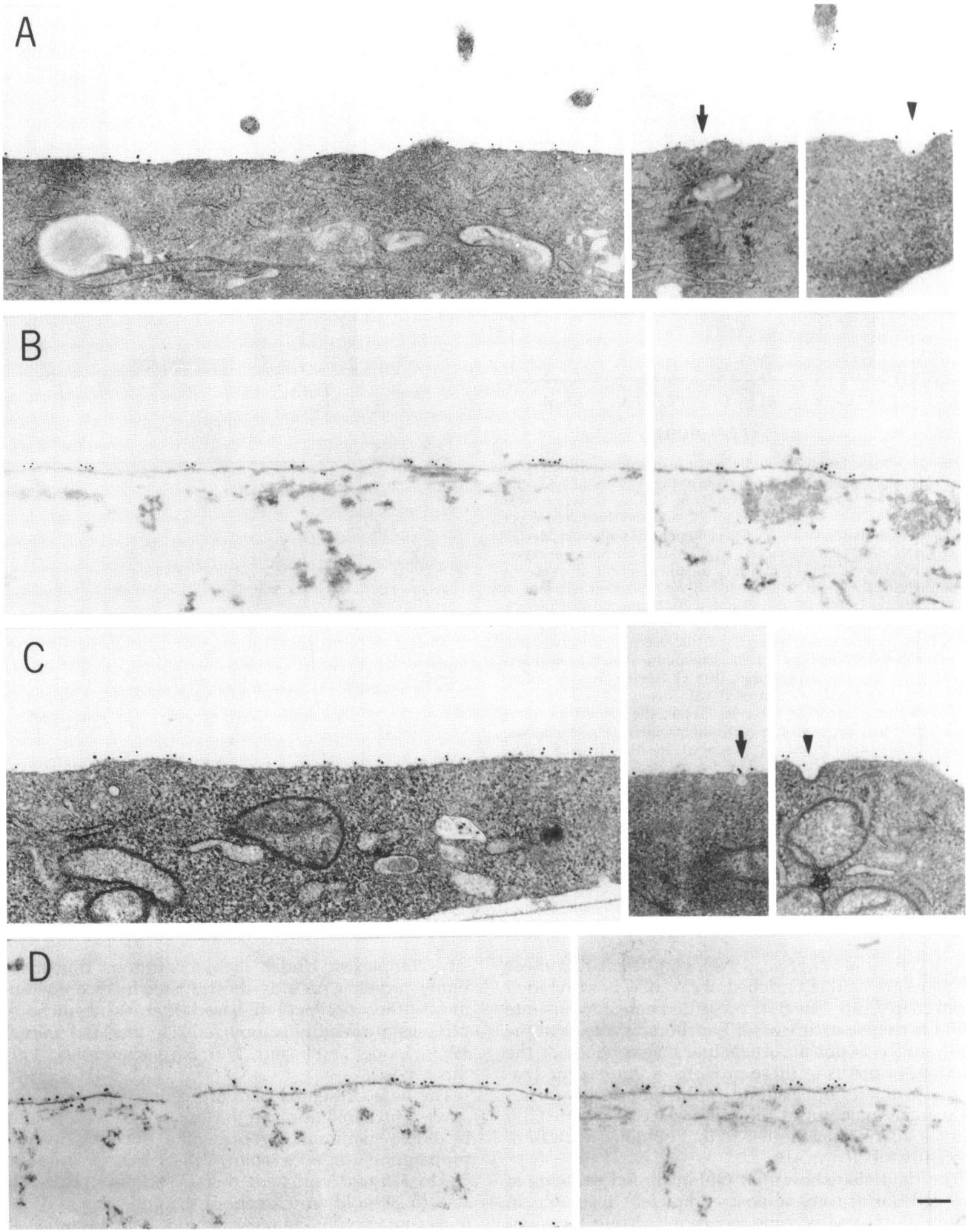

Figure 6.

