Clustered Folate Receptors Deliver 5-Methyltetrahydrofolate to Cytoplasm of MA104 Cells

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Abstract. Previously, a high affinity, glycosylphosphatidylinositol-anchored receptor for folate and a caveolae internalization cycle have been found necessary for potocytosis of 5-methyltetrahydrofolate in MA104. We now show by cell fractionation that folate receptors also must be clustered in caveolae for potocytosis. An enriched fraction of caveolae from control cells retained 65–70% of the [3H]folic acid bound to cells in culture. Exposure of cells to the cholesterol-binding drug, filipin, which is known to uncluster receptors, shifted ~50% of the bound [3H]folic acid from the caveolae fraction to the noncaveolae membrane fraction and markedly inhibited internalization of [3H]folic acid. An mAb directed against the folate receptor also shifted ~50% of the caveolae-associated [3H]folic acid to noncaveolae membrane, indicating the antibody perturbs the normal receptor distribution. Concordantly, the mAb inhibited the delivery of 5-methyl[3H]tetrahydrofolate to the cytoplasm. Receptor bound 5-methyl[3H]tetrahydrofolate moved directly from caveolae to the cytoplasm and was not blocked by phenylarsine oxide, an inhibitor of receptor-mediated endocytosis. These results suggest cell fractionation can be used to study the uptake of molecules by caveolae.

There is now considerable evidence that the receptor-mediated delivery of 5-methyltetrahydrofolate to the cell cytoplasm occurs by a unique endocytic pathway called potocytosis (4). First, the folate receptor is anchored by glycosylphosphatidylinositol (GPI) rather than a transmembrane domain and localizes to caveolae instead of clathrin-coated pits (24). Second, transfer of membrane-bound 5-methyltetrahydrofolate to the cytoplasm appears to occur from a compartment that is associated with the plasma membrane rather than from an internal, endosomal compartment (11-13). Third, the kinetics of folate receptor recycling are distinctly different from those receptors that recycle using clathrin-coated pits (11). Fourth, inhibition of caveolae internalization either by depleting membrane cholesterol (6), treatment with PMA (29), or exposure to histamine (31) specifically blocks 5-methyltetrahydrofolate uptake. Finally, chimeric folate receptors that contain a coated pit targeting sequence deliver folate to the cytoplasm less efficiently than normal receptors and, in addition, lack the ability to regulate folate accumulation (22).

Several aspects of the potocytosis pathway remain unclear because there is not a ligand suitable for visualizing the internalization step with the electron microscope. Without this tool, investigators must rely on immunocytochemistry for information about the distribution of folate receptors (18, 24). After the application of primary and secondary antibodies, the folate receptor is clearly associated with caveolae. There is disagreement about whether the antibodies induce the migration of these molecules into caveolae (18) or, instead, detect their native distribution (24). Without an EM marker, it is also not possible to determine the mechanism of sequestration. Do caveolae actually bud from the membrane and form a vesicle that migrates to other compartments in the cell or do they remain associated with the plasma membrane and close transiently during internalization?

Another way to obtain basic information about the caveolae membrane traffic pattern, and potocytosis in general, is to use cell fractionation. This approach has provided critical information about membrane recycling during receptor-mediated endocytosis (1). Initially, caveolae were isolated from tissue-culture cells by taking advantage of the Triton X-100 insolubility of this membrane (26). While this method continues to be a valuable tool (16, 31), Triton X-100 can remove resident proteins from caveolae (7). A new purification scheme circumvents the use of detergents (32) and has the added advantage that one can follow bound ligands (32) and enzyme activities (28) associated with this membrane. We have now used this method to study the internalization process. The results suggest that ~70% of folate receptors are naturally clustered on the cell surface. Incubation of cells in the...
presence of mAb anti-folate receptor IgG stimulates unclustering of half of these receptors and inhibits the delivery of 5-methyltetrahydrofolate to the cytoplasm. In addition, open caveolae (external folate receptors) and closed caveolae (internal receptors) are always in the same caveolae fraction isolated from the plasma membrane. Cell fractionation promises to be a valuable tool for studying cotranscytosis in many different cells and tissues.

