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Clustering of GPI-Anchored Folate Receptor Independent of Both Cross-Linking and Association with Caveolin

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Abstract. The distribution of the glycosyl-phosphatidylinositol (GPI)-anchored folate receptor (FR) in a diffuse pattern vs. functional clusters associated with caveolae has been debated. The equivocal nature of direct localization studies is due to possible experimental artifacts such as cross-linking of the protein by the antibody probes prior to fixation and alternatively the use of a disruptive fixation method. Such studies have also been complicated by the use of cells that vastly overexpress FR. In this study a monovalent probe, i.e., a biotinylated folate affinity analogue was used to covalently label FR. Cells expressing moderate levels of FR, i.e., JAR epithelial cells expressing FR-α and recombinant CHO fibroblasts expressing FR-β, were used. The affinity label and either caveolin or antigenic sites on FR were localized by electron microscopy using colloidal gold conjugated antibody probes post-embedding in the relatively permeable LR White resin. The method avoided both receptor cross-linking and early fixation steps and also enabled the use of transport permissive conditions while labeling FR at the cell surface. The results indicate that in steady-state FR is not significantly colocalized with caveolin. However, the receptor molecules occur predominantly in clusters, independent of cross-linking, providing a physical basis for the observed kinetics of receptor internalization and recycling during folate transport. Evidence is also presented to suggest that early mild fixation will disrupt the clustering of FR.

Key words: Folate receptor — Caveolin — Caveolae — Folate transport — Glycosyl-phosphatidylinositol

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Introduction

The folate receptor (FR) is the only known transport protein that is anchored to the plasma membrane by glycosyl-phosphatidylinositol (GPI) (Cross, 1990). The receptor is N-glycosylated with a polypeptide molecular weight of approximately 28,000 and binds one molecule of folate per receptor molecule (Antony, 1992). The receptor mediates energy dependent uptake of folate compounds into cells. Multiple tissue specific isoforms of the receptor, whose primary structures are 70–80 percent identical have been identified (Ratnam et al., 1989; Lacey et al., 1989; Elwood, 1989; Brigle et al., 1991; Shen et al., 1994, 1995; Ross, Chaudhuri & Ratnam, 1994). FR-α (epithelial tissues) and FR-β (nonepithelial tissues) are GPI anchored (Yan et al., 1995) while FR-γ is a secretory protein (Shen et al., 1995).

The completely extracellular orientation of FR implied by a GPI membrane anchor has led to the proposal of a novel mechanism for FR mediated folate transport and is termed potocytosis (Anderson et al., 1992; Anderson, 1993). The hypothesis involves recycling of FR molecules clustered in caveolin-coated (Rothberg et al., 1992) pits (termed caveolae) between the extracellular surface and an invaginated compartment; the acidity of this compartment causes receptor-bound folates to be released at a high local concentration within the vesicle (Kamen et al., 1988, 1989; Rothberg et al., 1990). A V-type proton pump has been implicated in the acidification step (Prasad et al., 1994). A second membrane carrier is invoked to explain folate transport across the vesicle membrane into the cytoplasm; this carrier is identified by the inhibitory effect of high concentrations of probenecid on the transmembrane folate transport step (Kamen, Smith & Anderson, 1991). Furthermore, such a

mechanism has been proposed (Anderson et al., 1992; Anderson, 1993) for the internalization of other small molecules including potential signaling molecules generated from glycosyl-phosphatidylinositols in response to peptide hormones and growth factors (Low & Saltiel, 1988; Saltiel, 1991; Misek & Saltiel, 1992). A variety of GPI-anchored proteins have been proposed as candidates for potocytosis by virtue of their proposed association with caveolae, which are known to occur in a variety of cell types. Cell surface localization of GPI-anchored proteins is also important in view of the proposed association of these proteins with the Src family of nonreceptor tyrosine kinases Lck and Fyn, trimeric G proteins and small G proteins (Stephanova et al., 1991; Cinek & Horejsi, 1992; Shenoy-Scaria et al., 1992, 1993; Brown, 1993; Lisanti et al., 1994) and the potential significance of such noncovalent detergent insoluble complexes in GPI-protein mediated signal transduction events. However, biochemical studies demonstrating the presence of GPI-protein enriched detergent resistant membrane fractions cannot exclude the possibility of formation of such complexes as an artifact of the detergent extraction procedures (Casey, 1995; Mayor & Maxfield, 1995).

Direct electron microscopic studies examining the possible clustering and association of GPI-anchored proteins with caveolae are also subject to potential experimental artifacts. The initial data (Rothberg et al., 1990; Ying, Anderson & Rothberg, 1992) on direct localization of FR in caveolae was attributed (Mayor, Rothberg & Maxfield, 1994) to cross-linking and mobilization of FR molecules by the primary, secondary and tertiary (colloidal gold conjugated) antibody probes prior to fixing the cell surface proteins. It was shown that by introducing an additional fixation step (3% paraformaldehyde and 0.5% glutaraldehyde) prior to addition of secondary antibody, the clustering of FR molecules was lost; instead, the localizing gold particles appeared to be diffusely distributed on the cell surface (Mayor et al., 1994). The latter method may in turn be criticized because of the potential disruption of receptor clusters due to the additional early fixation step.

The present study was designed to directly localize FR molecules in relation to one another and to caveolin by a method that would avoid mobilization of receptor molecules in a manner that could potentially cause either receptor clustering or dissipation of molecules from preexisting receptor clusters. Furthermore, it was of interest to localize FR molecules by labeling at the cell surface under transport permissive conditions. It was also desirable to avoid potential mistargeting of receptor molecules that may be expected in overexpressing systems (Rijnboutt et al., 1996). These objectives were accomplished by using a monovalent biotinylated folate affinity analog (Biotin-SS-Folate) and a post-embedding method of probing with antibodies. Different cell types i.e., epithelial cells (JAR cells expressing FR-α) and fibroblasts (recombinant CHO cells expressing FR-β) (CHO-FR-β) were chosen for the studies based on their moderate FR expression levels.