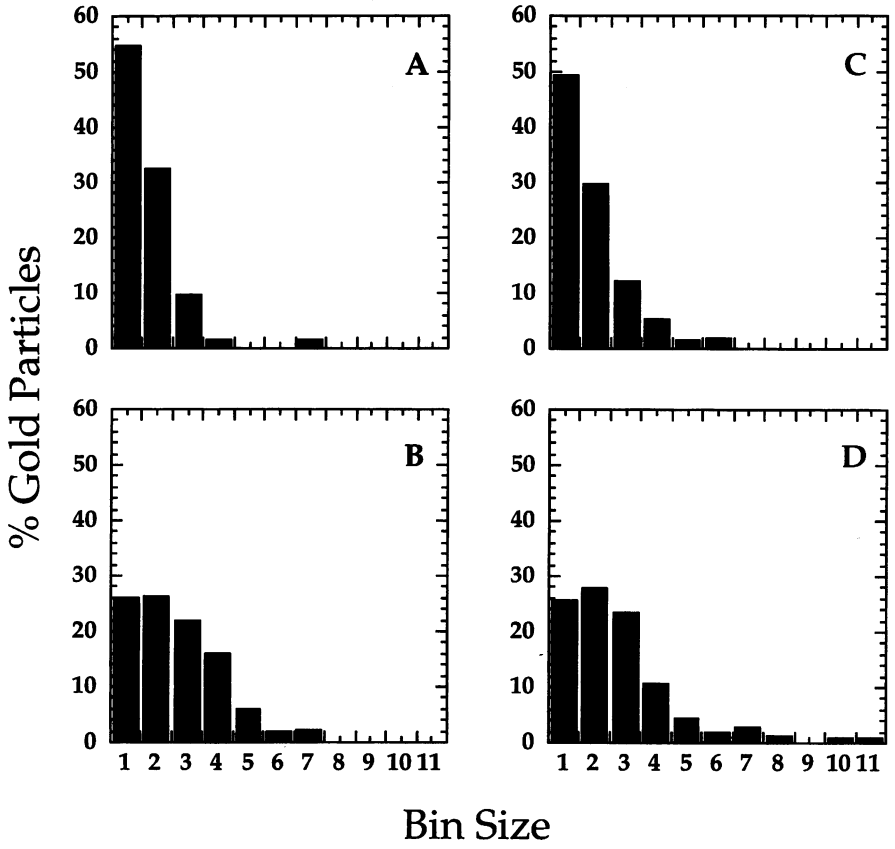

Figure 7. Analyses of the clustering of GPI-anchored proteins at the cell surface after Triton X-100 treatment. Folate receptor–expressing MA104 cells (A and B) and DAF-expressing CHO cells (C and D) were treated as described in Figure 6 and the distribution of gold particles on the cell surface was quantitated in each case as described in Table 1. Bin size refers to the number of gold particles per cluster, and % gold particles refers to the percentage of gold particles found in a particular size cluster with respect to the total number at the cell surface.

1993; Sargiacomo *et al.*, 1993; Chang *et al.*, 1994). The almost complete insolubility of the GPI-anchored proteins along with their lack of enrichment in the relatively small punctate areas occupied by caveolae after detergent treatment shows that these proteins are detergent insoluble, independent of their association with caveolae. The lack of association with caveolin/VIP-21 is consistent with recent studies in lymphocytes in which GPI-anchored Thy-1 and the ganglioside GM1 were detergent insoluble and almost quantitatively formed low-density complexes even though the cells lack detectable caveolin/VIP-21 or caveolae (Fra *et al.*, 1994). As suggested by Fra *et al.* (1994) this could be due to the formation of specific detergent-insoluble glycolipid domains. Alternatively, these proteins may be intrinsically detergent-insoluble in the milieu of the plasma membrane (see below).

We have previously shown that cross-linking diffusely distributed GPI-anchored proteins specifically enriched these proteins in caveolae (see also Parton *et al.*, 1994). However, multiple GPI-anchored proteins on the same cell did not co-cluster or get recruited to caveolae unless they were independently cross-linked, indicating that the GPI-anchored proteins are not associated with each other at the cell surface (Mayor *et al.*, 1994). Similar observations have been made in lymphocytes in which Thy-1 and GM-1 did not co-cluster after cross-linking with multivalent agents (Fra *et al.*, 1994). This is in contradiction with the detection and significant enrichment of multiple GPI-anchored proteins and glycolipids in the detergent-resistant complexes (Lisanti *et al.*, 1993; Sargiacomo *et al.*, 1993; Fra *et al.*, 1994; Lisanti *et al.*, 1994b; Chang *et al.*, 1994), which has been interpreted to suggest that GPI-anchored proteins are associated with each other (or a common receptor) in these complexes.

