Cholesterol Controls the Clustering of the Glycophospholipid-anchored Membrane Receptor for 5-Methyltetrahydrofolate

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Abstract. The folate receptor is a glycosyl-phosphatidylinositol (GPI)-anchored membrane protein that mediates the delivery of 5-methyltetrahydrofolate to the cytoplasm of MA104 cells. Ordinarily the receptor is sequestered into numerous discrete clusters that are associated with an uncoated pit membrane specialization called a caveola. By using two different methodological approaches, we found that the maintenance of both receptor clusters and caveolae depends upon the presence of cholesterol in the membrane. These results suggest that cholesterol plays a critical role in maintaining the caveola membrane domain and modulates the interaction of GPI-anchored membrane proteins via their phospholipid anchors.

THE functional specialization of a cell is reflected in the spatial organization of its macromolecular substructure. Not only do differentiated cells express genes that distinguish them from other cells, but often the products of these genes are differentially distributed within the cell and at the cell surface. The generation and maintenance of these organizational patterns requires the faithful sorting of newly synthesized molecules into the correct transport pathway and their delivery to the proper target. In many instances the information required for these sorting reactions is not known.

In the case of cell membranes, the sorting step involves the sequestration of molecules into vesicular vehicles that carry them to the correct destination. For maximal efficiency of transport, these molecules must first be segregated from other molecules in the membrane to form domains that are enriched in that molecular species. This clustering reaction has been well documented for molecules that participate in receptor mediated endocytosis (20) but presumably also occurs at different stages in the exocytic pathway as well (15). The clustering together of molecular subsets is also required for the formation of cell–cell junctions (40), cell–substratum contact sites (11), clathrin coated pits (2), and microvilli (31).

The clustering of a subset of membrane molecules often depends upon the interaction of a transmembrane, integral membrane protein with a specific extrinsic membrane protein, and these interactions can occur at either side of the membrane. For example, receptors involved in endocytosis are thought to rely on the cytoplasmic portion of the molecule for clustering in coated pits (2), whereas acetylcholine receptor clustering is mediated by the interaction of extrinsic proteins with both the external (14) and cytoplasmic (32, 34) portions of the receptor. Molecules might also cluster by virtue of their self associative properties. This is thought to be the mechanism for the formation of glycosphingolipid microdomains in the membrane (43) and cholesterol-rich domains in liposomes (35, 36, 44). Self-association may also be responsible for the segregation of certain membrane proteins.

Most integral membrane proteins have a hydrophobic transmembrane domain and a cytoplasmic tail. The glycosylphosphatidylinositol (GPI)[1]-linked integral membrane proteins, however, are anchored in the membrane by a covalently attached glycoinositol phospholipid (16). Recently we reported that the GPI-linked membrane receptor for 5-methyltetrahydrofolate is highly clustered on the surface of MA104 cells and that these clusters are enriched over caveola, a membrane pit that lacks a clathrin coat (37). Each receptor cluster contains ~750 receptors with a density of ~30,000 molecules per μm^2, which is 50 times the density of LDL receptors in clathrin coated pits (37). We now report that the maintenance of folate receptor clusters is dependent on the presence of cholesterol in the membrane, which suggests that the GPI anchor is responsible for the highly aggregated state of this membrane protein.

Materials and Methods

Materials

Goat anti-rabbit IgG (65-6100) and goat anti-rabbit IgG conjugated to FITC (62-6111) were from Zymed Laboratories (South San Francisco, CA.). Polyclonal anti-LDL receptor IgG was prepared as previously described (1). Rabbit anti-goat IgG conjugated to 10-nm gold (BC-RAG-10) was from Energy Beam Sciences (Agawam, MA). Medium 199 with Earle's salts with and without folic acid was prepared in the laboratory by standard protocols. Glutamine (320-5030), trypsin-EDTA (610-5300), and penicillin/streptomycin (600-5145AE) were from Gibco Laboratories (Grand Island, NY). Fetal calf serum (12-10378) was from Hazleton Research

1. *Abbreviation used in this paper*: GPI, glycosyl-phosphatidylinositol.

Products, Inc. (Lenexa, KS). T-75 culture flasks and 35-mm culture dishes were from Corning Glass Works (Corning, NY). Human lipoprotein-deficient serum ($d > 1.215$ g/ml) was prepared as previously described (19). Mevalonic acid lactone (69761), magnesium chloride (63065), and paraformaldehyde (76240) were from Fluka (Ronkonkoma, NY). Hepes (H-3375), crystalline bovine serum albumin (A-7638), Triton X-100 (T-6878), digitonin (D-1407), polymyxin B sulfate (P-1004), and filipin (F-9765) were from Sigma Chemical Co. (St. Louis, MO). Ammonium chloride (A-661) was from Fisher Scientific Co. (Fairlawn, NJ). Nystatin (100416) was from ICN Biomedicals Inc. (Cleveland, OH). Compactin was obtained as previously described (10). Rabbit anti-folate receptor IgG was obtained from rabbits that were immunized with purified human placental folate binding protein (5). The purified IgG from these animals immunoprecipitated a single 38-kD band from radiolabeled MA104 cells (data not shown). All other reagents were obtained as previously described (24, 37).