**Materials and Methods**

**Materials**

Medium 199 with Earle's salts minus folate acid was prepared by standard methods. FCS was from Hazleton Research Products, Inc. (Lexenra, KS). Glutamine, trypsin-EDTA, and penicillin/streptomycin were from GIBCO BRL (Gaithersburg, MD). Percoll was from Pharmacia Fine Chemicals (Piscataway, NJ). OptiPrep was from GIBCO BRL. [H]Folate acid (sp act 27 Ci/mmol) and 5'-[3H]methyltetrahydrofolate (sp act 21 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). 125I-diferric transferrin (human) (sp act 0.78 µCi/µg) was purchased from Du-Pont-New England Nuclear (Wilmington, DE). Antibodies were obtained from the following sources: anti-caveolin IgG from Transduction Labs (Lexington, KY); anti-folate receptor IgG (mAb, Mov18) and (mAb, Pont-New England Nuclear (Wilmington, DE). Antibodies were obtained from Sigma Chemical Co. (St. Louis, MO).

**Buffers.** Buffer A: 0.25 M sucrose, 1 mM EDTA, 20 mM tricine pH 7.8; buffer B: 0.25 M sucrose, 6 mM EDTA, 120 mM tricine, pH 7.8; buffer C: 50% OptiPrep in buffer B.

**Methods**

**Cell Culture.** The monkey kidney epithelial cell line, MA104, was grown as a monolayer in folate-free medium 199 supplemented with 5% (vol/vol) FCS and 100 U/ml penicillin/streptomycin. Cells for each experiment were set up according to a standard format. On day 0, cells were seeded into T-25 (1.5 x 10^5 cells) or T-75 (3 x 10^5 cells) culture flasks and cultured for 5 d without further feeding. For folate-binding studies, the medium was replaced with folate-free M199 containing 20 mM Hepes (pH 7.4) which was added to each flask. Cells were preincubated in the absence or presence of PMA (1 µM) or phenylarsine oxide (20 µM) for 30 min at 4°C. 125I-transferrin (0.5 µCi/µl, sp act 0.25 µCi/µg) was added to each dish, and the cells were incubated at 37°C for the indicated time. The flasks were chilled on ice for 20 min, and 1.5 ml of lysis buffer (10 mM Tris, pH 8.0, containing 1 µM unlabeled transferrin) was added to each flask. The cells were placed at 80°C for 15 min and thawed on ice. The suspension was collected, and the flask was washed with 1.0 ml of lysis buffer at 4°C. The two were combined and centrifuged for 20 min at 100,000 g to separate the membrane (pellet) from the cytosol (supernatant). Radioactivity in each was measured in a gamma counter.

**OptiPrep gradient (6 ml) (prepared by diluting buffer C with buffer A) was prepared on top of the sample, and the centrifugation was at 52,000 g for 90 min in a Sorvall TH641 swinging bucket rotor. The top 5 ml of the gradient (fractions 1 to 7) was collected, placed in a fresh TH641 centrifuge tube, and mixed with 4 ml of buffer C. The sample was overlaid with 1 ml of 15% OptiPrep and 0.5 ml of 5% OptiPrep (prepared by diluting buffer C with buffer A) and centrifuged at 52,000 g for 90 min at 4°C. A distinct opaque band was present at both interfaces. The band at the 5% interface was collected and designated caveolar membranes. Typically, we obtained 10–20 µg of protein in this band.

**Folate Receptor Sequestration.** A standard [3H]folic acid binding assay was used to measure internal and external folate receptors (10). After the indicated treatments, MA104 cells were incubated in the presence of 5 nM [H]folic acid for 1 h at 37°C. External [H]folic acid corresponded to the amount released when cells were incubated in 100-fold excess of acid saline (0.15 M NaCl, adjusted to pH 3.0 with glacial acetic acid). Internal folate was the amount of [H]folic acid that remained associated with the acid-saline–treated cells. The latter was collected by adding 0.1 N NaOH to the flask to dissolve the cells. Radioactivity was measured by liquid scintillation counting using a liquid scintillation analyzer (Tri-carb 1900A; Packard Instrument Co., Inc., Downers Grove, IL). Nonspecific binding, which was measured by adding 100-fold excess unlabeled folic acid, was <5% of specific binding.

