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Folate receptors targeted to clathrin-coated pits cannot regulate vitamin uptake

(caveolae/glycosyl-phosphatidylinositol/potocytosis/endocytosis/5-methyltetrahydrofolate)

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ABSTRACT **Potocytosis is an endocytic process that is specialized for the internalization of small molecules. Recent studies on the uptake of 5-methyltetrahydrofolate by the folate receptor have suggested that the glycosyl-phosphatidylinositol anchor on this protein causes it to cluster and be internalized by caveolae instead of coated pits. To test this hypothesis directly, we have constructed a chimeric folate receptor that has the glycosyl-phosphatidylinositol anchor replaced with the transmembrane domain and cytoplasmic tail of the low density lipoprotein receptor. The cells with wild-type receptors delivered 5-methyltetrahydrofolate to the cytoplasm more rapidly than did cells expressing the chimeric receptor. This suggests that efficient delivery to the cytoplasm depends on caveolae. In sharp contrast to cells with wild-type folate receptors, cells internalizing folate by clathrin-coated pits were unable to decrease vitamin uptake when they were either folate replete or confluent.**

Cells use specific membrane receptors to concentrate various types of molecules before they are sequestered and delivered to the interior of the cell. There are at least two different sets of receptors: those that govern the uptake of macromolecules such as low density lipoprotein (LDL) and those that handle small molecules such as 5-methyltetrahydrofolate (5-MeTHF). Cells take up macromolecules by receptor-mediated endocytosis (1), using receptors that are internalized by clathrin-coated pits. Many of these receptors use a tight β-turn motif (2, 3) in their cytoplasmic domain to cluster in coated pits (4, 5) prior to internalization. Small molecules, by contrast, appear to enter cells through caveolae by a process called potocytosis (6, 7). The receptors in this latter group are linked to the membrane by glycosyl-phosphatidylinositol (GPI) and it is the lipid anchor that mediates receptor clustering in association with caveolae (8–13).

The molecules that are internalized by these two pathways have quite different fates. Clathrin-coated pits bud from the membrane and form vesicles that merge by a series of regulated fusion reactions with endosomes, lysosomes, and portions of the trans-Golgi network (14, 15). The principal function of this pathway is to deliver macromolecules by lysosomes for hydrolytic processing or to transport them across polarized cells by transcytosis (16). Caveolae, on the other hand, seal off from the plasma membrane but appear to remain separate from other endocytic compartments. The small molecules that are concentrated within each closed caveola reach the cytoplasm by diffusing across the membrane through water-filled channels.

The existence of two separate endocytic pathways, operating side-by-side in the same cell, suggests that caveolae are better able to deliver small molecules to the cytoplasm than are coated pits (6). Clearly it is not the initial internalization step that is advantageous, because the rate of ligand sequestration by caveolae is about 5 times slower (17) than that by clathrin-coated pits (18). Nothing is known, however, about how the two pathways might differ in either the efficiency of ligand delivery to the cytoplasm or the regulation of folate accumulation in the cytoplasm. We have addressed these two questions by constructing a chimeric folate receptor that is internalized by clathrin-coated pits and the comparing uptake of 5-MeTHF by this receptor with uptake by the wild-type receptor in transfected cells.

MATERIALS AND METHODS

Plasmids. The plasmid pG4ZF56 contained a cDNA coding for the human folate receptor (8). The plasmid pLDLR2 contained the cDNA for the human LDL receptor (19) and was used as template for the polymerase chain reaction under plasmid construction. The plasmid pJB20 (gift of Pamela Beck, Texas Biotechnology Corporation, Houston) was used for permanent transfections and was derived from pCMV1 (provided by David W. Russell, University of Texas Southwestern Medical Center, Dallas) (20). pJB20 contained a selection marker for neomycin resistance and the simian virus large tumor 40 (T)-antigen intron in the polycloning site.