Cell Culture

MA104 cells, a monkey kidney epithelial cell line, were grown as previously described (24, 37). 1.5×10^5 cells were seeded onto coverslips in 35-mm dishes and grown for 5 d in folic acid-free medium 199 supplemented with 5% (vol/vol) fetal calf serum and 100 U/ml penicillin/streptomycin (medium A). In experiments to detect LDL receptor, cells were transferred to folic acid-free medium 199 supplemented with 5% lipoprotein deficient serum on day 2 of cell growth. Cells were depleted of cholesterol by growing them in 5% lipoprotein deficient serum that contained 25 μM compactin and 200 μM mevalonate for 60 h before the experiment. The low concentration of mevalonate was required to maintain cell viability during the 60-h incubation.

Indirect Immunofluorescence

Cells were chilled to 4°C for 30 min, rinsed with ice-cold buffer A (folic acid-free medium 199, 20 mM Hepes, pH 7.4, 0.1% crystalline BSA) and incubated with 25 μg/ml of polyclonal rabbit anti-folate receptor IgG, diluted in buffer A, for 1 h at 4°C. Cells were rinsed with buffer B (2.68 mM KCl, 1.46 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 200 mM NaCl, 0.49 mM $MgCl_2$, pH 7.4, 0.1% crystalline BSA) and then fixed with 3% formaldehyde in buffer C (buffer B minus the BSA) for 30 min. After the indicated incubation, cells were rinsed with buffer B and processed to localize rabbit IgG with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate as previously described (37).

Immunogold-labeling Procedure

Cells were processed as described for the immunofluorescence experiments except that anti-folate receptor IgG was used at 100 μg/ml. After treatment with 7.5 μg/ml of filipin at the indicated temperature, cells were incubated with goat anti-rabbit IgG (25 μg/ml) for 1 h at 4°C, followed by a 1-h incubation at 4°C with a 1:30 dilution of rabbit anti-goat IgG conjugated to gold. Cells were then fixed, dehydrated, and embedded in Epon (37). For live cells, cells were chilled to 4°C, incubated with 5 μg/ml of filipin for 15 min, fixed, and processed to localize the folate receptor using anti-folate receptor IgG as previously described (37).

Other Methods

The total cholesterol content of cells grown in the presence and absence of 25 μM compactin was measured by the method of Gamble et al. (18). Quantitation of gold particle distribution was performed as previously described (37).

Results

Sterol Binding Agents Uncluster the Folate Receptor

The folate receptor mediates the delivery of 5-methyltetrahydrofolate to the cytoplasm of MA104 cells (24). Clustered folate receptors are easily detected by indirect immunofluorescence (37). When cells were incubated at 4°C with an antibody to the receptor, fixed with formaldehyde, incubated at 4°C for 30 min, and processed for indirect immunofluorescence, the apical surface of the MA104 cells displayed numerous bright foci corresponding to clusters of bound immunoglobulin (Fig. 1 *A*). If, however, the cells were incubated at 4°C in the presence of 5 μg/ml of filipin after the fixation, there was a marked reduction in the size of the foci and the appearance of bound immunoglobulin that was diffusely distributed on the cell surface (Fig. 1 *B*). Unclustering was not temperature dependent because when fixed cells bearing bound anti-folate receptor IgG were incubated in the presence of filipin at 37°C, the unclustered anti-receptor IgG staining pattern was also evident (Fig. 1 *C*).

Filipin is a polyene antibiotic, a class of drugs that bind cholesterol (8, 29). Fig. 2 shows that both nystatin (Fig. 2 *A*), another polyene antibiotic (22), and digitonin (Fig. 2 *B*),

