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

Eric J. Smart, Chieko Mineo, and Richard G.W. Anderson

Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9039

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 [^{3}H]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 [^{3}H]folic acid from the caveolae fraction to the noncaveolae membrane fraction and markedly inhibited internalization of [^{3}H]folic acid. An mAb directed against the folate receptor also shifted ~50% of the caveolae-associated [^{3}H]folic acid to noncaveolae membrane, indicating the antibody perturbs the normal receptor distribution. Concordantly, the mAb inhibited the delivery of 5-methyl[^{3}H]tetrahydrofolate to the cytoplasm. Receptor bound 5-methyl[^{3}H]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)[1] 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

Address all correspondence to Richard G.W. Anderson, Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX 75235-9039. Tel.: (214) 648-2346. Fax: (214) 648-7577.

1. *Abbreviations used in this paper*: GPI, glycosylphosphatidylinositol; PAO, phenylarsine oxide; PNS, postnuclear supernatant fraction.

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 potocytosis in many different cells and tissues.

Materials and Methods

Materials

Medium 199 with Earle's salts minus folic acid was prepared by standard methods. FCS was from Hazleton Research Products, Inc. (Lenexa, 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. [^{3}H]folic acid (sp act 27 Ci/mmol) and 5-[^{3}H]methyltetrahydrofolate (sp act 21 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). ^{125}I-diferric transferrin (human) (sp act 0.78 μCi/μg) was purchased from DuPont-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, Mov19) from Centacor Corp. (Malvern, PA); rabbit anti–mouse IgG from Zymed Laboratories, Inc. (South San Francisco, CA). The anti-clathrin IgG was developed in the laboratory. The phenylarsine oxide and unlabeled transferrin were 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 (1.5×10^5 cells) or T-75 (3×10^5 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) without serum, and additions were made directly to the culture flasks.

Purification of Caveolae. The caveolae isolation procedure was a modification of the method developed by Smart et al. (32). All steps were carried out at 4°C. Purified plasma membranes were prepared from 10 100-mm dishes or T-75 flasks of confluent tissue-culture cells (8–10 mg of total protein) by Percoll gradient centrifugation. Each dish/flask was washed twice with 5 ml of buffer A, and the cells were collected by scraping in 3 ml of buffer A. The cells were pelleted by centrifugation for 5 min at 1,400 *g* in an IEC Centra-4B centrifuge (International Equipment Co., Needham Heights, MA). The cells were resuspended in 1.0 ml of buffer A, placed in a 2-ml tissue grinder (No. 0841416A; Wheaton Instruments, Millville, NJ), and homogenized with 20 strokes of the teflon homogenizer. We transferred the suspension to a 1.5-ml centrifuge tube and centrifuged at 1,000 *g* for 10 min in an Eppendorf 5415C centrifuge (Eppendorf North American, Inc., Madison, WI). The postnuclear supernatant fraction (PNS) was removed and stored on ice. The pellet from each tube was resuspended in 1.0 ml of buffer A, homogenized, and centrifuged at 1,000 *g* for 10 min again. The two post nuclear supernatant fractions were combined (~4 mg of total protein), layered on the top of 23 ml of 30% Percoll in buffer A, and centrifuged at 84,000 *g* for 30 min in a rotor (Ti 60; Beckman Instruments, Inc., Palo Alto, CA). The plasma membrane fraction was a visible band ~5.7 cm from the bottom of the centrifuge bottle. The membrane fraction, which contained ~0.6 mg of protein, was collected with a Pasteur pipette, adjusted to 2.0 ml with buffer A, and placed in a centrifuge tube (TH641; Sorvall Instruments Division, DuPont Co., Newton, CT) on ice. We placed a 3-mm-diam sonication probe equidistant from the bottom of the tube and the top of the solution and sonicated the sample six successive times (total power of 50 J each time), using the automatic settings of a Vibra Cell sonicator (model VC60S; Sonics & Materials Inc., Danbury, CT.). An aliquot of the sonicate was saved before mixing the remainder with 1.84 ml of buffer C and 0.16 ml of buffer A (final OptiPrep concentration, 23%) in the bottom of the same TH641 tube. A linear 20–10% OptiPrep gradient (6 ml) (prepared by diluting buffer C with buffer A) was poured on top of the sample, and then centrifuged 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 [^{3}H]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 [^{3}H]folic acid for 1 h at 37°C. External [^{3}H]folic acid corresponded to the amount released when cells were incubated on ice for 30 s in the presence of acid saline (0.15 M NaCl, adjusted to pH 3.0 with glacial acetic acid). Internal folate was the amount of [^{3}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 1.5 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. ^{125}I-transferrin (0.32 μg/ml, sp act 0.25 μCi/ml) 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). The radioactivity in each was measured in a gamma counter. Nonspecific binding of ^{125}I-transferrin was <20%.

To measure the migration of transferrin to internal membrane compartments, cells were incubated in the presence of ^{125}I-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 Immunoblots. 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× 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 (15) before transferring to polyvinylidene difluoride membranes. The membrane was blocked in TBST (20 mM Tris, pH 7.6, 137 mM NaCl, 0.5% Tween-20) plus 5% dry milk for 1 h at room temperature. Primary antibodies were diluted in TBST + 1% dry milk 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. The membrane was then washed and processed to visualize reactive proteins by the chemiluminescence method.