Schroeder *et al.* (1994) have recently shown that the insolubility of GPI-anchored proteins and membrane

Figure 6 cont. Electron microscopic analyses of GPI-anchored proteins after detergent extraction. Folate receptor–expressing MA104 cells (A and B) and DAF-expressing CHO cells (C and D) were incubated with mAb to the folate receptor (MOv19), and DAF (1A10), respectively. The cells were then treated with medium 1 (A and C) or medium 1 containing 1% Triton X-100 (B and D) for 20 min at 0°C, fixed with 3% paraformaldehyde and 0.5% glutaraldehyde, and processed for electron microscopy as described in MATERIALS AND METHODS. Small arrows represent caveolae, whereas arrowheads represent coated pits. Bar, 200 nm.

Table 1. Quantitative analyses of the distribution of folate receptors on MA104 cells and DAF on CHO cells

Cells (condition)[a]	Membrane length (μm)	Number of		Number of gold particles[b]	% gold particles in clusters[c]	% gold particles associated[d] with	
		caveolae	coated pits			caveolae (relative conc.)[e]	coated pits (relative conc.)[e]
MA104 (−TX-100)	228	37	40	488	3	4.7 (1.9)	5.4 (1.2)
MA104 (+TX-100)	197	—	—	910	26	—	—
DAF-CHO (−TX-100)	174	41	28	926	9	3.6 (1.0)	5.6 (1.4)
DAF-CHO (+TX-100)	195	—	—	1237	23	—	—

Cells were processed for electron microscopy as described in Fig. 6. 50–60 nm sections were used for quantitative analyses. Analysis of negatives (20,000 × magnification) was carried out using a 10 × ocular lens. More than 50 negatives of randomly selected fields were analyzed for each condition in each experiment.

[a] After labelling with primary monoclonal antibodies, the cells were treated in the presence (+TX-100) or absence of detergent (−TX-100) and processed for electron microscopy as described in Figure 6 and MATERIALS AND METHODS.

[b] Nonspecific binding of gold particles to the cells was determined for the two conditions using an irrelevant IgG as the primary antibody. <0.02 gold particles per μm were detected in all cases.

[c] A cluster was defined to contain more than three gold particles separated by less than 50 nm from the nearest neighbor.

[d] Gold particles (or clusters) less than 50 nm from the edge of caveolae or coated pits were considered to be associated with them.

[e] Relative concentration is the ratio of the observed gold particle density in caveolae or coated pits to the average gold particle density over all membrane. The average membrane length associated with caveolae and coated pits was 0.15 μm and 0.25 μm, respectively.

lipids in Triton X-100 can be reconstituted in the absence of any special structures or protein(s). The primary requirement for detergent insolubility is the presence of a significant mole fraction of high-melting temperature lipids such as saturated acyl chain-containing phospholipids as well as an optimal concentration of cholesterol or neutral glycolipids. Furthermore, detergent insolubility of GPI-anchored proteins requires the presence of the appropriate lipid milieu in the same bilayer as these proteins (D. Brown, personal communication; Schroeder *et al.*, 1994). Thus, the insolubility of GPI-anchored proteins in Triton X-100 depends mainly on the acyl or alkyl chain composition of the membrane lipids, cholesterol or neutral glycolipid content, and probably the degree of saturation of the acyl or alkyl moiety of the GPI-anchor. Saturated alkyl/acyl chains appear to be the predominant components of the lipid portion of GPI-anchors (McConville and Ferguson, 1993).

Fluidity measurements of the detergent-resistant fraction from plasma membrane and from reconstituted membrane preparations showed that the detergent-resistant membranes were considerably less fluid than the untreated membrane preparations (Schroeder *et al.*, 1994). As discussed by Brown and colleagues (Brown, 1992; Schroeder *et al.*, 1994), these results suggest the potential for membrane domains with more and less fluid regions in the untreated membrane. However, the a priori existence of membrane domains enriched in high-melting temperature lipids has only been shown in artificial membranes (e.g. at relatively high mole fractions of neutral glycolipid in phospholipid bilayers; reviewed in Thompson and Tillack, 1985). In a separate study using a multivalent ligand, cholera toxin binding subunit-colloidal gold complexes, Parton (1994) has shown that the ganglioside GM1 is enriched in caveolae in freeze-substituted preparations of A431 cells and is further enriched upon warming up at 37°C in the presence of cholera toxin.