**Transferrin Uptake.** MA104 cells were washed once with PBS before 1 ml of M199 medium containing 20 mM Hepes (pH 7.4) was added to each flask. Cells were preincubated in the presence or absence of PMA (1 µM) or phenylarsine oxide (20 µM) for 30 min at 4°C. 125I-transferrin (0.5 µCi/µl, sp act 0.25 µCi/µg) was added to each dish, and the cells were incubated at 37°C for the indicated time. The flasks were chilled on ice for 20 min, and 1.5 ml of lysis buffer (10 mM Tris, pH 8.0, containing 1 µM unlabeled transferrin) was added to each flask. The cells were plated at 80°C for 15 min and thawed on ice. The suspension was collected, and the flask was washed with 1.0 ml of lysis buffer at 4°C. The two were combined and centrifuged for 20 min at 100,000 g to separate the membrane (pellet) from the cytosol (supernatant). The radioactivity in each was measured in a gamma counter. Nonspecific binding of 125I-transferrin was <20%.

To measure the migration of transferrin to internal membrane compartments, cells were incubated in the presence of 125I-transferrin as described above before preparing a postnuclear fraction and separating cytosol, plasma membrane, and internal membranes on Percoll gradients. The cytosol fraction corresponded to the top 2 ml of the gradient, while the plasma membrane fraction was the visible band in fraction 4. The remaining fractions, which contained the intracellular membranes, were pooled and centrifuged at 185,000 g for 1.5 h at 4°C to obtain a pellet.

**Electrophoresis and Immunoblotting.** Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Richmond, CA). Proteins were concentrated by TCA precipitation, washed in acetone, and resuspended in distilled water before the addition of 5 x Laemmli sample buffer and heating at 95°C for 2 min. Protein samples were loaded onto 12.5% SDS polyacrylamide gels and separated by the method of Laemmli et al. (1970) to obtain the appropriate secondary antibody conjugated to HRP was diluted 1:30,000 and added to the incubation mixture for 1 h at room temperature. After the primary antibody incubation, the membrane was washed four times, 10 min each in TBST + 1% dry milk. The appropriate secondary antibody conjugated to HRP was diluted 1:30,000 and added to the incubation mixture for 1 h at room temperature. After the primary antibody incubation, the membrane was washed four times, 10 min each in TBST + 1% dry milk. The appropriate secondary antibody conjugated to HRP was diluted 1:30,000 and added to the incubation mixture for 1 h at room temperature. After the primary antibody incubation, the membrane was washed four times, 10 min each in TBST + 1% dry milk. The appropriate secondary antibody conjugated to HRP was diluted 1:30,000 and added to the incubation mixture for 1 h at room temperature. After the primary antibody incubation, the membrane was washed four times, 10 min each in TBST + 1% dry milk. The appropriate secondary antibody conjugated to HRP was diluted 1:30,000 and added to the incubation mixture for 1 h at room temperature.

**Results**

A membrane receptor is said to be clustered when mor-
phologic methods detect multiple receptors in a restricted area of the cell surface (3). The isolated region of membrane also contains a high concentration of the receptor relative to the remainder of the plasma membrane (19). Previously, we used immunoblotting to show that the caveolae fraction isolated from MA104 cells is enriched in folate receptors (32). A more quantitative method for assessing enrichment is to measure the amount of receptor-bound [3H]folic acid in the caveolae fraction. Folic acid binds with such a high affinity that it cannot dissociate from the receptor and move to the cytoplasm of the cell after internalization (11). Folate-depleted MA104 cells were incubated in the presence of 5 nM [3H]folic acid for 1 h at 37°C to label both internal and external folate receptors. Isolated plasma membranes, which contained all of the cell-associated [3H]folic acid, were sonicated and separated on the first of the two OptiPrep gradients used to purify caveolae (Fig. 1 A). The gradient was divided into fifteen fractions, and each was either assayed for the amount of protein (□) and the specific binding of [3H]folic acid (○) or immunoblotted with the indicated antibody. Greater than 90% of the protein was in fractions 8–15, but these fractions contained just 34% of the [3H]folic acid (Table I). The bulk of the [3H]folic acid was in the light

![Figure 1](image.png)