Quantification. For the folate receptor sequestration, 5-methyltetrahydrofolate uptake and transferrin uptake experiments, ligand binding was determined either by liquid scintillation or a gamma radiation counting. Each time point had three to six independent determinations per experiment. In addition, each experiment was conducted at least three times. Although the absolute numbers varied between experiments, the results were always the same. Representative experiments are shown. For the quantification of radiation in the different OptiPrep 1 gradients, 50 μl from each fraction was measured. Each fractionation experiment was conducted at least four times with similar results.

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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]folic acid (Table I). The bulk of the [^{3}H]folic acid was in the light

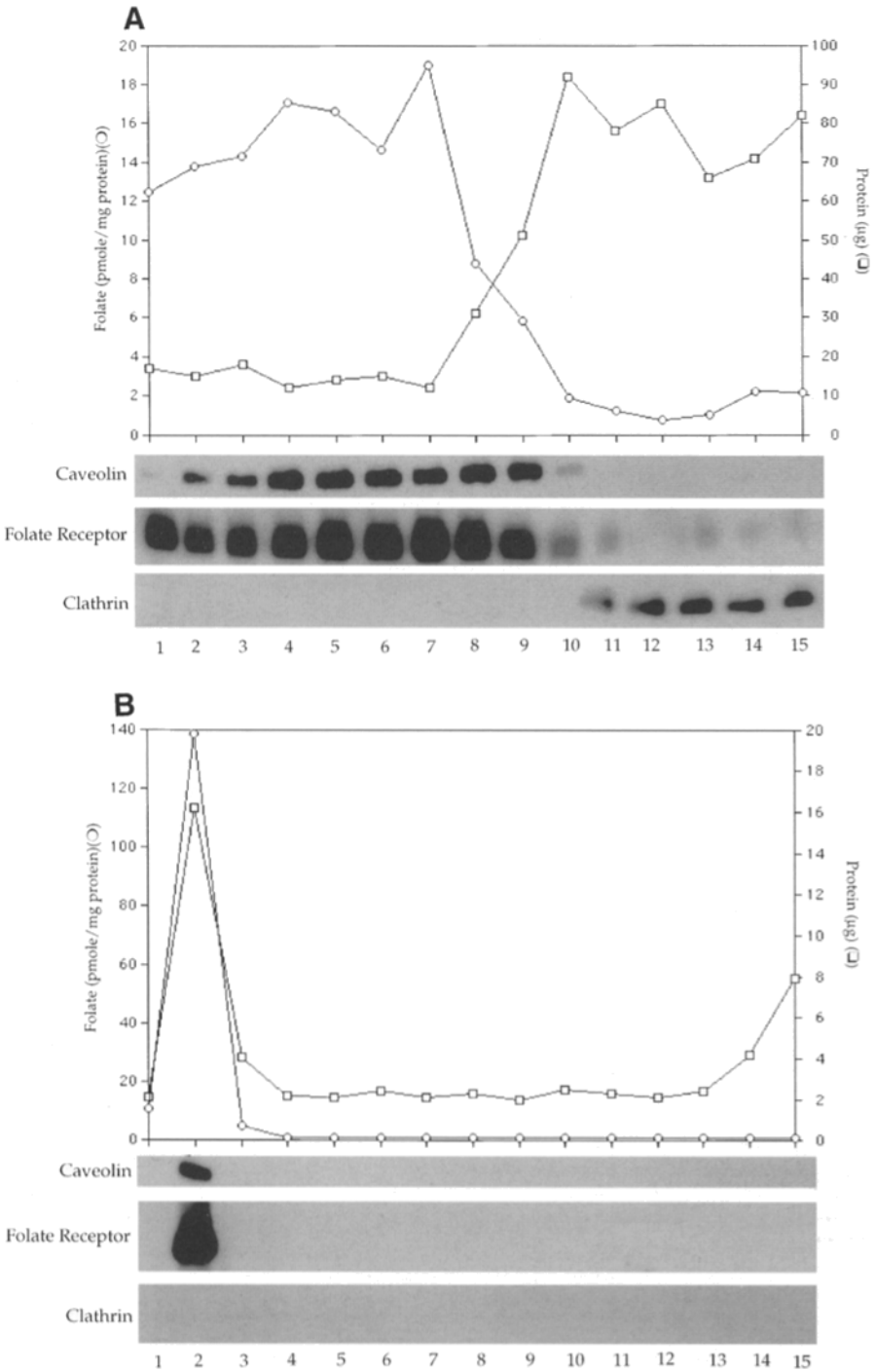

Figure 1. Distribution of folate receptor and receptor-bound [^{3}H]folic acid during caveolae purification on the first (*A*) and second (*B*) OptiPrep gradients. MA104 cells were incubated for 1 h at 37°C in the presence of 5 nM [^{3}H]folic acid. They were then chilled to 4°C, washed, and processed to purify caveolae. Each fraction was assayed for either total protein and bound [^{3}H]folic acid (*graph*) or immunodetectable 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.