Plasmid Constructions. Appropriate oligonucleotides were used to amplify a fragment of the human LDL receptor cDNA corresponding to the anchor/tail domain. The sequence of the sense primer was 5′-AAACTGCAGAGAAGAAGCCCAG-TAGCGTGAGGCTCTG-3′. The terminal G of the *Pst* I site (underlined) is the first base coding for Glu-760 of the LDL receptor. The sequence of the antisense primer was 5′-AAACCCGGGTCATCACGCCACCCCGTCTACCTCCAG-ACTGACCAT-3′. Amplification introduced a *Sma* I site (underlined), and there is a second stop codon immediately downstream of the native stop codon. The amplified fragment was subcloned between the *Pst* I and *Sma* I sites of pGEM-4Z (Promega) and sequenced by the dideoxy chain-termination method (21). The cDNA for the chimeric folate receptor (coated-pit targeted) was constructed by ligating the *Eco*RI–*Pst* I fragment of the folate receptor cDNA (pG4ZF56) (8) and the *Pst* I–*Sma* I fragment containing the LDL receptor anchor domain into pSL2. The wild-type folate receptor expression vector (GPI anchor) was constructed similarly. The constructs were then subcloned into pJB20 to make vectors for selecting cell lines.

Cells in Culture. CHO ldl-A7 is a mutant Chinese hamster ovary (CHO) cell line that lacks both folate and LDL receptors and was kindly provided by Michael S. Brown and Joseph L.

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Abbreviations: GPI, glycosyl-phosphatidylinositol; LDL, low density lipoprotein; 5-MeTHF, 5-methyltetrahydrofolate.

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Goldstein (University of Texas Southwestern Medical Center, Dallas) (22). The cells were transfected by the Polybrene/dimethyl sulfoxide method (23, 24). We cloned permanent lines by selection in standard (folate-rich) Ham's F12 medium supplemented with 10% fetal bovine serum (GIBCO; no. 200-6140PJ), penicillin (50 units/ml) and streptomycin (50 μg/ml) (GIBCO, no. 600-5070PG), glutamine (292 μg/ml) (GIBCO, no. 300-2403AD), and Geneticin (G418, 1 mg/ml; GIBCO, no. 860-1811IJ), termed selection medium. Subsequently, the cells were grown in folate-free F12 medium supplemented with 15 mM Hepes, 10% fetal bovine serum, glutamine, penicillin, and streptomycin, referred to as complete F12.

Indirect Immunofluorescence. Cells were stained with monoclonal anti-folate receptor IgG (25). To stain surface receptors, both cell lines were incubated for 60 min at 4°C with monoclonal anti-folate receptor IgG (10 μg/ml), washed, and incubated for 60 min with rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate. To identify the destination of internalized receptors, both cell types were incubated identically with anti-folate receptor IgG and warmed to 37°C for 30 min. Internalization was examined by fixing with 3% paraformaldehyde, permeabilizing with 0.1% Triton X-100, and incubating with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG for 60 min at room temperature. All the cells were then mounted in DABCO (1,4-diazabicyclo[2.2.2]octane; Aldrich) and photographed with a Zeiss photomicroscope III.

Folate Binding Assay. Acid-labile/acid-resistant [^{3}H]folic acid binding assays were performed by a modification of the method of Kamen *et al.* (26) to track receptor movement between surface (acid-labile) and internalized (acid-resistant) compartments. Folic acid was used because it binds to receptors and is not transported or metabolized but remains bound to receptors. In brief, we performed binding assays by incubating the cells at 37°C in folate-depleted F12 medium supplemented with 20 nM [^{3}H]folic acid. Three plates of cells were used for each time point, two with only [^{3}H]folic acid and a third with 100-fold excess unlabeled ligand to determine nonspecific labeling. Results represent the mean of two experimental values less the nonspecific control. Five milliliters of prewarmed medium was added to each 60-mm dish containing 0.5–1.5 million cells and incubated for various time periods. Distribution of ligand between surface and internal compartments was determined by acid washing. After incubation with [^{3}H]folic acid, the cells were placed on ice, the medium was removed, and the cells were washed with ice-cold phosphate-buffered saline. The cells were then washed for 30 sec with ice-cold acid saline (150 mM NaCl adjusted to pH 3.0 with glacial acetic acid). The acid wash was collected, and the cells were washed once with 1 ml of phosphate-buffered saline. The acid and post-acid washes were then combined for scintillation counting to measure the number of receptors on the cell surface. The cells were harvested with 1 ml of trypsin/EDTA. Each plate received two subsequent washes with phosphate-buffered saline, and the entire collection of trypsinized cells and washes were subjected to scintillation counting to determine the number of internalized receptors.