**Figure 1.** Distribution of folate receptor and receptor-bound [3H]folic acid during caveolae purification on the first (A) and second (B) OptiPrep gradients. MA104 cells were incubated for 1 h at 37°C in the presence of 5 nM [3H]folic acid. They were then chilled to 4°C, washed, and processed to purify caveolae. Each fraction was assayed for either total protein and bound [3H]folic acid (graph) or immunodeectable caveolin (Caveolin), folate receptor (Folate Receptor), and clathrin (Clathrin). For the immunoblots, each lane was loaded with all of the protein in the fraction. (A) Analysis of fractions from the first OptiPrep gradient. (B) Analysis of fractions from the second OptiPrep gradient. The data presented in A and B were generated in separate experiments.
membrane fractions (1–7). Immunoblotting showed that these same fractions contained nearly all the caveolin (Caveolin) and folate receptor (Folate Receptor) but no clathrin (Clathrin). A further enrichment in bound folate was obtained when we pooled fractions 1–7 from a second experiment and used them to prepare the caveolea fraction (Fig. 1B). While protein was detected in most of the fractions (C), the highest amount was in fraction 2. Fraction 2 contained all of the detectable [3H]folate acid (C), caveolin (Caveolin), and folate receptor (Folate Receptor). In a third experiment, we tabulated the amount of protein and [3H]folate acid in each step of the purification protocol (Table I). The caveolea fraction typically contained ~65% of the original plasma membrane–bound [3H]folate acid with a specific binding of 140–150 pmol/mg of protein, which corresponds to a 15-fold enrichment relative to the plasma membrane.

The quantity of bound [3H]folate acid in the caveolea fractions suggests that both cell surface and internalized receptors are present. This was confirmed (Fig. 2) by measuring the specific [3H]folate acid in caveolea fractions isolated from cells labeled with [3H]folate acid under conditions that detect either internal or external populations of receptor (11). Caveolea fractions from cells incubated at 37°C for 1 h had a specific [3H]folate acid binding of 150 pmol/mg of protein and contained 69% of the folate acid initially associated with the membrane fraction (Control). If the incubation was carried out at 4°C, which only labels surface receptors (11), the specific binding was reduced by 50% (4°C Binding), but the fraction still contained the same percentage of total membrane associated [3H]folate acid (68%). The caveolea fraction from cells incubated at 37°C, chilled, and acid stripped (Acid Strip) to remove all [3H]folate acid bound to external receptors also had 50% of the control [3H]folate acid and the same percentage of membrane-associated label (64%). Finally, the amount of [3H]folate acid in the caveolea fraction (70% of membrane bound) was unchanged in cells stripped of surface-bound [3H]folate acid but warmed to 37°C (Acid Strip + 37°C) for 1 h.

**Clustered Receptors and 5-Methyltetrahydrofolate Delivery**

The concentration of [3H]folate acid in the caveolea fraction was assessed by immunofluorescence. Cells were incubated in the presence of 5 nM [3H]folate acid for 1 h at 37°C. Cells were then processed to isolate caveolea as described. Each fraction was assayed for the amount of protein and the quantity of [3H]folate acid. NCM, postnuclear supematant; CYTO, cytosol (fractions 1–3 Percoll gradient); IM, internal membranes (fractions 5–25 Percoll gradient); PM, plasma membrane (fraction 4 Percoll gradient); NCM, noncaveolea membrane (fractions 8–15, OptiPrep 1); CM, caveolea membrane (fraction 2, OptiPrep 2).

**Table I. Distribution of [3H]Folic Acid during Fractionation**

| Sample | Protein | Folate | Protein yield | DPM yield (PNS) | DPM yield (PM) |
|--------|---------|--------|---------------|-----------------|----------------|
| NCM    | 0.39    | 3.64   | 9.33          | 16              | 50             |
| CYTO   | 1.78    | 0.11   | 0.06          | 71              | 1.5            |
| IM     | 0.2     | 0.94   | 4.7           | 8               | 13             |
| PM     | 0.4     | 5.43   | 13.6          | 16              | 75             |
| NCM    | 0.39    | 3.64   | 9.33          | 16              | 50             |
| CM     | 0.025   | 1.77   | 71            | 1               | 24             |

MA104 cells were incubated in the presence of 5 nM [3H]folate acid for 1 h at 37°C. Cells were then processed to isolate caveolea as described. Each fraction was assayed for the amount of protein and the quantity of [3H]folate acid. NCM, postnuclear supernatant; CYTO, cytosol (fractions 1–3 Percoll gradient); IM, internal membranes (fractions 5–25 Percoll gradient); PM, plasma membrane (fraction 4 Percoll gradient); NCM, noncaveolea membrane (fractions 8–15, OptiPrep 1); CM, caveolea membrane (fraction 2, OptiPrep 2).