Table I. Distribution of [^{3}H]Folic Acid during Fractionation

Sample	Protein	Folate	Protein	Protein yield	DPM yield (PNS)	DPM yield (PM)
	mg	*pmol*	*pmol/mg*	*%*	*%*	*%*
PNS	3.08	8.85	2.87	100	100	
CYTO	2.16	0.22	0.1	70	2.5	
IM	0.315	1.27	4.03	10	14	
PM	0.506	5.49	10.85	16	62	100
NCM	0.461	1.86	4.03	15	21	34
CM	0.023	3.44	150	0.7	39	63

MA104 cells were incubated in the presence of 5 nM [^{3}H]folic acid for 1 h at 37°C. Cells were then processed to isolate caveolae as described. Each fraction was assayed for the amount of protein and the quantity of [^{3}H]folic acid. *PNS*, 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*, noncaveolae membrane (fractions 8–15, OptiPrep 1); *CM*, caveolae membrane (fraction 2, OptiPrep 2).

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 caveolae fraction (Fig. 1 *B*). While protein was detected in most of the fractions (□), the highest amount was in fraction 2. Fraction 2 contained all of the detectable [^{3}H]folic acid (○), caveolin (*Caveolin*), and folate receptor (*Folate Receptor*). In a third experiment, we tabulated the amount of protein and [^{3}H]folic acid in each step of the purification protocol (Table I). The caveolae fraction typically contained ~65% of the original plasma membrane–bound [^{3}H]folic 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 [^{3}H]folic acid in the caveolae fractions suggests that both cell surface and internalized receptors are present. This was confirmed (Fig. 2) by measuring the specific [^{3}H]folic acid in caveolae fractions isolated from cells labeled with [^{3}H]folic acid under conditions that detect either internal or external populations of receptor (11). Caveolae fractions from cells incubated at 37°C for 1 h had a specific [^{3}H]folic acid binding of 150 pmol/mg of protein and contained 69% of the folic 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 [^{3}H]folic acid (68%). The caveolae fraction from cells incubated at 37°C, chilled, and acid stripped (*Acid Strip*) to remove all [^{3}H]folic acid bound to external receptors also had 50% of the control [^{3}H]folic acid and the same percentage of membrane-associated label (64%). Finally, the amount of [^{3}H]folic acid in the caveolae fraction (70% of membrane bound) was unchanged in cells stripped of surface-bound [^{3}H]folic acid but warmed to 37°C (*Acid Strip + 37°C*) for 1 h.

Clustered Receptors and 5-Methyltetrahydrofolate Delivery

The concentration of [^{3}H]folic acid in the caveolae fraction is consistent with previous morphologic observations showing clustered folate receptors associated with invaginated caveolae (24). Cells with unclustered receptors should have a reduced amount of bound [^{3}H]folic in isolated caveolae. Cholesterol-binding drugs such as filipin partially uncluster folate receptors on the surface of formaldehyde-fixed cells (23). Therefore, we identified a concentration of filipin that was not toxic to cells, as judged by trypan blue exclusion. We incubated cells in the presence of filipin for 15 min before adding [^{3}H]folic acid to the dish and further incubating 1 h at 37°C. Filipin had no effect on total [^{3}H]folic acid binding, indicating the receptors were not inactivated by the drug (compare *PM* in Table I with Table II). By contrast, we observed a >50% loss of bound [^{3}H]folic acid from the caveolae fraction and a reciprocal

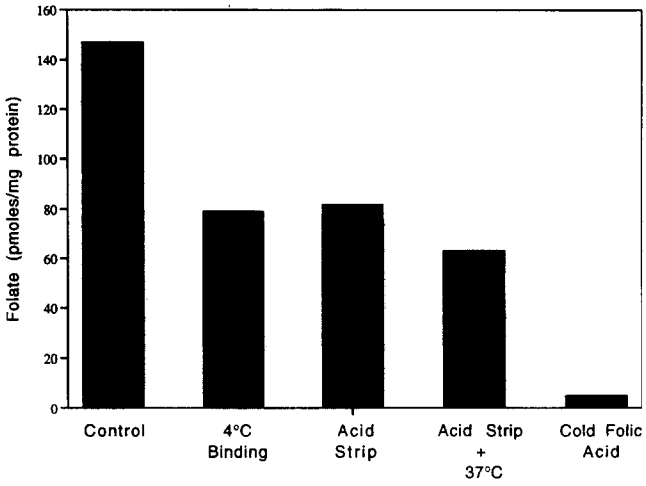

Figure 2. Both internal and external folate receptors are in the caveolae fraction. MA104 cells were incubated either at 37°C (*Control, Cold Folic Acid, Acid Strip, Acid Strip+37°C*) or at 4°C (*4°C Binding*) for 1 h in the presence of 5 nM [^{3}H]folic acid. Cells were either used immediately (*Control* 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 [^{3}H]folic acid in the respective caveolae fractions was: control, 69%; 4°C binding, 68%; acid stripped, 64%; acid stripped+37°C, 70%; and cold folic acid, 70%.