Materials and Methods

Chemicals

[^{3}H]folic acid was purchased from Moravek Biochemicals, Brea, CA. Folate free RPMI 1640 medium, Dulbecco's Modified Eagle Medium (D-MEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), penicillin-streptomycin, glutamine, L-proline, lipofectin and geneticin were obtained from GIBCO BRL, Grand Island, NY. RPMI 1640 medium was purchased from Irvine Scientific, Santa Ana, CA. Methotrexate (MTX), adenosine, deoxyadenosine, and thymidine were from Sigma Chemical, St. Louis, MO.

Antibodies

Purified placental FR was immobilized on AH-Sepharose (Pharmacia) and the fraction of the IgG in a rabbit antiserum to FR that specifically bound to the immobilized FR was purified as described (Ratnam et al., 1989). Affinity purified polyclonal rabbit anti-caveolin antibody was purchased from Transduction Laboratories, Lexington, KY. EM grade gold conjugates, goat anti-rabbit IgG (5 nm gold), and goat anti-biotin (10-nm gold) were obtained from Ted Pella, Redding, CA, while goat anti-rabbit IgG (10 nm gold) was from Amersham Life Science, Arlington Heights, IL.

Cell Culture

All culture media were supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cells were purchased from American Type Culture Collection (Rockville, MD) unless otherwise indicated. JAR cells were grown in RPMI 1640 media. CHO cells deficient in dihydrofolate reductase (CHO-dhfr$^-$ cells) were grown in D-MEM supplemented with 150 mg/L of L-proline, 10 mg/L of adenosine, 10 mg/L of deoxyadenosine, and 10 mg/L of thymidine. CHO cell transfactants containing the neomycin resistance gene were grown in the above medium containing 1 mg/ml geneticin (active concentration: 0.7 mg/ml). Transfectants containing both the neomycin resistance gene and the dihydrofolate reductase gene were grown in the above medium with 2 μM MTX instead of nucleosides. JAR cells and recombinant CHO cells were alternately grown in folate free RPMI 1640 supplemented with the appropriate reagents, as described above.

Production of Recombinant CHO Cells

The cDNA for FR-β was placed at the XbaI site in the polylinker region of the expression vector pcDNAIneo (Invitrogen), and the resulting plasmid (FR-β-pcDNAIneo) was amplified in *Escherichia coli MC1061/p3.* CHO-dhfr$^-$ cells were transfected with FR-β-pcDNAIneo using lipofectin. Transfectants expressing the neomycin resistance gene were selected in the presence of geneticin (1 mg/ml) for 20 days. The cells were then transfected with a second plasmid (pSV2) containing the dhfr gene (pSV2-dhfr). This plasmid has a DNA sequence that is in part homologous to that of pCDNAIneo. The genes in the trans-

fected cells were amplified by stepwise selection in MTX (10 nM-2 μM). Stable CHO cell transfectants were cloned by serial dilution.

Transport Measurements of [^{3}H]Folic Acid

Cells were grown in folate free-RPMI 1640 medium for at least 48 hr before this procedure. Cells were first washed successively at 4°C with Hank's balanced salt solution (HBSS), acidic buffer (10 mM sodium acetate, pH 4.7/150 mM sodium chloride/7 mM D-glucose) and HBSS in order to remove endogenous receptor bound folate. The cells were incubated at 37°C and 5% CO_2 for 2 hr in folate-free RPMI 1640 medium containing 5% Norit A charcoal treated fetal bovine serum and 50 nM [^{3}H]folic acid. The cells were then acid washed as above and trypsinized. The radioactivity due to internalized folate was determined by liquid scintillation counting. Specificity of uptake of the labeled compounds via FR was ensured using negative controls in which the cells were incubated for 10 min with excess unlabeled folic acid prior to the addition of the labeled compounds.

Synthesis and Activation of Biotin-SS-Folate

Biotin-SS-Folate was synthesized as described previously (Fan et al., 1991). For activation, the compound (0.8 mg) was dissolved in 100 μl of dimethylsulfoxide (DMSO), diluted in 20 ml deionized H_2O (pH adjusted to 7.5), and loaded on a C_{18} cartridge; the bound compound was protonated by passing 2 L of 2% acetic acid through the cartridge, and eluted with 0.05% trifluoroacetic acid/50% acetonitrile; the compound was dried and dissolved in 100 μl DMSO; 1 μl of 100 mM N-hydroxysuccinimide (NHS) and 1 μl of 100 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) were added to 3 μl of the protonated probe, mixed thoroughly, and incubated in the dark for 1 hr at room temperature before use.

Determination of Covalent Labeling Efficiency of Activated Biotin-SS-Folate

Crude membrane preparations from CHO-FR-β and JAR cells were prepared as previously described (Wang et al., 1992). Membranes equivalent to 1.1 pmol of FR-β (CHO-FR-β cells) or 2.9 pmol of FR-α (JAR cells) were incubated in 0.5 ml of HBSS at 37°C with or without 16 μl of activated Biotin-SS-Folate for 2 hr. The membranes were then sedimented, and resuspended in 0.5 ml of acid buffer (10 mM sodium acetate pH 3.5, 150 mM sodium chloride) to remove noncovalently bound probe. The membranes were sedimented, resuspended in HBSS containing 4 μM [^{3}H]folic acid and incubated for 15 min at 37°C. The membranes were then sedimented and dissolved in 0.5 ml PBS (10 mM sodium phosphate buffer, pH 7.5/150 mM sodium chloride) containing 1% Triton X-100 and the radioactivity was measured by liquid scintillation counting.