The labeling phase of experiments that measured cytoplasmic accumulation was identical to those above except that [^{3}H]5-MeTHF was the ligand. 5-MeTHF is the natural ligand for the folate receptor and can undergo transport to the cytoplasm and modification by cytoplasmic enzymes. At the indicated times, the cells were removed from the incubator, washed with ice-cold phosphate-buffered saline, and treated with 1 ml of hypotonic lysis buffer (10 mM Tris, pH 7.5) before subjection to three cycles of rapid freezing at −90°C and thawing at 4°C. The fractured cells were collected and sedi-

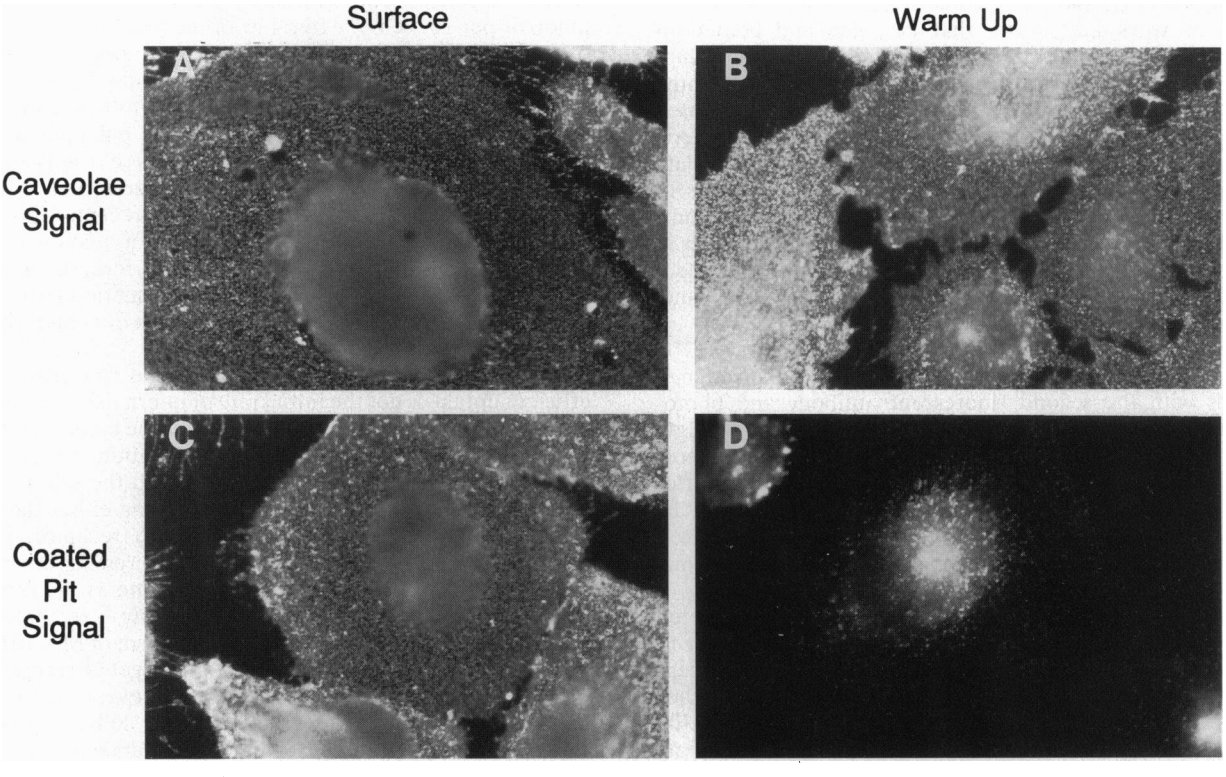

FIG. 1. Internalization of anti-folate receptor IgG by cells expressing either wild-type (caveolae-targeted) (*A* and *B*) or chimeric (coated-pit-targeted (*C* and *D*) folate receptors. Matched sets of cells were chosen that had the same total number of folate receptors, and they were maintained in culture under folate-depleted conditions (27). To stain surface receptors, both sets of cells (*A* and *C*) were incubated for 60 min at 4°C in the presence of monoclonal anti-folate receptor IgG (10 μg/ml), washed, and incubated for 60 min with rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate. For comparison, both sets of cells (*B* and *D*) were incubated identically with anti-folate receptor IgG but then warmed to 37°C for 30 min. The cells (*B* and *D* only) were then fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated for 60 min at room temperature with anti-mouse IgG conjugated to fluorescein isothiocyanate.

mented at 100,000 × *g* for 80 min. The supernatant was harvested and subjected to liquid scintillation counting to measure the number of 5-MeTHF molecules delivered to the cytoplasm per unit time. Results are the mean of two experimental determinations less a nonspecific control. All scintillation counting was performed with Ecolite scintillation cocktail (ICN, no. 882475).

RESULTS

Plasmids that express the chimeric receptor were constructed from the wild-type folate receptor cDNA by replacing the carboxyl-terminal 26-aa signal (aa 232–257) for addition of the GPI anchor with aa 760–838 of the LDL receptor (19). Thus, the chimeric protein has the LDL receptor transmembrane domain and cytoplasmic tail beginning at aa 760 extending from the carboxyl terminus of the