The detergent extraction of membranes is a very complex process and the mechanisms of lipid redistribution and insolubility are poorly understood (Helenius and Simons, 1975; Lichtenberg *et al.*, 1983). The selective solubilization of various lipid classes in Triton X-100 have been documented extensively (Yu *et al.*, 1973; Helenius and Simons, 1975; Streuli *et al.*, 1981; Brown and Rose, 1992; Fiedler *et al.*, 1993). The data show that the insoluble residue consisting mainly of glycolipids, sphingolipids, cholesterol, and some glycerophospholipids could represent at least 50% of the total plasma membrane surface area if the detergent-insoluble lipids are mainly derived from the plasma membrane as in the case of red blood cell ghosts (Yu *et*

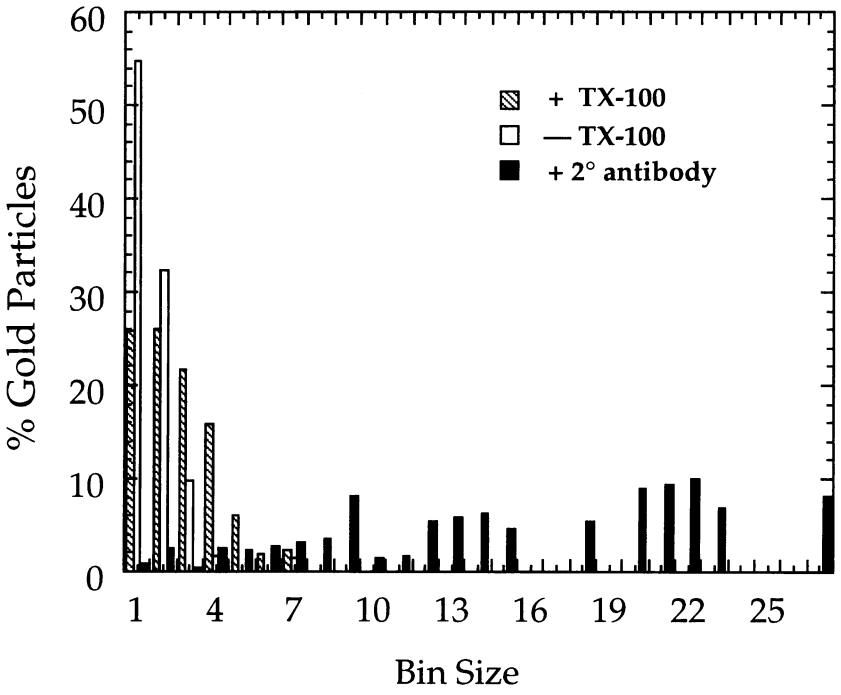

Figure 8. Comparison of the clustering of GPI-anchored proteins at the cell surface after Triton X-100 treatment and antibody-mediated cross-linking. Cluster size distribution of gold particles obtained after Triton X-100 treatment (hatched bars), secondary antibody-mediated cross-linking (solid bars) (as described in Mayor *et al.*, 1994), or no treatment (open bars) of MOv19-labeled folate receptors at the MA104 cell surface as described in Figure 6.

al., 1973). This fraction may be higher than 50% considering the asymmetrical distribution of the insoluble lipids in the membrane bilayer (reviewed in Dawidowicz, 1987; Devaux, 1991). It is possible that detergent extraction of the plasma membrane bilayer could result in the coalescence of micro-domains, or even create these less fluid regions de novo by the differential extraction of lipid classes found in natural membranes (Yu *et al.*, 1973). Our data show that before detergent extraction a very small fraction (3–9%) of the cell surface GPI-anchored proteins is present in clusters of more than three gold particles whereas after detergent extraction a much larger fraction of the total surface GPI-anchored proteins (22–26%) is clustered. These observations suggest that membrane domains enriched in GPI-anchored proteins do not exist before detergent extraction, or if they do, they contain a relatively low concentration of GPI-anchored proteins with respect to the rest of the lipid milieu. It is possible that the detergent extraction–induced redistribution serves to concentrate the GPI-anchored proteins in the plane of the membrane leading to an association with other detergent insoluble components (Glenney and Zokas, 1989; Kurzchalia *et al.*, 1992). However, there appears to be no detectable enrichment of the GPI-anchored proteins in the punctate caveolin-staining areas at the level of light microscopy in the detergent-insoluble membranes.