Western Blot Analysis

Crude membrane preparations from CHO-FR-β and L1210 cells were prepared as previously described (Wang et al., 1992). The total membrane proteins from CHO-FR-β cells (0.5 μg) or L1210 cells (10 μg) were electrophoresed on a 12% SDS polyacrylamide gel. A human endothelial cell lysate (provided by Transduction Laboratories) was used as the positive control. After transfer to nitrocellulose, the filter was probed with the affinity purified polyclonal anti-caveolin antibodies (1:2000 dilution) and secondary alkaline phosphatase conjugated goat anti-rabbit antibodies according to the procedure described previously (Wang et al., 1992).

Electron Microscopy

Cells were first washed successively at 4°C with HBSS, acidic buffer (pH 4.7) and HBSS. Activated Biotin-SS-Folate (24 μg) was added to 1 ml of HBSS in a 35-mm tissue culture well containing 1–2 × 10^6 cells. After incubation at 37°C for 2 hr, cells were washed with cold acidic buffer to remove the noncovalently bound probe, followed by washing with HBSS. The cells were then fixed with 3% glutaraldehyde for 30 min. After washing with 0.2 M sodium cacodylate (pH 7.2) and alcohol dehydration, the cells were embedded in LR-white resin, and polymerized at 55°C for 2 days. The sections were first treated with PBS containing 1% bovine serum albumin (BSA) for 15 min. To detect Biotin-SS-Folate, the sections were probed with 10 nm gold conjugated goat anti-biotin antibody (1:100 dilution) for 1.5 hr. Caveolin was detected by incubating with rabbit anti-caveolin antibody (1:250 dilution) for 2.5 hr, followed by incubation with 5 nm gold conjugated goat anti-rabbit IgG (1:100 dilution) for 1.5 hr. To detect antigenic sites on FR, sections were probed with affinity purified rabbit polyclonal antibodies to FR-β for 2.5 hr, followed by the 5 nm gold conjugated goat anti-rabbit IgG for 1.5 hr. All of the antibodies were diluted in PBS containing 1% BSA, and the grids were washed 5 times with the same buffer between antibody incubations. The grids were examined under a CM-10 Philips electron microscope. The primary antibody probes were omitted in certain negative controls.

Two alternative methods were also used to visualize FR molecules by electron microscopy. In one, the cells were initially fixed with 3% paraformaldehyde/0.5% glutaraldehyde followed by incubation in HBSS for 1 hr at room temperature. The cells were then fixed with 3% glutaraldehyde and embedded in LR White resin as previously described. The sections were first treated with PBS containing 1% bovine serum albumin (BSA) for 15 min. To detect antigenic sites on FR, sections were probed with affinity purified rabbit polycolonal antibodies to FR-β for 2.5 hr, followed by the 10 nm gold conjugated goat anti-rabbit IgG for 1.5 hr as described above.

In the second procedure, the cells were incubated with the primary antibodies for 30 min at room temperature, followed by the secondary (10 nm gold conjugated) antibodies for 30 min at room temperature. The antibodies were diluted in HBSS containing 30 mM NaN_3 and 1% BSA, and the cells were washed in the same buffer 3 times between antibody incubations. The cells were then fixed with 3% glutaraldehyde and embedded in LR White resin. The sections were examined under a CM-10 Philips electron microscope.

Results and Discussion

Functionality of FR in CHO-FR-β and JAR Cells

Prior to undertaking studies of the localization of FR in CHO-FR-β and JAR cells it was necessary to establish that the FRs in these cells are capable of transporting folate. Specific receptor-mediated folate uptake was measured in the cells by measuring the amount of [^{3}H]folic acid that was internalized in 2 hr at 37°C at a physiologically relevant concentration (50 nM) and that could be inhibited by excess unlabeled folic acid (Fig. 1). In addition, the receptor mediation of [^{3}H]folic acid uptake in CHO-FR-β cells was established by comparison with

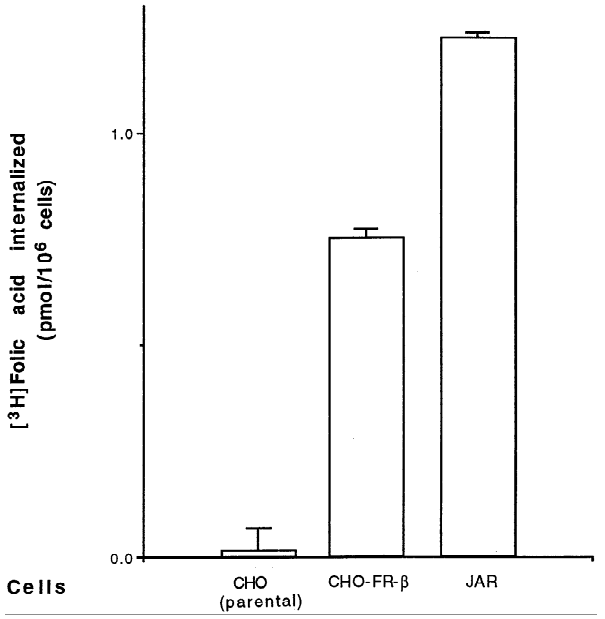

Fig. 1. [^{3}H]Folic acid uptake in parental CHO cells, CHO-FR-β cells and JAR cells. The cells were incubated with 50 nM [^{3}H]folic acid in folate free RPMI 1640 medium with 5% Norit A charcoal treated FBS at 37°C in 5% CO_2 for 2 hr. The cell surface bound [^{3}H]folic acid was removed by treatment at low pH, and the intracellular radioactivity was measured as described under Methods. The negative control samples were treated with excess unlabeled folic acid.

the uptake by parental CHO cells which was relatively insignificant (Fig. 1). These results demonstrate that both FR-β in CHO-FR-β cells and FR-α in JAR cells are functional.