folate receptor ectodomain. This method of construction preserved the folate-binding domain (aa 1–231) in each of the proteins. Both cDNAs were inserted into a cytomegalovirus-based plasmid (pJB20) and used to transfect CHO cells, which have neither folate receptors nor LDL receptors (22). Stable cell lines that expressed equal numbers of receptors were selected for study. Previously, we showed that binding affinities for the wild-type and chimeric receptors were 2.1 nM and 3.5 nM, respectively (27).

It was necessary to ascertain whether the intended amino acid sequence of the anchor domain had been preserved during the introduction of restriction sites by the polymerase chain reaction. To confirm this, we sequenced each construct in both directions (21, 24). Phosphatidylinositol-specific phospholipase C removed nearly all the folate receptors from the membranes of cells with caveolae-targeted (wild-type) receptors but had no effect on membranes derived from cells expressing coated-pit targeted receptors (27). Therefore, each receptor possessed the intended clustering signal.

Previous work has shown that caveolae and coated-pit receptors have distinct appearances by indirect immunofluorescence (25). We used a similar method to assess the staining patterns of folate receptors targeted to caveolae and coated pits (Fig. 1). Cells with wild-type (Fig. 1 *A* and *B*) and chimeric (Fig. 1 *C* and *D*) receptors were incubated with anti-folate receptor IgG for 30 min on ice. We washed all the cells to remove the antibody and then divided them into two groups (surface and warm up). We promptly stained both cell types in the surface group (Fig. 1 *A* and *C*) with a second fluorescent antibody. With this method, both cell lines stained in a punctate surface pattern.

We warmed up the second group (Fig. 1 *B* and *D*) to 37°C for 30 min and then permeabilized the cells. We then stained the cells with the second fluorescent antibody and photographed the cells to show internalized anti-folate receptor IgG (Fig. 1 *B* and *D*). The cells with caveolae-targeted receptors continued to exhibit a punctate surface staining pattern after warm up. The cells with coated-pit-targeted receptors, by contrast, cleared the anti-folate receptor IgG from the cell surface and delivered it to perinuclear vesicles in a lysosomal pattern.