Table II. Effects of Filipin on Distribution of Folate Receptor

Sample	Protein	Folate	Protein	Protein yield	DPM yield (PNS)	DPM yield (PM)
	mg	*pmol*	*pmol/mg*	*%*	*%*	*%*
PNS	2.51	7.24	2.88	100	100	
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	100
NCM	0.39	3.64	9.33	16	50	67
CM	0.025	1.77	71	1	24	32

MA104 cells were preincubated in the presence of 10 μg/ml filipin for 15 min before 5 nM [^{3}H]folic acid was added, and the cells were incubated an additional 1 h at 37°C. Cells were then processed to isolate caveolae as described. Each fraction was assayed for the amount of protein and the quantity [^{3}H]folic acid. *PNS*, 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*, noncaveolae membrane (fractions 8–15, OptiPrep 1); *CM*, caveolae membrane (fraction 2, OptiPrep 2).

increase of [^{3}H]folic acid in the noncaveolae 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 caveolae fraction. Therefore, two different assays (immunoblotting and folic acid binding) detected filipin-induced movement of folate receptors from the caveolae fraction to fractions containing the bulk of the plasma membrane.

Both functional folate receptors (22) and a caveolae 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 [^{3}H]folic acid internalization. Ordinarily, the ratio of internal to external bound [^{3}H]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 caveolae 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

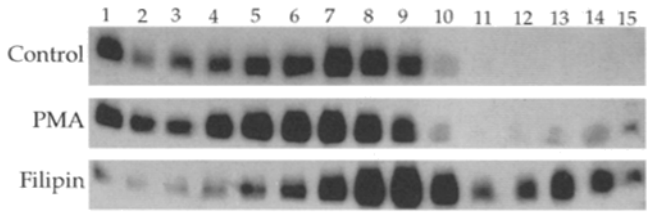

Figure 3. Filipin shifts the folate receptor from caveolae to the bulk membrane fractions. MA104 cells were either not treated (*Control*), incubated for 15 min in the presence of 10 μg/ml filipin (*Filipin*), or incubated in the presence of 1 μM PMA for 1 h at 37°C. At the end of the treatment, plasma 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.

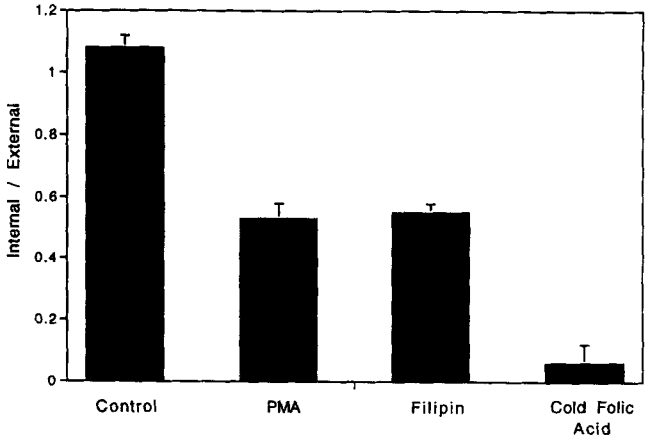

Figure 4. Filipin inhibits internalization of [^{3}H]folic acid. MA104 cells were incubated in the presence of media alone for 1 h (*Control*), 1 μM PMA (*PMA*) for 1 h, or 10 μg/ml filipin for 15 min before the addition of 5 nM [^{3}H]folic acid and an additional 1-h incubation at 37°C. At the end of the incubations, the ratio of internal to external receptors was measured as described. All values are the average of six trials ±SD.

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. 5 *A*), cells were incubated in the presence or absence of different combinations of antibodies for 1 h 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 [^{3}H]folic acid, MA104 cells were incubated for 1 h at 37°C in the presence or absence of Mov19 before adding [^{3}H]folic acid to the dish for an additional 1 h (Fig. 5 *B*). Caveolae were isolated and the amount of bound [^{3}H]folic acid was measured. Untreated cells (*Control*) had a specific binding of ~150 pmol/mg of protein in the caveolae 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 [^{3}H]folic acid lost from the caveolae fraction was shifted to the noncaveolae fraction (Table III). If the cells were incubated sequentially with Mov19 and anti-mouse IgG (*Mov19 + 2°Ab*), the specific binding of [^{3}H]folic acid in the caveolae fraction returned to control values. The anti-mouse IgG alone had no effect (*2°Ab*), and neither did Mov18 (*Mov18*).