**Figure 2.** Both internal and external folate receptors are in the caveolea fraction. MA104 cells were incubated either at 37°C (Control, Cold Folic Acid, Acid Strip, Acid Strip + 37°C) or at 4°C (4°C Binding) for 1 h in the presence of 5 nM [3H]folate acid. Cells were either used immediately (Control and 4°C Binding) or chilled to 4°C and acid stripped (Acid Strip, Acid Strip + 37°C). One set of acid-stripped cells was incubated further at 37°C for 1 h. The percentage of recovery of membrane-bound [3H]folate acid in the respective caveolea fractions was: control, 69%; 4°C binding, 68%; acid stripped, 64%; acid stripped + 37°C, 70%; and cold folic acid, 70%.
increase of [3H]folic acid in the noncaveolar fraction (compare Table I with Table II). Filipin did not affect the amount of protein recovered in each fraction.

Immunoblotting also detected an altered distribution of folate receptors in filipin-treated cells (Fig. 3). This was best seen in immunoblots of the fractions from the first OptiPrep gradient where all of the protein in each fraction is loaded on the gel. In untreated cells, the majority of the receptor migrated in the light membrane fractions (fractions 1–7) used to purify and concentrate caveolae (Control). Filipin treatment caused the receptor distribution to shift toward the bottom of this gradient (Filipin) into fractions containing the bulk of the membrane proteins (see Fig. 1). Previously, we showed that immunogold labeling detected clustered receptors in PMA-treated cells (29). In contrast to filipin, PMA treatment did not reduce the number of folate receptors (PMA) in the caveolar fraction. Therefore, two different assays (immunoblotting and folic acid binding) detected filipin-induced movement of folate receptors from the caveolar fraction to fractions containing the bulk of the plasma membrane.

Both functional folate receptors (22) and a caveolar internalization cycle (29) appear to be required for the uptake of physiologic concentrations of 5-methyltetrahydrofolate. An essential function for clustered receptors has not been determined. The ability of filipin to uncluster the folate receptor provided a potential tool for assessing this requirement (Fig. 4). Cells were either not treated (Control) or incubated in the presence of filipin (Filipin) before measuring [3H]folic acid internalization. ordinarily, the ratio of internal to external bound [3H]folic acid in MA104 cells is ~1 (Control). In filipin-treated cells, however, this ratio was reduced by ~50%. A similar inhibition of receptor internalization was seen in cells exposed to PMA (PMA), a drug that prevents caveolae internalization without unclustering receptors (PMA; Fig. 3).

Another activity of cholesterol-binding drugs is to disassemble the caveolar coat (25). A more specific tool for unclustering GPI-anchored receptors may be anti-receptor mAbs. Jemmerson and Agree (9) showed that the choice of mAb used to localize GPI-anchored alkaline phosphatase affects the localization of the protein. Some antibodies give a dispersed distribution, while others give a clustered pattern. Mayor et al. (18) reported that cells incubated in the presence of mAb anti-folate receptor Mov19 had dispersed receptors, but after further incubation in the presence of an anti-mouse IgG, the receptors appeared clustered over caveolae. To measure the effect of Mov19 on receptor distribution (Fig. 5A), cells were incubated in the presence or absence of different combinations of antibodies for 1 h at 37°C, and the receptor distribution was assayed by immunoblotting fractions from the first OptiPrep gradient. Compared to untreated cells (Control), Mov19 (Mov19) shifted a substantial number of the receptors to the bulk membrane fractions. Cells incubated in the presence of both Mov19 and anti-mouse IgG (Mov19 + 2°Ab), by contrast, had the same receptor distribution as untreated cells (compare with control). The anti-mouse IgG alone (2°Ab) did not affect the receptor distribution. Finally, another mAb anti-folate receptor designated Mov18 did not shift the receptor distribution (Mov18).