The staining patterns after warm up for receptors targeted to caveolae and coated pits (Fig. 1 *B* and *D*) were similar to patterns observed by Rothberg *et al.* (25) for the folate receptor and LDL receptor, respectively. Therefore, the GPI anchor appears to direct folate receptors to caveolae whereas the transmembrane/tail anchor targets chimeric folate receptors to coated pits. These observations demonstrate that these matched cell lines constitute a system for comparing regulation of folate uptake by caveolae and coated pits.

Using [^{3}H]folic acid—which will not dissociate from the receptor after binding (17)—as the ligand, we measured the rates of internalization for the two receptors. When we incubated cells expressing either the wild-type (Fig. 2*A*) or chimeric (Fig. 2*B*) receptor in the presence of [^{3}H]folic acid, the external receptor pool rapidly became saturated (■). This was followed (◇) by a very fast internalization of ligand by the chimeric receptor (15.1 pmol/hr per 10^6 cells) and a comparatively slow rate of sequestration by the wild-type receptor (2.3 pmol/hr per 10^6 cells). After 4 hr of incubation at 37°C, most of the [^{3}H]folic acid bound to chimeric receptors was inside the cell (Fig. 2*B*; 7.5 pmol per 10^6 cells inside and 1.5 pmol per 10^6 cells outside), while the majority of the [^{3}H]folic acid bound to wild-type receptor was outside (Fig. 2*A*, 1.5 pmol per 10^6 cells inside and 7 pmol per 10^6 cells outside). These results indicate that the chimeric folate receptor internalized folate with kinetics that are similar to those of the LDL receptor in human fibroblasts (1) whereas the wild-type receptor behaved like the native receptor in MA 104 cells (17). Therefore, the matched sets of transfected cells expressing the chimeric or wild-type receptor, respectively, reflect the behaviors of coated-pit and caveolar receptors.

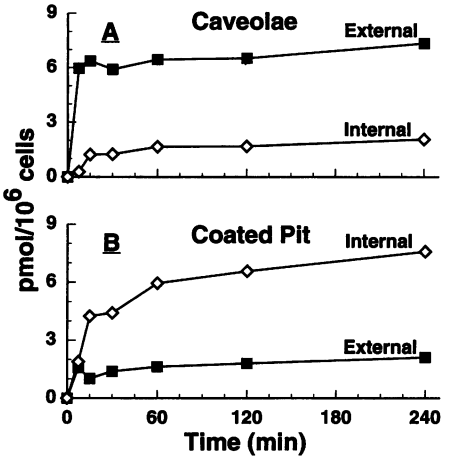

FIG. 2. Distribution of sequestered (◇) and external (■) folate receptors at various times after cells expressing either wild-type (*A*) or chimeric (*B*) receptors were incubated in the presence of [^{3}H]folic acid. [^{3}H]Folic acid, a high-affinity ligand of the folate receptor that remains bound during the recycling of the receptor (17), was used to track the receptor in each type of cell. Cells were grown in folate-depleted medium, incubated in the presence of 20 nM [^{3}H]folic acid (≈5000 cpm/pmol) for the indicated time, and then chilled and assayed for the amount of radioactivity that was sequestered in the cell (internal) and the amount that was exposed on the cell surface (external) by means of a standard acid wash procedure (8). Each point represents the average of duplicate samples less a nonspecific control.

For a small molecule like a vitamin, the true measure of efficient uptake is not the initial rate of internalization but how effectively the sequestered ligand reaches the cytoplasm of the cell where it is used. To make this determination, we compared the rate of receptor sequestration (◇) with the rate of cytoplasmic delivery (■) in CHO cells expressing either the wild-type (Fig. 3*A*) or the chimeric (Fig. 3*B*) receptor. [^{3}H]5-MeTHF reached the cytoplasm of cells expressing the caveolae-targeted receptor at nearly the same rate as the receptor was sequestered (Fig. 3*A*), which is exactly how the native receptor delivers the vitamin to the cytoplasm of MA 104 cells (28). In cells expressing the coated-pit-targeted receptor, by contrast, there was a large difference between the rate of sequestration and the rate of cytoplasmic delivery (Fig. 3*B*, compare ◇ with ■ at 7.5 min). The vitamin reached the cytoplasm far slower than expected from the initial sequestration rates. During the first 15 min of uptake, on average 6–10 molecules of 5-MeTHF were sequestered for each one that reached the cytoplasm. These results suggest that caveolae are more effective than coated pits at cytoplasmic delivery. The difference in efficiency between the two pathways would be more pronounced in cells that express fewer receptors.