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

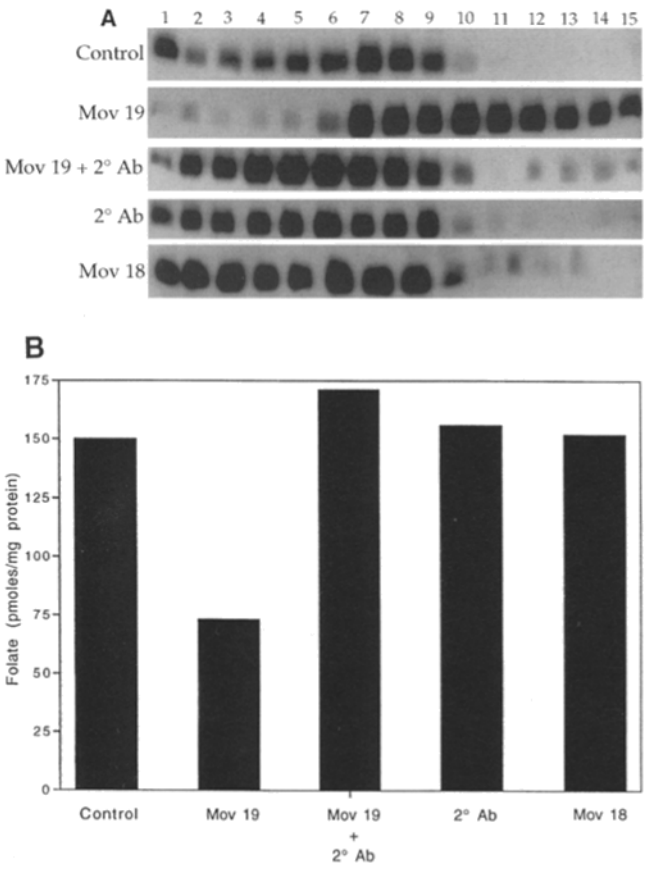

Figure 5. Effect of Mov19 on the distribution of either the folate receptor (*A*) or bound [^{3}H]folic acid in the plasma membrane (*B*). MA104 cells were incubated in the absence (*Control*) or presence of 10 μg/ml of the indicated antibody for 1 h at 37°C. (*A*) Plasma membranes were prepared, sonicated, and separated on OptiPrep 1 gradient. The total protein from each fraction was loaded on gels and immunoblotted with mAb anti-folate receptor Mov19. (*B*) Cells were incubated in the presence of 5 nM [^{3}H]folic acid for 1 h at 37°C before caveolae fractions were collected from OptiPrep 2 gradients.

anti–mouse IgG alone (*2°Ab*), or Mov18 (*Mov18*) before assaying for the internal to external bound [^{3}H]folic acid ratio. Only Mov19 inhibited internalization. Mov19 also blocked the delivery of 5-[^{3}H]methyltetrahydrofolate to the cytoplasm (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 Cytoplasm 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-[^{3}H]methyltetrahydrofolate moves directly from the plasma membrane to the cytosol of the cell, and the blockage of this step by monensin (11). To be sure we could detect movement of a ligand to internal membranes with this procedure, we tested ^{125}I-transferrin. MA104 cells were incubated in the presence of ^{125}I-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 ^{125}I-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 ^{125}I-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-[^{3}H]methyltetrahydrofolate through endosomes if this were the route to the cytoplasm.

We used ^{125}I-transferrin and 5-[^{3}H]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 ^{125}I-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 ^{125}I-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 ^{125}I-transferrin had 50% less radiolabel associated with the membrane fraction (△), 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 ^{125}I-transferrin, it had no effect on the internalization of 5-[^{3}H]methyltetrahydrofolate (Fig. 8 *B*). Neither the internalization of membrane-bound vitamin (compare △ with ○) nor the delivery to the cytoplasm (compare ■ with ▲ on the ordinate) was affected by PAO.

Table III. Effects of Mov19 on Distribution of Folate Receptor

Sample	Protein	Folate	Protein	Protein Yield	DPM yield (PNS)	DMP yield (PM)
	mg	*pmol*	*pmol/mg*	*%*	*%*	*%*
PNS	2.76	8.69	3.15	100	100	
CYTO	1.98	0.13	0.07	71	1.5	
IM	0.21	1.08	5.14	8	12	
PM	0.44	6.52	14.82	16	75	100
NCM	0.44	4.44	10.09	16	51	68
CM	0.029	2.12	73	1	24	32

MA104 cells were preincubated in the presence of 10 μg/ml Mov 19 for 60 min before 5 nM [^{3}H]folic acid was added, and the cells were incubated an additional 1 h at 37°C. Cells were then processed to isolate caveolae as described. Each fraction was assayed for the amount of protein and the quantity of [^{3}H]folic acid. *PNS*, 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*, noncaveolae membrane (fractions 8–15, OptiPrep 1); *CM*, caveolae membrane (fraction 2, OptiPrep 2).