Efficiency of Covalent Labeling with Biotin-SS-Folate

The N-hydroxysuccinimide ester of Biotin-SS-Folate (Fig. 2) was used in this study to covalently label FR over a 2-hr period at 37°C following specific binding to the receptor at the cell surface. To determine the labeling efficiency under these conditions, membrane preparations from CHO-FR-β and JAR cells were used (Fig. 3). In this experiment the extent of covalent labeling of FR was determined by measuring residual [^{3}H]folic acid binding sites at the end of the reaction. At saturating concentrations of the affinity reagent 23 and 40 percent respectively of the FR from CHO-FR-β cells and JAR cells were labeled (Fig. 3).

Specificity of the Anticaveolin Antibodies to Caveolin

When the total membrane proteins in CHO-FR-β cells were probed on a western blot using the affinity purified polyclonal antibodies to caveolin, a single protein band of apparent Mr 22 kDa was obtained (Fig. 4). The band showed the expected mobility for caveolin in endothelial cells (positive control); the protein was also absent in the membranes of L1210 lymphocytes that do not contain caveolae (negative control) (Fig. 4). Further, as discussed below the antibodies did not interact with cellular proteins in L1210 cells under the experimental conditions used for electron microscopy in this study. The results demonstrate the specificity of the antibody probe for caveolin.

Localization of FR in Relation to Caveolin

Folate receptor molecules accessible at the cell surface were labeled covalently with the monovalent Biotin-SS-Folate under transport permissive conditions. The cells were then fixed with 3% glutaraldehyde as in the procedure of Rothberg et al. (1990) followed by embedding in LR White resin because this medium is relatively permeable to colloidal gold conjugated antibody probes. The sections were probed with antibodies without prior osmication to preserve antigenicity and because osmication is known to result in reactivity with LR White resin. Treatment with uranyl acetate was also avoided because it rapidly generated background staining that hindered visualization of colloidal gold particles. The biotinylated affinity label was localized directly using 10 nm gold conjugated anti-biotin antibody. Either FR or caveolin were localized using the appropriate affinity purified polyclonal antibody followed by 5 nm gold particle conjugated secondary antibody. Although this post-embedding technique using LR White did not allow morphological characterization of cellular substructures including caveolae, it was suitable for examining possible spatial relationships among FR molecules and between FR and caveolin. Despite the experimental limitations described above, the method used in this study for localizing FR and caveolin provided a unique advantage in that experimental procedures in previous studies that could potentially cause receptor mobilization could be excluded.

The JAR epithelial cell line which naturally expresses FR-α and is a suggested candidate for potocytosis (Prasad et al., 1994) was used in these experiments. Since no established nonepithelial cell lines are known to express a significant amount of FR, recombinant CHO fibroblasts expressing FR-β were used in this study. Both of these cell lines express moderate levels of FR i.e., 3.4×10^5 and 5.3×10^5 receptors per cell for the CHO-FR-β and JAR cell lines, respectively. The moderate expression of FR in these cells will presumably ensure against possible random localization of receptor molecules as observed in certain established cell lines e.g., KB cells ($2–3 \times 10^7$ receptors per cell) and recom-

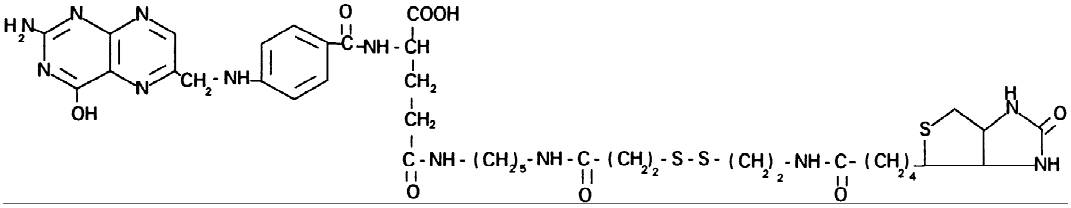

Fig. 2. Structure of Biotin-SS-Folate.

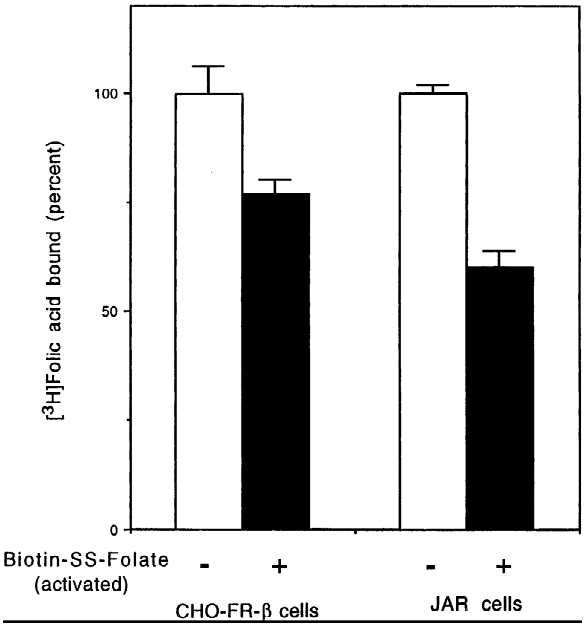

Fig. 3. Covalent labeling efficiency of Biotin-SS-Folate. Membranes from CHO-FR-β and JAR cells were incubated in HBSS with (solid bar) or without (open bar) NHS-activated Biotin-SS-Folate for 2 hr. The membranes were then washed with acidic buffer to remove non-covalently bound probe followed by incubation with 4 μM [^{3}H]folic acid for 15 min at 37°C. The membranes were sedimented and the bound [^{3}H]folic acid was measured by liquid scintillation counting.