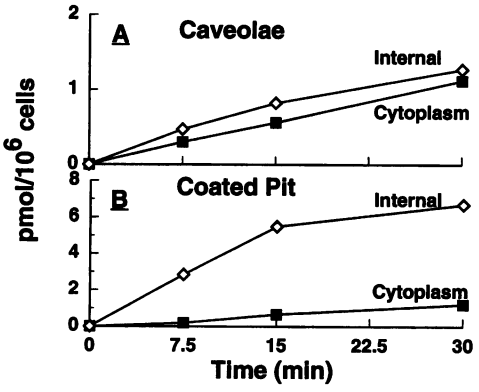

FIG. 3. Rate of receptor sequestration (◇) verses rate of 5-MeTHF delivery to the cytoplasm (■) in cells expressing either wild-type (*A*) or chimeric (*B*) folate receptors. To measure sequestration rates, cells were incubated for the indicated time in the presence of 20 nM [^{3}H]folic acid, chilled to 4°C, and acid washed to remove externally bound ligand. The amount of [^{3}H]folic acid that remained in the cell represents the sequestered pool (◇). 5-MeTHF delivery to the cytoplasm was measured by incubating cells for the indicated time in the presence of [^{3}H]5-MeTHF and chilling the cells to 4°C. The cells were then homogenized and the membrane and cytoplasmic fractions were separated as described (28). Fractions were subjected to scintillation counting. Each point represents the average of duplicate samples less a nonspecific control.

Folate uptake in MA 104 cells is tightly regulated. Folate-depleted cells incubated in the presence of 5-MeTHF stop accumulating the vitamin when the cytoplasmic concentration reaches physiological levels. The number of folate receptors does not change and receptor recycling proceeds normally (17). To determine whether the route of internalization affects this regulatory process, rapidly dividing CHO cells expressing the receptors were incubated in the presence of [^{3}H]5-MeTHF for 4 hr and the amount of folate that reached the cytoplasm was measured. In cells expressing the caveolae-targeted receptor, [^{3}H]5-MeTHF accumulation stopped after the cytoplasm accumulated physiological levels (≈3 pmol per 10^6 cells; Fig. 4*A*, ■). By contrast, the cells expressing the coated-pit-targeted receptor accumulated the vitamin at a linear rate throughout the experiment (◇). In this experiment the wild-type receptor delivered 5-MeTHF to the cytoplasm faster than the chimeric receptor (Fig. 4*A*, initial slope of the curve = 8.7 versus 2.6 pmol/hr per 10^6 cells).

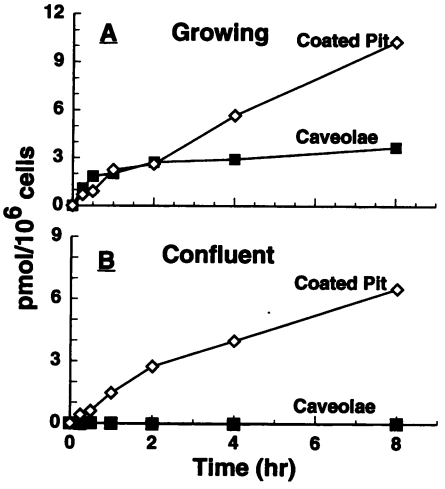

FIG. 4. Effect of folate repletion and cell growth on the accumulation of 5-MeTHF in the cytoplasm of cells expressing either wild-type (◇) or chimeric (■) receptors. Growing cells (*A*) had 10^6 and confluent cells (*B*) had ≈5 × 10^6 cells per 60-mm dish. Each set of cells was incubated for the indicated time in the presence of 20 nM [^{3}H]5-MeTHF and then assayed for the quantity of radioactivity that reached the cytoplasm, as described in Fig. 3. The fraction of the [^{3}H]5-MeTHF that was polyglutamylated was measured by HPLC (29). Each point represents the average of duplicate samples less a nonspecific control.