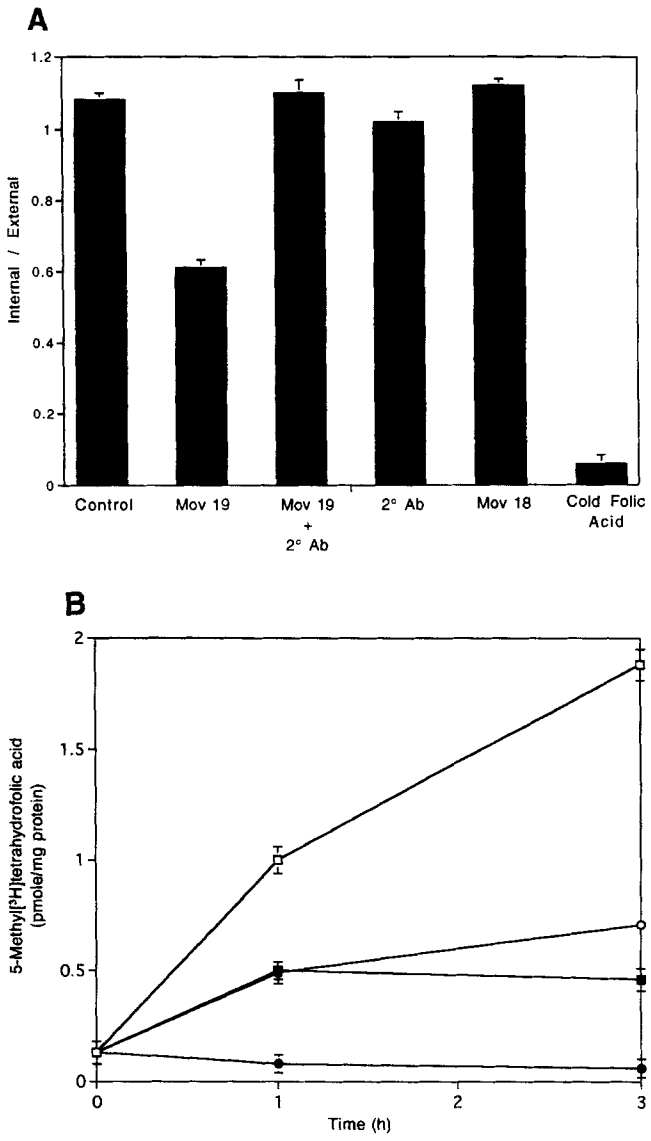

Figure 6. Effect of Mov19 on the internal to external folate receptor ratio (*A*) and the uptake of 5-[^{3}H]methyltetrahydrofolate (*B*). (*A*) MA104 cells were incubated in the absence (*Control*) or presence of 10 μg/ml of the indicated antibody for 1 h at 37°C. [^{3}H]folic acid (5 nM) was added to the dish and the cells were incubated an additional 1 h. At the end of the incubation, cells were chilled to 4°C and assayed for acid resistant (internal) and acid labile (external receptors) [^{3}H]folic acid. (*B*) MA104 cells were incubated in media alone (□) or in media that contained either 10 μg/ml of Mov19 (■) or 1 μM PMA (○). 5-[^{3}H]methyltetrahydrofolate (10 nM) was added to the dish, and the cells were incubated for the indicated time. The presence of excess cold 5-methyltetrahydrofolate blocked uptake of 5-[^{3}H]methyltetrahydrofolate (●). All values are the average of six trials ±SD.

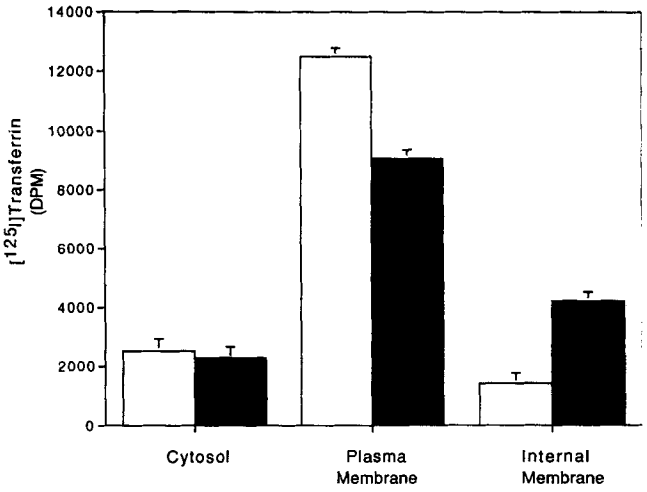

Figure 7. ^{125}I-transferrin migrates to internal membranes. MA104 cells were incubated for 30 min in the presence of 0.32 μg/ml ^{125}I-transferrin at 4°C. The cells were washed and either not warmed (*open column*) or warmed for 30 min (*solid column*). Cells were processed to isolate cytosol, plasma membrane, and internal membranes from Percoll gradients as described. The amount of ^{125}I-transferrin in each of these fractions was then measured. All values are the average of four trials ±SD.

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 ^{125}I-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).