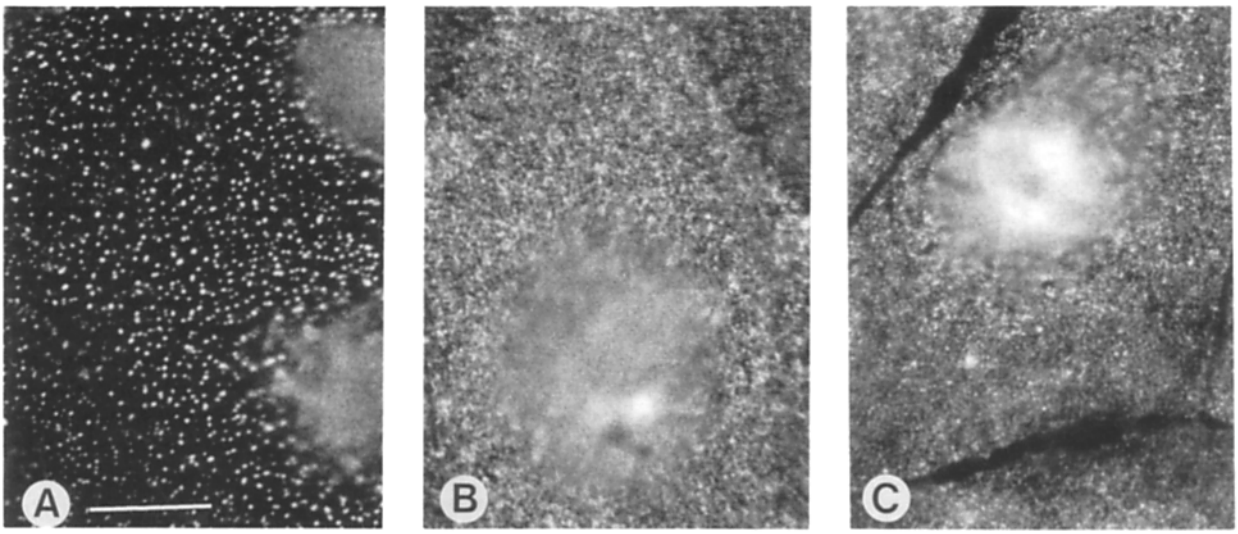

Figure 1. Effect of control treatment (*A*) or treatment with 5 μg/ml of filipin (*B* and *C*) at either 4 (*A* and *B*) or 37°C (*C*) on the distribution of anti-folate receptor IgG binding sites in MA104 cells. Cells were grown for 5 d in folic acid-free medium, chilled to 4°C for 30 min, and incubated with 25 μg/ml of polyclonal rabbit anti-folate receptor IgG for 1 h at 4°C. Cells were rinsed, and then fixed with 3% formaldehyde. Cells were treated for 15 min with either 0.1% DMSO (*A*) or DMSO plus 5 μg/ml filipin (*B* and C) at the indicated temperature and then processed to localize rabbit IgG as described in Materials and Methods. Bar, 10 μm.

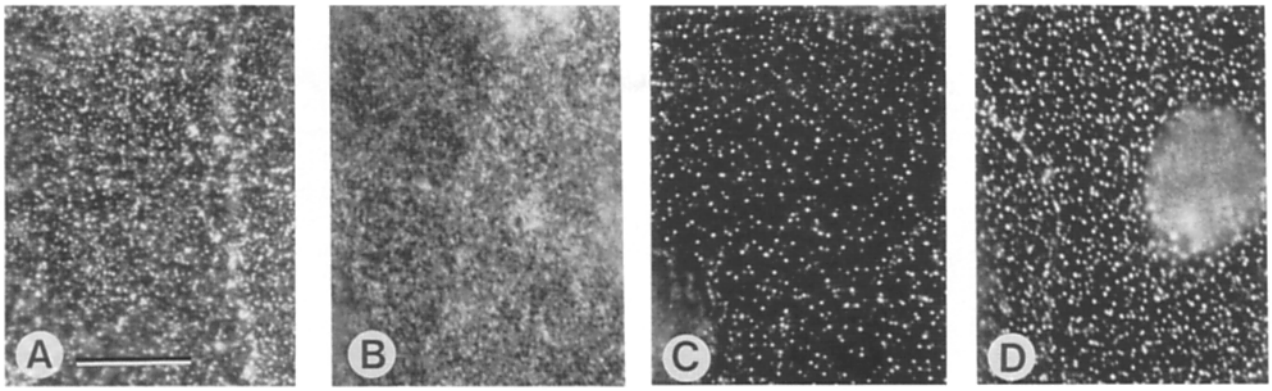

Figure 2. Effect of nystatin (*A*), digitonin (*B*), Triton X-100 (*C*), and polymyxin B sulfate (*D*) on the distribution of anti-folate receptor IgG binding sites. Cells were prepared and incubated with anti-folate receptor IgG as described in Fig. 1. Three sets of dishes were fixed and incubated for 15 min (*A–C*) with either 100 μg/ml nystatin at 37°C (*A*), 100 μg/ml digitonin at 4°C (*B*), or 1 mg/ml Triton X-100 at 4°C (*C*). A fourth dish (*D*) was incubated at 37°C for 30 min with 4 mM polymyxin B sulfate before fixation. All dishes then were processed for immunofluorescence localization of anti-folate receptor IgG. Bar, 10 μm.