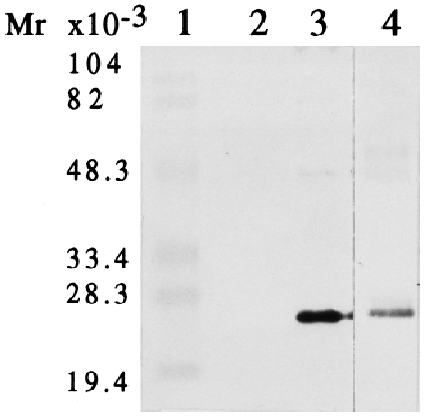

Fig. 4. Specificity of anticaveolin antibodies. Western blot analysis of crude membrane proteins from L1210 cells (lane 2) and CHO-FR-β cells (lane 3) and a human endothelial cell lysate (lane 4). Prestained molecular weight standards were loaded in lane 1. The blot was probed with affinity purified polyclonal rabbit anti-caveolin antibodies followed by alkaline phosphatase conjugated goat-anti-rabbit IgG as described in Materials and Methods.

binant cell lines which express FR at levels comparable to or greater than the levels of caveolin (Rijnboutt et al., 1996). In initial electron microscopy studies using FR specific antibody probes it was possible to observe either clustering or a diffuse distribution of FR by essentially following previously published procedures (Rothberg et al., 1990 and Mayor et al., 1994).

In CHO-FR-β cells and JAR cells, 52.7 and 62.3 percent respectively of the 10 nm colloidal gold particles localizing Biotin-SS-Folate occurred in clusters of ⩾3 gold particles; 27.5 and 25.5 percent respectively occurred in clusters of 2 gold particles; 19.8 and 12.2 percent respectively occurred singly (Figs. 5*A*, 6, 7*A* and Table 1). The specificity of covalent labeling of Biotin-SS-Folate for FR and its localization with anti-biotin antibody was confirmed by the following negative and positive controls in Figs. 5–7 and Table 1. (i) reduction in covalent labeling in the presence of competing folic acid (Figs. 5*B*, 7*C* and Table 1); (ii) detection of a very low level of the label in parental CHO cells compared to CHO cells expressing recombinant FR (Fig. 5*C*); (iii) inability to detect the label when Biotin-SS-Folate was used without activating it for covalent modification (Fig. 7*B*); and (iv) colocalization of the label and an FR specific antibody probe (Fig. 6); in this experiment, by random inspection, most of the labeled receptor molecules colocalized with the antibody probe (Table 2).

The possible effects of folate (or the affinity reagent) and low pH treatment on the clustering of FR molecules were also tested. CHO-FR-β cells were acid washed to remove endogenously bound folate followed by probing post-embedding with antibody to FR-β. Clusters of FR molecules were observed as above (*results not shown*). Similar results were obtained in the absence of the acid wash (*results not shown*) indicating that neither the low pH treatment nor ligand occupancy influenced the clustering of FR.

Most (76%) of the covalently attached Biotin-SS-Folate visualized in Fig. 6 was colocalized with FR detected by the antibody probe (Table 2). However, as expected, a smaller proportion (67%) of the FR clusters detected by antibodies were associated with the ligand probe (Table 2). This is understandable because the Bio-

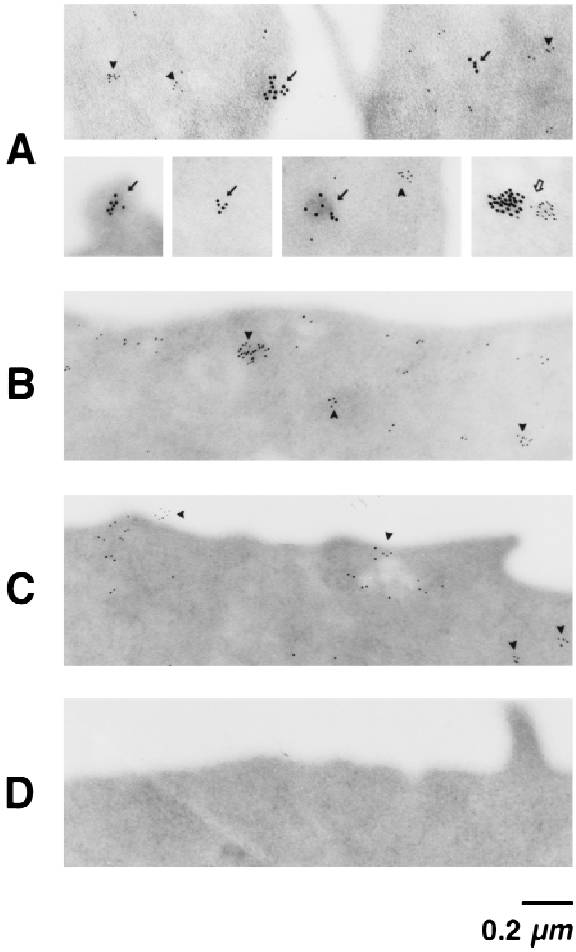

Fig. 5. Localization of Biotin-SS-Folate label and caveolin in CHO-FR-β cells. In *A*, CHO-FR-β cells were incubated for 2 hr with NHS-activated Biotin-SS-Folate under transport permissive conditions, fixed with 3% glutaraldehyde, embedded in LR White medium and sectioned; the sections were probed with goat anti-biotin antibody conjugated to 10 nm gold, and with rabbit anti-caveolin antibody followed by 5 nm gold conjugated goat anti-rabbit IgG. In *B*, the cells were preincubated with 1 μM folic acid and then treated as described for *A*. In *C*, parental CHO cells were used and treated as described for *A*. In *D*, CHO-FR-β cells were treated as described for *A* with the omission of the primary antibodies. Arrows indicate clusters of 10 nm gold; arrow heads indicate clusters of 5-nm gold; open arrow indicates clusters of 10 nm gold and 5-nm gold occurring within a distance of 50 nm.

tin-SS-Folate was introduced from the surface of whole cells while the antibodies may detect additional FR in intracellular compartments. After embedding, FR is immobilized and thus the introduction of antibody (either to biotin or directly to FR) at this stage cannot influence the localization of FR. The fact that both the biotin probe and the post-embedding anti-FR antibody probe colocalized with a high frequency strongly supports the validity of the FR localization in this study.