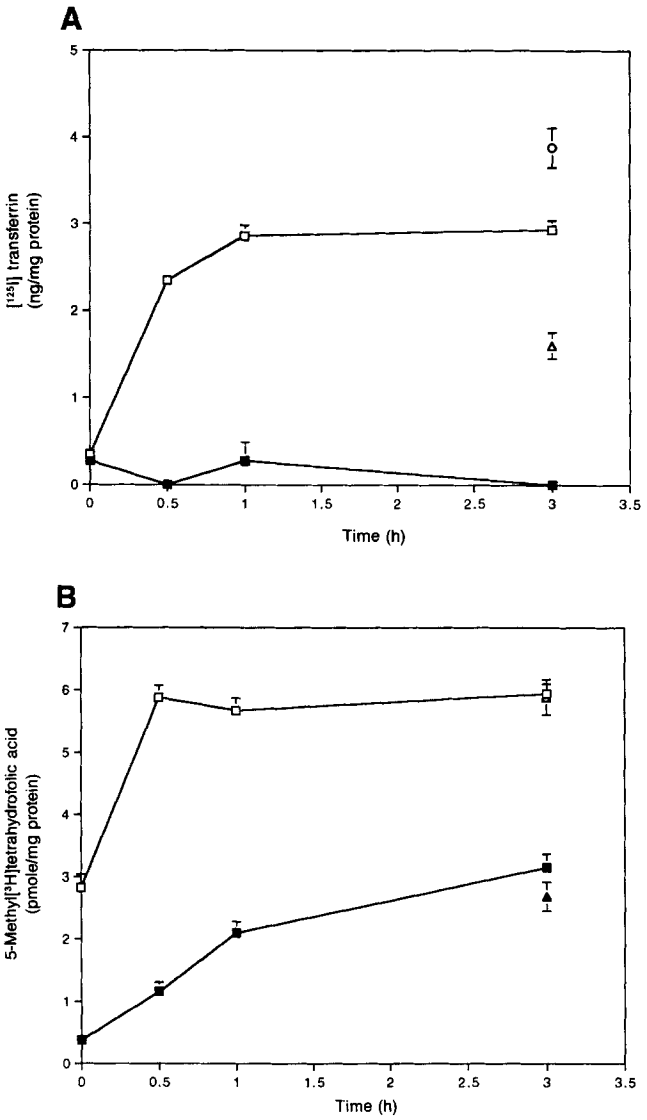

Figure 8. Effect of PAO and PMA on the uptake of either ^{125}I-transferrin (*A*) or 5-[^{3}H]methyltetrahydrofolate (*B*). (*A*) In the presence of 0.32 μg/ml ^{125}I-transferrin, MA104 cells were incubated in media that contained either no additions (□, ■), 1 μM PMA (○), or 20 μM PAO (△) for the indicated time at 37°C. At the end of the incubation, total membrane (□, ○, △) or cytosol associated (■) ^{125}I-transferrin was measured. (*B*) In the presence of 10 nM 5-[^{3}H]methyltetrahydrofolate, MA104 cells were incubated in media that contained either no additions (□, ■) or 20 μM PAO (△, ▲) for the indicated times. Membranes (□, △) were separated from cytosol (■, ▲), and the amount of 5-[^{3}H]methyltetrahydrofolate was measured in each fraction. All values are the average of six trials ±SD.

This means caveolae membrane must have physical characteristics that are independent of membrane shape. The flask-shaped membrane originally designated as caveolae (34) may correspond to the subset of caveolae membrane captured by the fixation process during internalization. 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 caveolae 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-[^{3}H]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 caveolae 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.

We thank William Donzell and Grace Liao for their valuable technical assistance, and Stephanie Baldock for administrative assistance.

This work was supported by grants from the National Institutes of Health (HL 20948, GM 43169, GM 15631) and the Perot Family Foundation.

Received for publication 27 February 1996 and in revised form 19 June 1996.