Many cytoplasmically oriented proteins such as PTKs and heterotrimeric G proteins have been found in detergent insoluble complexes (Stefanova *et al.*, 1991; Cinek and Horejsi, 1992; Bohuslav *et al.*, 1993; Lisanti *et al.*, 1993, 1994b; Chang *et al.*, 1994). As pointed out by Lisanti and co-workers, these proteins share a common lipid modification motif, Met-Gly-Cys, which is the site for N-myristylation (on the N-terminal Gly after cleavage of Met) and palmitylation (on the Cys) at the N-terminus and may direct association with the detergent-insoluble complexes (Lisanti *et al.*, 1994a). In confirmation of this hypothesis, the association of two PTKs, $p56^{lck}$ and $p59^{fyn}$, with the detergent-insoluble complexes was found to be due to the N-terminal myristylation at the Gly residue and palmitylation at the Cys 3 residue (Shenoy *et al.*, 1994). Furthermore, another PTK, $p60^{src}$, is N-myristylated but is not palmitylated (because it has the sequence Met-Gly-Ser at the N-terminus) and was found not to be associated with these detergent-insoluble complexes (Shenoy *et al.*, 1994). However, a mutant version of $p60^{src}$ that contains the Met-Gly-Cys motif at its N-terminus is N-myristylated at the Gly residue and palmitylated at Cys, and was found to be associated with the detergent-resistant complex. These data are consistent with the idea that association with the detergent-insoluble membranes requires closely juxtaposed saturated fatty acyl chains and the presence in the same bilayer of the various saturated acyl/alkyl chain–containing proteins and lipids (Yu *et al.*, 1973; Schroeder *et al.*, 1994).

These cytoplasmic lipid-modified proteins share the property of detergent insolubility with GPI-anchored

proteins and association with the low-density complexes. However, we have shown that in the case of the GPI-anchored proteins, this does not reflect an initial concentration in any specialized structures. Rather, it is a measure of their distribution into detergent-resistant structures after detergent treatment. It is possible that a similar redistribution occurs with these cytoplasmic proteins. These observations indicate that there is little evidence for the existence of complexes of GPI-anchored proteins and PTKs at the cell surface before detergent extraction.

It is possible that complexes similar to those formed after detergent extraction may be formed in cells as a regulated event and require physiological triggers. Antibody-mediated cross-linking of GPI-anchored proteins, although not entirely physiological, is a means for activating signalling functions of GPI-anchored proteins (reviewed in Robinson, 1991; Brown, 1993; Ferguson, 1994). The aggregated state of these lipid-linked proteins could generate a relatively stable membrane domain where signalling proteins such as N-terminal–myristylated and Cys-3–palmitylated PTKs could be recruited and consequently activated. It would be interesting to determine if such complexes may be formed in the membrane of caveolae because, after cross-linking, GPI-anchored proteins are relatively enriched in these structures in cells that have caveolae (Mayor *et al.*, 1994; Parton *et al.*, 1994). Further studies will be required to distinguish whether cross-linked GPI-anchored proteins could cause the formation of membrane domains that assemble signalling molecules or if GPI-anchored proteins and cytoplasmic signalling molecules exist in complexes in micro-domains that are unresolvable at the resolution of current localization techniques. Most importantly, our data indicate that GPI-anchored proteins are redistributed during detergent extraction to a more clustered distribution and suggest that any interpretation of the function of these proteins or their association with caveolae, and PTKs, based on detergent extraction, should be viewed with caution.

ACKNOWLEDGMENTS

This work was supported by a grant from the Helen Hay Whitney Foundation (S.M.) and National Institutes of Health grant DK-27083 (F.R.M.). We thank Dr. M. Low (Columbia University) for a generous gift of *B. thuringienses* PI-PLC; Drs. R.G.W. Anderson (University of Texas Southwestern Medical Center) and M. Davitz (New York University) for generously providing mAbs MOv19 and 1A10, respectively; Dr. M. Davitz for the transfection of DAF into TRVb-1 cells; and Dr. M. Ratnam (Medical College of Ohio, Toledo, OH) for providing the cDNA for the human folate receptor. We also thank Dr. Tim McGraw, Jules Park, and Amy Johnson (Columbia University) for helping with the cloning of the folate receptor–expressing TRVb-1 cells, Dr. Tim McGraw for his critical comments and suggestions on the manuscript, and Dr. Deborah Brown (State University of New York, Stony Brook, NY) for communicating results before publication. Finally, we thank Kristy Brown (Columbia University) for her invaluable assistance with electron microscopy. S.M. thanks F.F. Bosphorus, H.I. Bagel, and L.M. Silko for inspiration.