We next compared the ability of the two sets of cells to accumulate 5-MeTHF when they were confluent and, therefore, not rapidly dividing (Fig. 4*B*). Under these conditions, the wild-type receptor did not deliver any vitamin to the cytoplasm of the cell (■). Uptake in cells with the chimeric receptor, however, occurred at a linear rate just as in growing cells (◇). The 5-MeTHF that reached the cytoplasm was always polyglutamylated (data not shown), regardless of the route of internalization.

DISCUSSION

The results suggest that GPI-anchored receptors are more efficient at delivering small, undigestable molecules such as folate to the cytoplasm than are coated-pit-targeted receptors. This difference is probably due to the influence of separate endocytic pathways. Caveolae appear to sequester ligands bound to GPI-anchored membrane proteins and remain near the cell surface (25) without merging with other endocytic compartments (30). Therefore, caveolae would be able to maintain the initial concentration of any sequestered ligand, which is essential to drive transport into the cytoplasm of the cell. Coated pits, by contrast, bud from the membrane, lose the clathrin coat, and fuse to form endosomes that eventually develop into lysosomes (31). The endomembrane traffic required for this maturation process leads directly to an increase in the volume of the endocytic compartment. Any small molecule or ion sequestered by a coated-pit receptor would be rapidly diluted as it moved through the remainder of the pathway. As a consequence, many more molecules would have to be delivered to the endosomal system to achieve the same cytoplasmic delivery rate as by caveolae.

An unexpected finding during this study was that only GPI-anchored receptors seem to be able to regulate cytoplasmic 5-MeTHF levels. Previous work showed that in MA 104 cells, receptor binding and recycling remained the same after cells stop accumulating 5-MeTHF in the cytoplasm (29). It was proposed at the time that regulation probably occurred by controlling the enzyme that polyglutamylates 5-MeTHF. If this were the case, then accumulation should be regulated independently of the vehicle used for internalization. The current results, however, indicate that the route of entry is critical for regulation. Both GPI-anchored and transmembrane-anchored receptors have the same ability to internalize the vitamin, but only caveolae-targeted receptors are able to regulate cytoplasmic accumulation. This raises the possibility that caveolae not only sequester substances during import but may also be used to export these same molecules to regulate concentration in the cytoplasm.

Mayor *et al.* (32) recently proposed that potocytosis occurs through clathrin-coated pits rather than caveolae. This conclusion was based on the observation that wild-type folate receptors appeared to be randomly distributed in the membrane after a fluorescent anti-folate receptor IgG was applied to the cell. The application of a second antibody was required to see clustered receptors in caveolae. The differential behavior of the chimeric and wild-type folate receptors observed in the current study formally establishes that coated pits are not normally used for the endocytosis of folate. Furthermore, biochemical and morphological experiments have shown that while activators of protein kinase C do not inhibit endocytosis by coated pits (33), they completely inhibit the potocytosis of 5-MeTHF and block the invagination of caveolae (34). Another explanation for the observation of Mayor *et al.* (32) is that the primary antibody may cause GPI-anchored antigens to

uncluster. Thus, the addition of a second antibody may stabilize the natural, clustered organization of these proteins. This interpretation is consistent with the facts that these domains are known to be extremely dynamic (35, 36) and that during the biochemical purification of caveolae (12, 13, 37), the GPI-anchored proteins copurify with the organelle.

In summary, when the folate receptor is targeted to caveolae, cells can dynamically downregulate the accumulation of 5-MeTHF under two distinct conditions, folate repletion and confluence. If the folate receptor is targeted to coated pits, folate accumulation still occurs but these regulatory abilities are completely lost. These observations confirm that the GPI-anchored folate receptor does not deliver 5-MeTHF via coated pits and suggest that cells place receptors in caveolae to exploit their unique regulatory systems.

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