When the sections were probed for labeled FR (10 nm gold particles) and also for caveolin (5 nm gold particles), clusters of both 10 nm and 5 nm particles were observed (Figs. 5*A* and 7*A*); in negative controls, exclusion of the anti-caveolin antibody resulted in the absence of the 5 nm gold clusters as well (Fig. 5*D*). As an additional negative control, L1210 lymphocytic leukemia cells that do not contain caveolae were probed similarly with the anticaveolin antibodies and 5 nm gold-conjugated secondary antibody (Fig. 8). There was no significant localization of the gold particles in L1210 cells, further confirming the specificity of the primary antibody for caveolin in Figs. 5 and 7. FR clusters and caveolin clusters at the cell surface were clearly localized and the clusters apparently further removed from the cell surface may represent both internalized FR and overlapping portions of the plasma membrane. Nevertheless, when a sufficient number of 10-nm gold (FR) clusters were randomly counted in order to detect any significant association with caveolin, the frequency with which 5-nm gold particles (caveolin) occurred within a distance of 50 nm was low (Table 1). Furthermore, the occurrence of caveolin within 10 nm of an FR cluster was not observed. A far greater number of caveolin clusters were detected than FR (Table 1). This is presumably due to the relatively low expression levels of FR chosen for this study compared to other studies (Rijnboutt et al., 1996) and also because the intracellular caveolin may represent both endocytic membranes as well as the transgolgi network and transport vesicles. While caveolae may be expected to be revealed as caveolin clusters, it may be noted that caveolin itself is known to occur relatively abundantly in intracellular compartments and may be both clustered and unclustered.

The results of this study are in partial agreement with previous electron microscopic studies (Rothberg et al., 1990; Ying et al., 1992; Mayor et al., 1994). Thus while it is clear that FR molecules do not occur in caveolae to a relatively significant extent either in JAR cells or in the recombinant CHO cells, it also appears that the receptor molecules are not diffusely distributed on the cell surface. Rather, they exist predominantly in clusters in the absence of cross-linking. Such receptor clusters are possibly disrupted by certain fixation methods, leading to a diffuse distribution on the cell surface (Mayor et al., 1994). It may also be noted that the FR clusters are not significantly induced to associate with caveolin even under transport permissive conditions.

Influence of Alternate Procedures of Fixation or Probing on The Clustering of FR Molecules

The possible influence of the additional early mild fixation step (with 0.5% glutaraldehyde/3% paraformaldehyde) used in the pre-embedding electron microscopic procedure of Mayor et al. (1994) on the localization of FR molecules on the cell surface was further investi-

Table 1. Cellular distribution of folate receptor in relation to caveolin[a]

Cells	Number of 10-nm gold clusters[b] (particles)			Number of 5-nm gold clusters[c]
	Total[d]	Associated with 5-nm gold clusters		
		<50-nm distance	<10-nm distance	
CHO-FR-β	41 (298)	3	0	400
CHO-FR-β + 1μM folic acid	4 (29)	0	0	485
Parental CHO	4 (26)	0	0	493
JAR	40 (314)	2	0	351
JAR + 1μM folic acid	5 (41)	0	0	438
L1210 Lymphocytes	ND	ND	ND	7

[a] Ten cells were randomly chosen for counting for each condition. Similar results were obtained from three independent experiments.
[b] Localizing covalently attached Biotin-SS-Folate (≥3 gold particles per cluster).
[c] Localizing caveolin (≥3 gold particles per cluster).
[d] The total number of 10-nM gold particles occurring singly and in pairs were 144 and 118 respectively for CHO-FR-β and JAR cells.
ND; Not determined.

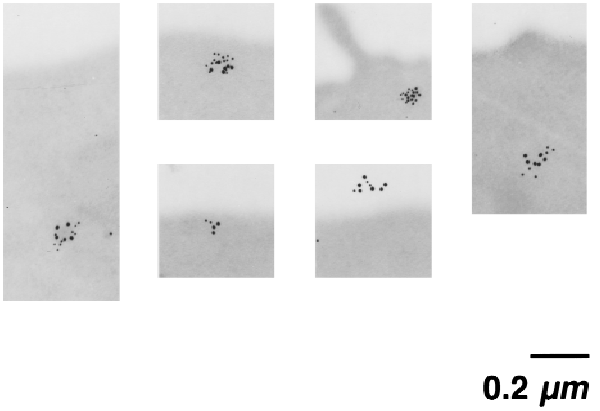

Fig. 6. Colocalization of Biotin-SS-Folate label and antigenic sites on FR in CHO-FR-β cells. Cells were incubated for 2 hr with NHS-activated Biotin-SS-Folate under transport permissive conditions, fixed with 3% glutaraldehyde, embedded in LR White medium and sectioned. Sections were probed with goat anti-biotin antibodies conjugated to 10 nm gold and also with affinity purified rabbit antibody to FR-β followed by 5-nm gold conjugated goat anti-rabbit IgG.