REFERENCES

Anderson, R.G. (1993). Caveolae: where incoming and outgoing messengers meet. Proc. Natl. Acad. Sci. USA *90*, 10909–10913.

Bohuslav, J., Cinek, T., and Horejsi, V. (1993). Large, detergent-resistant complexes containing murine antigens Thy-1 and Ly-6 and protein tyrosine kinase p56lck. Eur. J. Immunol. *23*, 825–831.

Brown, D. (1992). Interactions between GPI-anchored proteins and membrane lipids. Trends Cell Biol. *2*, 338–343.

Brown, D. (1993). The tyrosine kinase connection: how GPI-anchored proteins activate T cells. Curr. Opin. Immunol. *5*, 349–354.

Brown, D.A., and Rose, J.K. (1992). Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell *68*, 533–544.

Caras, I.W., Davitz, M.A., Rhee, L., Weddell, G., Martin, D.W., Jr., and Nussenzweig, V. (1987). Cloning of decay-accelerating factor suggests novel use of splicing to generate two proteins. Nature *325*, 545–550.

Chang, W.J., Ying, Y.S., Rothberg, K.G., Hooper, N.M., Turner, A.J., Gambliel, H.A., De, G.J., Mumby, S.M., Gilman, A.G., and Anderson, R.G. (1994). Purification and characterization of smooth muscle cell caveolae. J. Cell Biol. *126*, 127–138.

Cinek, T., and Horejsi, V. (1992). The nature of large noncovalent complexes containing glycosyl-phosphatidylinositol-anchored membrane glycoproteins and protein tyrosine kinases. J. Immunol. *149*, 2262–2270.

Coney, L.R., Tomassetti, A., Carayannopoulos, L., Frasca, V., Kamen, B.A., Colnaghi, M.I., and Zurawski, V.J. (1991). Cloning of a tumor-associated antigen: MOv18 and MOv19 antibodies recognize a folate-binding protein. Cancer Res. *51*, 6125–6132.

Cross, G.A.M. (1990). Glycolipid anchoring of plasma membrane proteins. Annu. Rev. Cell Biol. *6*, 1–34.

Davitz, M.A., Low, M.G., and Nussenzweig, V. (1986). Release of decay-accelerating factor (DAF) from the cell membrane by phosphatidylinositol-specific phospholipase C (PIPLC): selective modification of a complement regulatory protein. J. Exp. Med. *163*, 1150–1161.

Dawidowicz, E.A. (1987). Dynamics of membrane lipid metabolism and turnover. Annu. Rev. Biochem. *56*, 43–61.

Devaux, P.F. (1991). Static and dynamic lipid asymmetry in cell membranes. Biochemistry *30*, 1163–1172.

Dupree, P., Parton, R.G., Raposo, G., Kurzchalia, T.V., and Simons, K. (1993). Caveolae and sorting in the trans-Golgi network of epithelial cells. EMBO J. *12*, 1597–1605.

Englund, P.T. (1993). The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. Annu. Rev. Biochem. *62*, 121–138.

Ferguson, M.A.J. (1994). What can GPI do for you? Parasitol. Today *10*, 48–52.

Ferguson, M.A.J., and Williams, A.F. (1988). Cell-surface anchoring of proteins via glycosyl phosphatidylinositol structures. Annu. Rev. Biochem. *57*, 285–320.

Fiedler, K., Kobayashi, T., Kurzchalia, T.V., and Simons, K. (1993). Glycosphingolipid-enriched, detergent-insoluble complexes in protein sorting in epithelial cells. Biochemistry *32*, 6365–6373.

Field, M.C., and Menon, A.K. (1992). Glycolipid-anchoring of cell surface proteins. In: Lipid Modification of Proteins. ed. M.J. Schlesinger, Boca Raton, FL: CRC Press, 83–134.