References

1. Anderson, R.G.W. 1991. Molecular motors that shape endocytic membrane. *In* Intracellular Trafficking of Proteins. C.J. Steer and J.A. Hanover, editors. Cambridge University Press, London. 13–47.
2. Anderson, R.G.W., J.L. Goldstein, and M.S. Brown. 1976. A mutation that impairs the ability of lipoprotein receptors to localize in coated pits on the cell surface of human fibroblasts. *Nature (Lond.).* 270:695–699.
3. Anderson, R.G.W., M.S. Brown, and J.L. Goldstein. 1977. Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell.* 10:351–364.
4. Anderson, R.G.W., B.A. Kamen, K.G. Rothberg, and S.W. Lacey. 1992. Potocytosis: sequestration and transport of small molecules by caveolae. *Science (Wash. DC).* 255:410–411.
5. Andreasen, P.A., L. Sottrup-Jensen, L. Kjoller, A. Nykjær, S.K. Moestrup, C.M. Petersen, and J. Gliemann. 1994. Receptor-mediated endocytosis of plasminogen activators and activator/inhibitor complexes. *FEBS Lett.* 338:239–245.
6. Chang, W.-J., K.G. Rothberg, B.A. Kamen, and R.G.W. Anderson. 1992. Lowering the cholesterol content of MA104 cells inhibits receptor-mediated transport of folate. *J. Cell Biol.* 118:63–69.
7. Chang, W.-J., Y.-S. Ying, K.G. Rothberg, N.M. Hooper, A.J. Turner, H.A. Gambliel, J. De Gunzburg, S.M. Mumby, A.G. Gilman, and R.G.W. Anderson. 1994. Purification and characterization of smooth muscle cell caveolae. *J. Cell Biol.* 126:127–138.
8. Davis, C.G., M.A. Lehrman, D.W. Russell, R.G.W. Anderson, M.S. Brown, and J.L. Goldstein. 1986. The J.D. mutation in familial hypercholesterolemia: amino acid substitution in cytoplasmic domain impedes internalization of LDL receptors. *Cell.* 45:15–24.
9. Jemmerson, R., and M. Agree. 1987. Monoclonal antibodies to different epitopes on a cell-surface enzyme, human placental alkaline phosphatase, effect different patterns of labeling with protein A-colloidal gold. *J. Histochem. Cytochem.* 35:1277–1284.
10. Kamen, B.A., and A. Capdevila. 1986. Receptor-mediated folate accumulation is regulated by the cellular folate content. *Proc. Natl. Acad. Sci. USA.* 83:5983–5987.
11. Kamen, B.A., M.T. Wang, A.J. Streckfuss, X. Peryea, and R.G.W. Anderson. 1988. Delivery of folates to the cytoplasm of MA104 cells is mediated by a surface membrane receptor that recycles. *J. Biol. Chem.* 263:13602–13609.
12. Kamen, B.A., C.A. Johnson, M.T. Wang, and R.G.W. Anderson. 1989. Regulation of the cytoplasmic accumulation of 5-methyltetrahydrofolate in MA104 cells is independent of folate receptor regulation. *J. Clin. Invest.* 84:1379–1386.
13. Kamen, B.A., A.K. Smith, and R.G.W. Anderson. 1991. The folate receptor works in tandem with a probenecid-sensitive anion carrier. *J. Clin. Invest.* 87:1442–1449.
14. Kaplan, J., D.M. Ward, and H.S. Wiley. 1985. Phenylarsine oxide-induced increase in alveolar macrophage surface receptors: evidence for fusion of internal receptor pools with the cell surface. *J. Cell Biol.* 101:121–129.
15. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
16. Liu, P., and R.G.W. Anderson. 1995. Compartmentalized production of ceramide at the cell surface. *J. Biol. Chem.* 45:27179–27185.
17. Liu, P., Y.-S. Ying, Y.-G. Ko, and R.G.W. Anderson. 1996. Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. *J. Biol. Chem.* 271:10299–10303.
18. Mayor, S., K.G. Rothberg, and F.R. Maxfield. 1994. Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. *Science (Wash. DC).* 264:1948–1951.
19. Mello, R.J., M.S. Brown, J.L. Goldstein, and R.G.W. Anderson. 1980. LDL receptors in coated vesicles isolated from bovine adrenal corex: binding sites unmasked by detergent treatment. *Cell.* 20:829–837.
20. Mineo, C., G.L. James, E.J. Smart, and R.G.W. Anderson. 1996. Localization of EGF stimulated Ras/Raf-1 interaction to caveolae. *J. Biol. Chem.* 271:11930–11935.
21. Parton, R.G., B. Joggerst, and K. Simons. 1994. Regulated internalization of caveolae. *J. Cell Biol.* 127:1199–1215.
22. Ritter, T.E., O. Fajardo, H. Matsue, R.G.W. Anderson, and S.W. Lacey. 1995. Folate receptors targeted to clathrin-coated pits cannot regulate vitamin uptake. *Proc. Natl. Acad. Sci. USA.* 92:3824–3828.
23. Rothberg, K.G., Y.-S. Ying, B.A. Kamen, and R.G.W. Anderson. 1990. Cholesterol controls the clustering of the glycophospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. *J. Cell Biol.* 111:2931–2938.
24. Rothberg, K.G., Y.-S. Ying, J.F. Kolhouse, B.A. Kamen, and R.G.W. Anderson. 1990. The glycophospholipid-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytic pathway. *J. Cell Biol.* 110:637–649.
25. Rothberg, K.G., J.E. Heuser, W.C. Donzell, Y.-S. Ying, J.R. Glenney, and R.G.W. Anderson. 1992. Caveolin, a protein component of caveolae membrane coats. *Cell.* 68:673–682.
26. Sargiacomo, M., M. Sudol, Z. Tang, and M.P. Lisanti. 1993. Signal transducing molecules and GPI-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J. Cell Biol.* 122:789–808.
27. Schroeder, R., E. London, and D. Brown. 1994. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins. *Proc. Natl. Acad. Sci. USA.* 91: 12130–12134.
28. Shaul, P.W., E.J. Smart, L.J. Robinson, Z. German, Y. Ying, R.G.W. Anderson, and T. Michel. 1996. Acylation targets endothelial nitric oxide synthase to plasmalemmal caveolae. *J. Biol. Chem.* 271:6518–6522.
29. Smart, E.J., D.C. Foster, Y.-S. Ying, B.A. Kamen, and R.G.W. Anderson. 1994. Protein kinase C activators inhibit receptor-mediated potocytosis by preventing internalization of caveolae. *J. Cell Biol.* 124:307–313.
30. Smart, E.J., Y.-U. Ying, P.A. Conrad, and R.G.W. Anderson. 1994. Caveolin moves from caveolae to the Golgi apparatus in response to cholesterol oxidation. *J. Cell Biol.* 127:1185–1197.
31. Smart, E.J., Y.-S. Ying, and R.G.W. Anderson. 1995. Hormonal regulation of caveolae internalization. *J. Cell Biol.* 131:929–938.
32. Smart, E.J., Y.-S. Ying, C. Mineo, and R.G.W. Anderson. 1995. A detergent free method for purifying caveolae membrane from tissue culture cells. *Proc. Natl. Acad. Sci. USA.* 92:10104–10108.
33. van den Berg, C.W., T. Cinek, M.B. Hallett, V. Horejsi, and B.P. Morgan. 1995. Exogenous glycosyl phosphatidylinositol-anchored CD59 associates with kinases in membrane clusters on U937 cells and becomes Ca^{2+}-signaling competent. *J. Cell Biol.* 131:669–677.
34. Yamada, E. 1955. The fine structure of the gall bladder epithelium of the mouse. *J. Biophys. Biochem. Cytol.* 1:445–458.