To see how Mov19 affected the distribution of bound [3H]folic acid, MA104 cells were incubated for 1 h at 37°C in the presence or absence of Mov19 before adding [3H]folic acid to the dish for an additional 1 h (Fig. 5B). Caveolae were isolated and the amount of bound [3H]folic acid was measured. Untreated cells (Control) had a specific binding of ~150 pmol/mg of protein in the caveolar fraction, while Mov19-treated cells had only ~75 pmol/mg of protein (Mov19) in this fraction. The antibody had no effect on the distribution of caveolin or the total protein content of the fractions (data not shown). As was observed for filipin treatment (Table II), the [3H]folic acid lost from the caveolar fraction was shifted to the noncaveolar fraction (Table III). If the cells were incubated sequentially with Mov19 and anti–mouse IgG (Mov19 + 2°Ab), the specific binding of [3H]folic acid in the caveolar fraction returned to control values. The anti–mouse IgG alone had no effect (2°Ab), and neither did Mov18 (Mov18).

Mov19 also inhibited [3H]folic acid internalization (Fig. 6A). Cells were incubated in the presence of either Mov19 (Mov19), Mov19 + anti–mouse IgG (Mov19 + 2°Ab),

Figure 3. Filipin shifts the folate receptor from caveolae to the bulk membrane fractions. MA104 cells were either not treated (Control), incubated for 15 min in the presence of 10 μg/ml filipin (Filipin), or incubated in the presence of 1 μM PMA for 1 h at 37°C. At the end of the treatment, plasma membranes were purified and processed for OptiPrep 1 gradients according to standard methods. The total protein from each fraction was loaded on gels and immunoblotted using mAb anti-folate receptor Mov19.

Figure 4. Filipin inhibits internalization of [3H]folic acid. MA104 cells were incubated in the presence of media alone for 1 h (Control), 1 μM PMA (PMA) for 1 h, or 10 μg/ml filipin for 15 min before the addition of 5 nM [3H]folic acid and an additional 1-h incubation at 37°C. At the end of the incubations, the ratio of internal to external receptors was measured as described. All values are the average of six trials ±SD.
anti–mouse IgG alone (2°Ab), or Mov18 (Mov18) before assaying for the internal to external bound [3H]folic acid ratio. Only Mov19 inhibited internalization. Mov19 also blocked the delivery of 5-[3H]methyltetrahydrofolate to the cytosol (Fig. 6 B). After a 3-h incubation in the presence (□) or absence (○) of the mAb, control cells had taken up ~2 pmol/mg of protein, while cells exposed to Mov19 only accumulated 0.4 pmol/mg. PMA inhibited uptake to the same extent (○).

**Folate Delivery to Cytosol by Caveolae**

Biochemical (11) and morphologic (24) studies have failed to detect folate receptors in endosomes of MA104 cells. Previously, we used Percoll fractionation to show that 5-[3H]methyltetrahydrofolate moves directly from the plasma membrane to the cytosol of the cell, and the blockage of this step by monensin (19). To be sure we could detect movement of a ligand to internal membranes with this procedure, we tested 125I-transferrin. MA104 cells were incubated in the presence of 125I-transferrin for 1 h at 4°C, and one set was assayed immediately while the other was warmed to 37°C for 0.5 h (Fig. 7). Initially, most of the cell-associated radioactivity was found in the plasma membrane fraction (Plasma Membrane; open bar). The small amount in the cytosol fraction most likely was 125I-transferrin that had dissociated from the receptor (Cytosol; open bar). After 0.5 h at 37°C, there was a 25% decline in the plasma membrane radiolabel (Plasma Membrane; compare open bar with solid bar) and a corresponding increase in the 125I-transferrin associated with the internal membrane fraction (Internal Membrane; compare open bar with solid bar). Therefore, the Percoll gradient procedure detects ligand movement from the plasma membrane to endosomes. This suggests that we would have detected the movement of 5-[3H]methyltetrahydrofolate through endosomes if this were the route to the cytoplasm.