gated. Accordingly, CHO-FR-β cells were treated with the mild fixative followed by incubation at room temperature in HBSS for 1 hr. Subsequent fixation, embedding in LR White resin and probing of the sections with anti-FR antibodies were carried out as described in the previous section. The cells were also probed by a pre-embedding procedure similar to that of Rothberg et al. (1992) in which incubation with the anti-FR antibodies and secondary gold conjugated antibody preceded fixing

Table 2. Colocalization of biotin-SS-folate label and antigenic sites of FR-β[a]

10-nm Gold clusters[b]		5-nm Gold clusters[c]	
Total	Colocalized with 5 nm gold	Total	Colocalized with 10 nm gold
42	32	49	33

[a] Ten cells were randomly chosen for counting. Similar results were obtained from three independent experiments.
[b] Localizing covalently attached Biotin-SS-Folate.
[c] Localizing FR molecules.

and embedding in LR White resin and visualization of the gold particles in the sections. As seen in Table 3, inclusion of the early mild fixation step resulted in a dramatic reduction in the clustering of FR molecules. Instead, the localizing gold particles were relatively disperse. On the other hand, the clustering of FR molecules observed under the conditions described in the previous section was not disrupted by probing with the antibodies pre-embedding (Table 3). The results in Tables 1–3 demonstrate the clustering of FR molecules both in the presence and in the absence of antibody cross-linking. They further strongly suggest that the diffuse appearance of FR in previous electron microscopic studies (Mayor et al., 1994) could be attributed to the use of the early mild fixation step. An explanation of these results in physical terms may be that mild fixation disrupts the ability of FR molecules to cluster without preventing their ability to dissipate by diffusion; on the other hand, harsh fixation

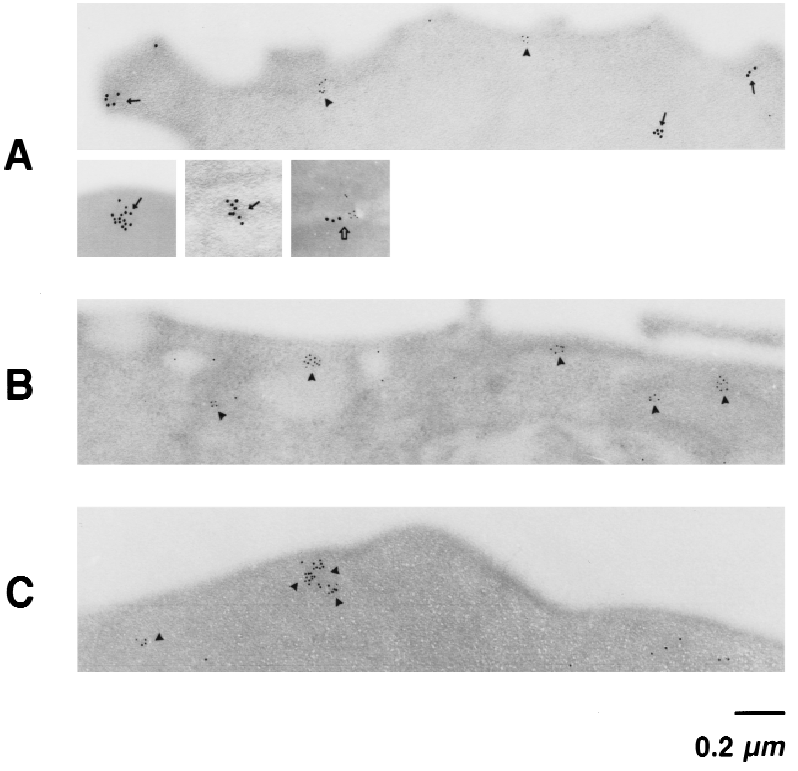

Fig. 7. Localization of Biotin-SS-Folate label and caveolin in JAR cells. In *A*, cells were incubated for 2 hr with NHS-activated Biotin-SS-Folate under transport permissive conditions, fixed with 3% glutaraldehyde, embedded in LR White medium and sectioned; the sections were probed with goat anti-biotin antibody conjugated to 10 nm gold, and with rabbit anti-caveolin antibody followed by 5 nm-gold conjugated goat anti-rabbit IgG. In *B*, cells were treated as described for *A* with the exception that the Biotin-SS-Folate used was not activated. In *C*, the cells were preincubated with 1 μM folic acid and then treated as described for *A*. Arrows indicate clusters of 10-nm gold; arrow heads indicate clusters of 5-nm gold; open arrow indicates clusters of 10-nm gold and 5-nm gold occurring within a distance of 50 nm.

Fig. 8. Probing of L1210 cells with anti-caveolin antibodies. L1210 cells were fixed with 3% glutaraldehyde, embedded in LR White medium and sectioned; the sections were probed with rabbit anti-caveolin antibodies followed by 5-nm gold conjugated goat anti-rabbit IgG as described in Materials and Methods.

Table 3. Effects of alternative experimental methods on the clustering of folate receptor molecules in CHO-FRβ cells[a]

Method of fixation/probing	Number of 10-nm gold clusters[b] (particles)
Addition of primary antibodies pre-embedding[c]	27 (370)
Early (mild) fixation[d]	6 (274)

[a] Ten cells were randomly chosen for counting.
[b] Localizing FR molecules (⩾3 gold particles per cluster).
[c] The cells were processed similar to the method of Rothberg et al. (1992), by the sequential addition of primary and secondary (gold conjugated) antibodies prior to fixation with 3% glutaraldehyde, with the exception that the samples were embedded in LR White resin (*see* Materials and Methods).
[d] The cells were subjected to an initial fixation step with 3% paraformaldehyde/0.5% glutaraldehyde as described by Mayor et al. (1994) followed by incubation at room temperature for 1 hr and a second fixation step using 3% glutaraldehyde as described by Mayor et al. (1994). The primary and secondary antibody probes were introduced after embedding in LR White resin (*see* Materials and Methods).

may rapidly precipitate the protein, ''freezing'' the receptor molecules within their pre-existing clusters. In any event, the results provide compelling evidence that FR molecules occur as clusters.