Fra, A.M., Williamson, E., Simons, K., and Parton, R.G. (1994). Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. J. Biol. Chem. *269*, 30745–30748.

Glenney, J.R., and Zokas, L. (1989). Novel tyrosine kinase substrates from rous sarcoma virus-transformed cells are present in the membrane skeleton. J. Cell Biol. *108*, 2401–2408.

Helenius, A., and Simons, K. (1975). Solubilization of membranes by detergents. Biochem. Biophys. Acta. *415*, 29–79.

Hooper, N.M., and Bashir, A. (1991). Glycosyl-phosphatidylinositol–anchored membrane proteins can be distinguished from transmembrane polypeptide-anchored proteins by differential solubilization and temperature-induced phase separation in Triton X–114. Biochem J. *280*, 745–751.

Hooper, N.M., and Turner, A.J. (1988). Ectoenzymes of the kidney microvillar membrane: differential solubilization by detergents can predict a glycosyl-phosphatidylinositol membrane anchor. Biochem. J. *250*, 865–869.

Jing, S., Spencer, T., Miller, K., Hopkins, C., and Trowbridge, I.S. (1990). Role of the human transferrin receptor cytosplasmic domain in endocytosis: localization of a specific signal sequence for internalization. J. Cell Biol. *110*, 283–294.

Johnson, L.S., Dunn, K.W., Pytowski, B., and McGraw, T.E. (1993). Endosome acidification and receptor trafficking: bafilomycin A1 slows receptor externalization by a mechanism involving the receptor's internalization motif. Mol. Biol. Cell *4*, 1251–1266.

Keller, G.-A., Siegel, M.W., and Caras, I.W. (1991). Endocytosis of glycophospholipid-anchored and transmembrane forms of CD4 by different endocytic pathways. EMBO J. *11*, 863–874.

Kurzchalia, T.V., Dupree, P., Parton, R.G., Kellner, R., Virta, H., Lehnert, M., and Simons, K. (1992). VIP21, a 21-kD membrane protein is an integral component of *trans*-Golgi-network–derived transport vesicles. J. Cell Biol. *118*, 1003–1014.

Lacey, S.W., Sanders, J.M., Rothberg, K.G., Anderson, R.G., and Kamen, B.A. (1989). Complementary DNA for the folate binding protein correctly predicts anchoring to the membrane by glycosylphosphatidylinositol. J. Clin. Invest. *84*, 715–720.

Lichtenberg, D., Robson, R.J., and Dennis, E.A. (1983). Solubilization of phospholipids by detergents: structural and kinetic aspects. Biochem. Biophys. Acta *737*, 285–304.

Lisanti, M.P., and Rodriguez-Boulan, E. (1990). Glycophospholipid membrane anchoring provides clues to the mechanism of protein sorting in polarized epithelial cells. Trends Biochem. Sci. *15*, 113–118.

Lisanti, M.P., Scherer, P.E., Tang, Z.L., and Sargiacomo, M. (1994a). Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. Trends Cell. Biol. *4*, 231–235.

Lisanti, M.P., Scherer, P.E., Vidugiriene, J., Tang, Z., Hermanowski, V.A., Tu, Y.H., Cook, R.F., and Sargiacomo, M. (1994b). Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: implications for human disease. J. Cell Biol. *126*, 111–126.

Lisanti, M.P., Tang, Z.L., and Sargiacomo, M. (1993). Caveolin forms a hetero-oligomeric protein complex that interacts with an apical GPI-linked protein: implications for the biogenesis of caveolae. J. Cell Biol. *123*, 595–604.

Low, M.G. (1989). The glycosyl-phosphatidylinositol anchor of membrane proteins. Biochim. Biophys. Acta. *988*, 427–454.

Mayor, S., and Menon, A.K. (1990). Structural analysis of the glycosylinositol phospholipid anchors of membrane proteins. Methods: A Companion to Methods in Enzymol. *1*, 297–305.

Mayor, S., Presley, J.P., and Maxfield, F.R. (1993). Sorting of membrane components from endosomes and subsequent recycling to the cell surface occurs by a bulk flow process. J. Cell Biol. *121*, 1257–1269.

Mayor, S., Rothberg, K.G., and Maxfield, F.R. (1994). Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. Science *264*, 1948–1951.