We used 125I-transferrin and 5-[3H]methyltetrahydrofolate to identify inhibitors that might distinguish between the two internalization pathways. In MA104 cells, uptake of 5-methyltetrahydrofolate is inhibited by PMA (29). Kaplan et al. (14) showed that transferrin internalization is inhibited by phenylarsine oxide (PAO). Cells were incubated in the presence of 0.32 μg/ml of 125I-transferrin for various times at 37°C before total cell membranes (plasma membrane and internal membranes) were separated from cytosol (Fig. 8 A). There was an immediate increase in 125I-transferrin associated with the membrane fraction that reached a plateau after 1 h of incubation (□). We did not detect a significant amount of radiolabel in the cytosol during the incubation (○). Cells that were exposed to PAO during the incubation with 125I-transferrin had 50% less radiolabel associated with the membrane fraction (Δ), indicating that receptor internalization and recycling was blocked. Replacing the PAO with PMA caused a slight increase in membrane-associated radioactivity (○). While PAO inhibited the uptake of 125I-transferrin, it had no effect on the internalization of 5-[3H]methyltetrahydrofolate (Fig. 8 B). Neither the internalization of membrane-bound vitamin (compare Δ with ○) nor the delivery to the cytoplasm (compare ■ with ▲ on the ordinate) was affected by PAO.
**Discussion**

One of the unresolved questions about potocytosis has been the exact route the receptor takes during the internalization cycle. We have used cell fractionation to better understand this pathway. A method for isolating caveolae was used that previously has been shown, by both immunoblotting (17, 20, 32) and PAGE (32), to yield a unique population of membranes. While no membrane purification procedure could ever yield an absolutely pure preparation of caveolae, the results we obtained with this method are consistent with previous biochemical (11) and morphologic (24) studies that localized the cycle to caveolae membrane in MA104 cells. We were able to recover ~65% of the bound folate in the caveolae fraction and detect both internal and external populations of receptor. Since all receptors appear to be functional in these cells (11), the 35% associated with noncaveolae membrane must be able to move into caveolae before internalization. Neither the folate receptor nor 5-methyltetrahydrofolate was found in endosomes during receptor recycling even though 125I-transferrin movement to this compartment was easily detected. Furthermore, inhibitors that interfere with the clathrin-coated pit pathway did not affect the internalization of folate. These results affirm (4) that potocytosis is a distinct endocytic pathway.

Ligand-binding studies detect opened and closed caveolae compartments, but electron microscopic immunogold labeling cannot distinguish between the two. Furthermore, immunoelectron microscopy failed to find folate receptors associated with endosomes in these cells (24). In the current study, caveolae with sequestered receptors (closed) and caveolae with receptors accessible at the cell surface (open) were in the same caveolae fraction (Fig. 2). Therefore, either the closed caveolae that internalize folate never detach from the plasma membrane or they become plasmalemmal vesicles with the same density as caveolae membrane and, as a consequence, migrate identically on the gradients. Treatment of cells with PMA stimulates the disappearance of invaginated caveolae and the return of internalized receptors to the cell surface (29). Yet, by cell fractionation, the caveolae membrane in these cells is indistinguishable from caveolae in untreated cells (Fig. 3).
fibroblast membranes showing many uninvaginated caveolae is supported by rapid-freeze, deep-etch images of the remaining caveolae, which have the same lipid and protein composition, cannot be distinguished in thin-section images from other segments of membrane. This conclusion is supported by rapid-freeze, deep-etch images of fibroblast membranes showing many uninvaginated caveola decorated with a caveolar coat (25). Caveolae membrane may constitute a much larger percentage of the plasma membrane than thin-section morphology indicates.

Filipin caused an ~50% decline in number of folate receptors in the caveolae fraction (Fig. 3). After this treatment, approximately two-thirds of the receptors were in the bulk membrane fraction. This suggests that the drug does not completely uncluster folate receptors. Immunofluorescence images also indicate filipin only partially unclusters the receptor in fixed cells (see Fig. 1) (23). Furthermore, we were forced to use a lower concentration of the drug in the current study to keep the cells alive. Therefore, the biochemical and the immunofluorescence results agree.