The EM data obtained in this study do not necessarily contradict the previously published (Mayor & Maxfield, 1995) data on the distribution of FR obtained by fluorescence microscopy. What is termed ''diffuse distribution'' of FR in fluorescence images of unfixed cells in that study could simply reflect a fundamental difference between the resolution afforded by fluorescence *vs.* EM techniques. Thus, relatively small clusters of FR molecules may be visualized by counting the number of associated gold particles (⩾3) by EM. The relatively small size and lateral mobility of the receptor clusters in unfixed cells may lead to a more diffuse appearance in fluorescence images. Under conditions of

antibody induced cross-linking, these clusters may come together to form much larger clusters of receptor molecules, that may also be less laterally mobile in unfixed cells and give rise to a distinctly punctate appearance in fluorescence images. Unfortunately, it will not be possible to meaningfully compare the sizes of the clusters obtained by the pre-embedding *vs.* the post-embedding methods. This is because the post-embedding method is additionally limited by the possible inaccessibility of receptor molecules deeper in the sections and also possible differences in the antigenicity of the receptor. Thus, the studies presented here do not contradict the core of the arguments put forth by Mayor et al. regarding the influence of antibodies on the distribution folate receptors on the cell surface. Rather, they point out that FR molecules occur in pre-existing clusters that may be unstable under their EM fixation conditions and relatively too small and mobile to be discerned clearly by fluorescence techniques. Perhaps, what was previously demonstrated as antibody-induced clustering of FR reflects the formation of larger clusters from smaller clusters.

Significance of Membrane Localization of FR

Several studies have equated GPI-protein rich microdomains with caveolae based on copurification of caveolin and GPI-proteins in relatively detergent insoluble membrane fractions (Kurzchalia et al., 1992; Sargiacomo, 1993; Brown & Rose, 1992; Lisanti et al., 1994) as well as the requirement of cholesterol for the integrity of caveolae (Rothberg et al., 1992). Although caveolin appears to be required for targeting GPI-anchored proteins to the apical surface of epithelial cells (Zurzolo et al., 1994), the biochemical data on cell surface association of GPI-proteins with caveolae in steady state is contradicted by observations that the detergent insoluble fractions can exist in certain cell types lacking caveolae (Fra et al., 1994; Gorodinsky & Harris, 1995). In a very elegant recent study, Schnitzer, Oh & McIntosh (1995), were able to obtain highly pure preparations of caveolae from rat endothelial cells by coating the outer surface of the cell membrane with colloidal silica which allowed physical separation of the invaginated caveolae from the silica coated plasma membrane. In yet another recent study (Schnitzer et al., 1996) caveolae were isolated as budded vesicles from plasma membranes in a cell-free system. While the studies showed that in the plasma membranes of these endothelial cells, the isolated caveolae did not contain GPI-anchored proteins, a curious observation was that an undetermined fraction of caveolae showed the occurrence of a GPI-protein rich region in their vicinity as a peripheral annulus. Similarly, in the present study, it was observed that infrequently (Fig. 4*A*, 6*A* and Table 1) in both JAR cells and the recombinant CHO cells, FR and caveolin occurred as distinct clusters within a distance of 50 nm. Such an association may represent a tendency for the two membrane microdomains to associate. In cells that are rich in caveolae, such as endothelial cells, or in cells vastly overexpressing GPI-anchored proteins, GPI-protein rich domains may be more frequently associated with caveolae. Thus in addition to similarity in the lipid composition between caveolae and membrane microdomains enriched in GPI-anchored proteins, the tendency for caveolae to peripherally associate with the latter domains may be dictated by the density of either membrane microdomain. It may be noted here that the results obtained in this study on the localization of FR molecules are not necessarily applicable to other GPI anchored proteins. The physical basis for the formation of FR clusters i.e., GPI anchor *vs.* polypeptide specific association, remains to be established.

In the potocytosis model for folate transport, the sequestered FR clusters are proposed to occur in association with caveolae. The results of the present study, using the folate affinity reagent introduced extracellularly under transport permissive conditions show that the internalized folate/FR is not associated with caveolin. This result rules out a role for caveolae in the internalization of FR. On the other hand, bulk membrane endocytosis of diffusely distributed FR molecules (Mayor et al., 1994) can not explain the observed kinetics of receptor-mediated folate transport, which entails quantitative sequestration and recycling of FR molecules to the cell surface within about 30 min (Kamen et al., 1988, 1989). Rather, the kinetic data on folate transport is consistent with the selective internalization and recycling of receptor rich membrane microdomains. Indeed, endocytosis and recycling of GPI-anchored proteins has been demonstrated to occur by a pathway independent of the clathrin-coated pit pathway in recombinant CHO cells and T-lymphocytes (Keller, Siegel & Caras, 1992; Bamezai, Goldmacher & Rock, 1992). In KB cells, the majority of FR within 15–60 min after internalization was present in multivesicular bodies (Turek, Leamon & Low, 1993). Furthermore, chimeric FR targeted to clathrin coated pits by replacing its GPI anchor with a polypeptide transmembrane domain was unable to efficiently transport 5-methyltetrahydrofolate suggesting the importance of GPI modification and its related membrane localization in receptor mediated folate transport (Ritter et al., 1995). Therefore, the fundamental concept of potocytosis based on the detailed kinetics of folate internalization and a recycling receptor may still provide a valid model for folate transport via membrane microdomains distinct from caveolae. Such regions of the plasma membrane may offer a means for receptor-mediated intracellular delivery of small molecules and efficient recycling of internalized receptor with the involvement of as yet unidentified vesicular proteins.

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