McAlinden, T.P., Hynes, J.B., Patil, S.A., Westerhof, G.R., Jansen, G., Schornagel, J.H., Kerwar, S.S., and Freisheim, J.H. (1991). Synthesis and biological evaluation of a fluorescent analogue of folic acid. Biochemistry *30*, 5674–5681.

McConville, M.J., and Ferguson, M.A. (1993). The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. Biochem. J. *294*, 305–324.

McGraw, T.E., Greenfield, L., and Maxfield, F.R. (1987). Functional expression of human transferrin receptor cDNA in Chinese hamster ovary cells deficient in endogenous transferrin receptor. J. Cell Biol. *105*, 207–214.

McGraw, T.E., and Maxfield, F.R. (1990). Human transferrin receptor internalization is partially dependent upon an aromatic amino acid on the cytoplasmic domain. Cell Regul. *1*, 369–377.

Medof, M.E., Lublin, D.M., Holers, V.M., Ayers, D.J., Getty, R.R., Leykam, J.F., Atkinson, J.P., and Tykocinski, M.L. (1987). Cloning and characterization of cDNAs encoding the complete sequence of decay-accelerating factor of human complement. Proc. Natl. Acad. Sci. USA *84*, 2007–2011.

Parton, R.G. (1994). Ultrastructural localization of gangliosides: GM1 is concentrated in caveolae. J. Histochem. Cytochem. *42*, 155–166.

Parton, R.G., Joggerst, B., and Simons, K. (1994). Regulated internalization of caveolae. J. Cell Biol. *127*, 1199–1215.

Robinson, P.J. (1991). Phosphatidylinositol membrane anchors and T-cell activation. Immunol. Today *12*, 35–41.

Rothberg, K.G., Heuser, J.E., Donzell, W.C., Ying, Y.-S., Glenney, J.R., and Anderson, R.G.W. (1992). Caveolin, a protein component of caveolae membrane coats. Cell *68*, 673–682.

Rothberg, K.G., Ying, Y.-S., Kolhouse, J.F., Kamen, B.A., and Anderson, R.G.W. (1990). The glycophospholipid-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytic pathway. J. Cell Biol. *110*, 637–649.

Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M.P. (1993). Signal transducing molecules and glycosyl-phosphatidylinositol–linked proteins form a caveolin-rich insoluble complex in MDCK cells. J. Cell Biol. *122*, 789–807.

Schroeder, R., London, E., and Brown, D.A. (1994). Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. Proc. Natl. Acad. Sci. USA *91*, 12130–12134.

Shenoy, S.A., Dietzen, D.J., Kwong, J., Link, D.C., and Lublin, D.M. (1994). Cysteine3 of Src family protein tyrosine kinase determines palmitoylation and localization in caveolae. J. Cell Biol. *126*, 353–363.

Simons, K., and Wandinger-Ness, A. (1990). Polarized sorting in epithelia. Cell *62*, 207–210.

Stefanova, I., Horejsi, V., Ansotegui, I.J., Knapp, W., and Stockinger, H. (1991). GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science *254*, 1016–1019.

Streuli, C.H., Patel, B., and Critchley, D.R. (1981). The cholera toxin receptor ganglioside GM_1 remains associated with Triton X-100 cytoskeletons of Balb/c-3T3 cells. Exp. Cell Res. *136*, 247–254.

Sugden, B., Marsh, K., and Yates, J. (1985). A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein-Barr virus. Mol. Cell. Biol. *5*, 410–413.

Thompson, T.E., and Tillack, T.W. (1985). Organization of glycosphingolipids in bilayers and plasma membranes of mammalian cells. Annu. Rev. Biophys. Chem. *14*, 361–386.

Wang, X., Shen, F., Freisheim, J.H., Gentry, L.E., and Ratnam, M. (1992). Differential stereospecificities and affinities of folate receptor isoforms for folate compounds and antifolates. Biochem. Pharmacol. *44*, 1898–1901.

Widnell, C.C., Schneider, Y.-J., Pierre, B., Baudhuin, P., and Trouet, A. (1982). Evidence for a continual exchange of 5′-nucleotidase between the cell surface and cytoplasmic membranes in cultured rat fibroblasts. Cell *28*, 61–70.

Yu, J., Fishman, D.A., and Steck, T.L. (1973). Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. J. Supramol. Struct. *1*, 233–248.