Clustered folate receptors appear to be required for the efficient internalization of folate (Figs. 4, 5 B, and 6, A and B). Two different treatments that partially unclustered the receptor inhibited the potocytosis of 5-[3H]methyltetrahydrofolate by decreasing the internalization of receptors. Receptor clustering has also been found to be required for receptor-mediated endocytosis by clathrin-coated pits (2). The percentage of folate receptors clustered in caveolae (65%) is nearly the same as the portion of low density lipoprotein receptors (50–70%) found in coated pits (3). Thus, the aggregation of receptors over a membrane domain specialized for internalization appears to be a common mechanism for guaranteeing efficient uptake of a ligand. The mechanism of folate receptor clustering, however, is distinctly different than for the low density lipoprotein receptor. The targeting of receptors to clathrin-coated pits depends on protein–protein interactions between a specific amino acid sequence in the cytoplasmic tail and clathrin-associated coat proteins (8). The clustering of GPI-anchored proteins in caveolae, by contrast, appears to depend on lipid–lipid interactions (23, 27). The lipid anchor may convey high mobility on GPI proteins when they are in noncaveolae membrane and low mobility in glycolipid/cholesterol-rich caveolae. As a consequence, the GPI protein spends more time in caveolae and, at the steady state, is predominantly clustered.

A dynamic GPI-anchored protein, shuttling between caveolae and noncaveolae membrane, would explain the unexpected effect of Mov19. Previous morphologic studies (18), together with the current biochemical experiments (Fig. 5 A), indicate that after this anti-folate receptor mAb binds, the receptors are partially dispersed in the plane of the membrane. The morphologic (18) and fractionation experiments (Fig. 5 A) also agree that when the cells are incubated in the presence of both the mAb and an anti-mouse IgG, the receptors are clustered in caveolae. Cell fractionation (Fig. 5 A) further shows that receptors from untreated cells are clustered in caveolae, which agrees with the recent observation that GPI proteins inserted into the plasma membrane of lymphocytes spontaneously cluster (33). Therefore, the Mov19 must alter the normal, clustered pattern of the folate receptor. The mAb could do this in two ways: (a) it may bind to receptors migrating in noncaveolae membrane and prevent their return to caveolae. (b) Alternatively, the mAb might bind to clustered receptors and cause them to disperse in the plane of the membrane. Regardless of the actual mechanism, not all mAbs against GPI proteins seem to uncluster (Fig. 5 A) (9).

The effect of the mAb anti-folate receptor on receptor
distribution predicts several characteristics of proteins with a GPI anchor. First, these proteins are not static on the cell surface. They can gain access to all membranes contiguous with the plasma membrane. This property endows the GPI protein with the ability to shuttle information between caveolae and other membrane compartments. Second, protein ligands may trigger the movement of specific GPI proteins to new locations in the plasma membrane. In some cases, the ligand may actually tether the GPI protein to a transmembrane protein located in a distant compartment (5). Finally, a lipid-dependent mechanism of clustering in caveolae obviates the need for homomeric protein–protein interactions to effect GPI-anchored organization on the cell surface. This may explain why clustered GPI proteins are difficult to detect in prefixed cells (18, 21): aldehyde-based fixatives are notoriously poor for immobilizing lipids in the plane of the membrane. Thus, the behavior of a GPI-anchored protein does not conform to standards previously established from studies of transmembrane proteins.

We have demonstrated how to use cell fractionation for studying potocytosis as well as the effects of a primary antibody on the membrane distribution of a GPI-anchored molecule. Our results are completely consistent with previous biochemical (6, 9, 26, 27) and morphologic (12, 18, 23, 33) results on the behavior of this class of proteins. The detergent-free caveola isolation procedure is gentle enough to retain ligands bound to caveolae in cultured cells. Combined with Percoll fractionation, the movement of a ligand or its receptor from caveolae to other cellular compartments can be followed. Ligands may move directly to the cytoplasm, as in the case of 5-methyltetrahydrofolate, or they may migrate to other membrane compartments such as the ER (30) or endosomes (21). The isolation procedure is rapid enough to allow kinetic measurements of ligand movement. Combined with morphologic and other biochemical assays, this fractionation procedure should be useful for identifying new molecules and ions that enter cells by potocytosis.

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