a cardiac glycoside that binds cholesterol (13, 21), also caused the folate receptor to uncluster. By contrast, detergents such as Triton X-100 (Fig. 2 *C*) had no effect. Benzyl alcohol, which fluidizes membranes (17), had no effect nor did 2.5 μM pentobarbital or 2.5 μM mepivacaine (data not shown), two anesthetics of opposite charge that intercalate into membrane bilayers and alter membrane shape (3, 12). Since many cholesterol binding drugs also form precipitates

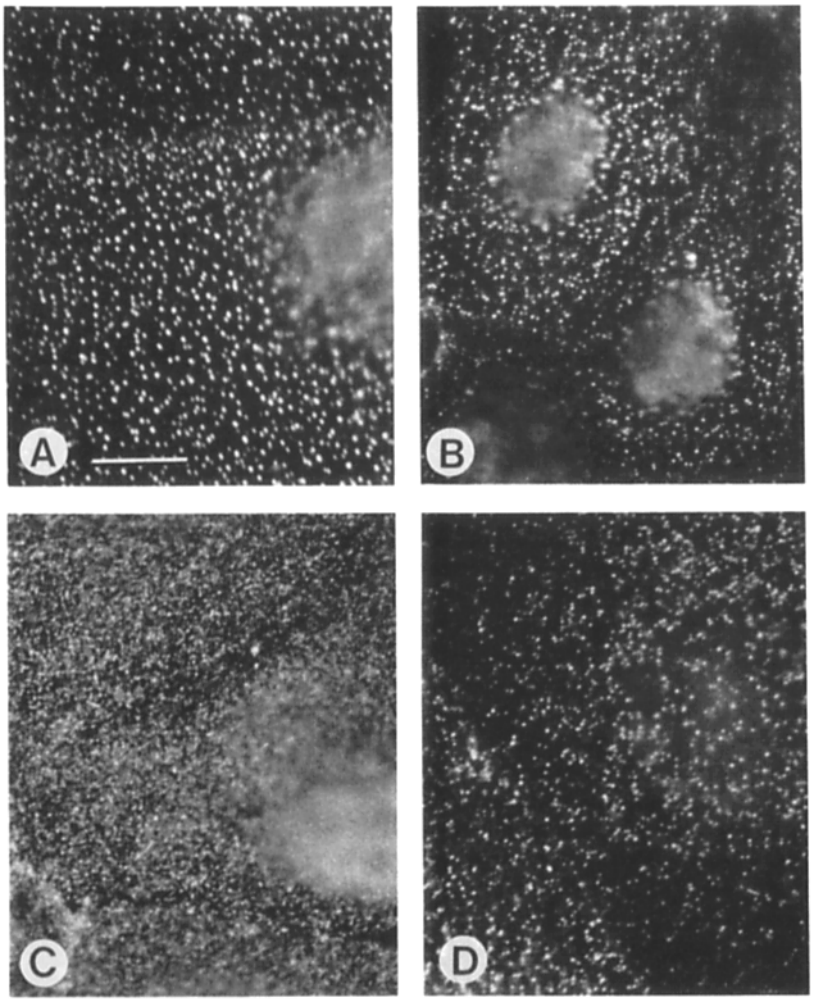

Figure 3. Effect of filipin on the distribution of anti-folate receptor IgG (*A* and *C*) and anti-LDL receptor IgG (*B* and *D*) binding sites. Cells were grown in medium A for 2 d and then transferred to folic acid-free medium containing 5% lipoprotein deficient serum. On day 5 cells were chilled to 4°C, incubated with anti-folate receptor IgG (*A* and *C*) or anti-LDL receptor IgG (*B* and *D*), then fixed and incubated with 10 μg/ml of filipin at 4°C before processing to localize bound IgG as described in Fig. 1. Bar, 10 μm.

in the plane of the membrane (13, 29), we tested polymyxin B, which forms morphologically distinct complexes with phospholipids in the membrane (6, 7). As seen in Fig. 2 *D*, cells treated with this agent had normal appearing receptor clusters. Therefore, of all the reagents we tested, only those that bind cholesterol caused unclustering of the folate receptor.

In contrast to the folate receptor, filipin had no effect on the clustering of the LDL receptor, an integral membrane protein that has transmembrane and cytoplasmic domains (20). In the absence of filipin both populations of receptors appeared to have a similar, clustered distribution (Fig. 3 *A* vs. Fig. 3 *B*). When MA104 cells were treated with 10 μg/ml of filipin, however, the folate receptor (Fig. 3 *C*) became dispersed but the LDL receptor (Fig. 3 *D*) remained clustered.

Receptor clustering can also be detected by immunogold labeling, where on average a folate receptor cluster has six gold particles (37). Fig. 4 *A* shows that without filipin treat-

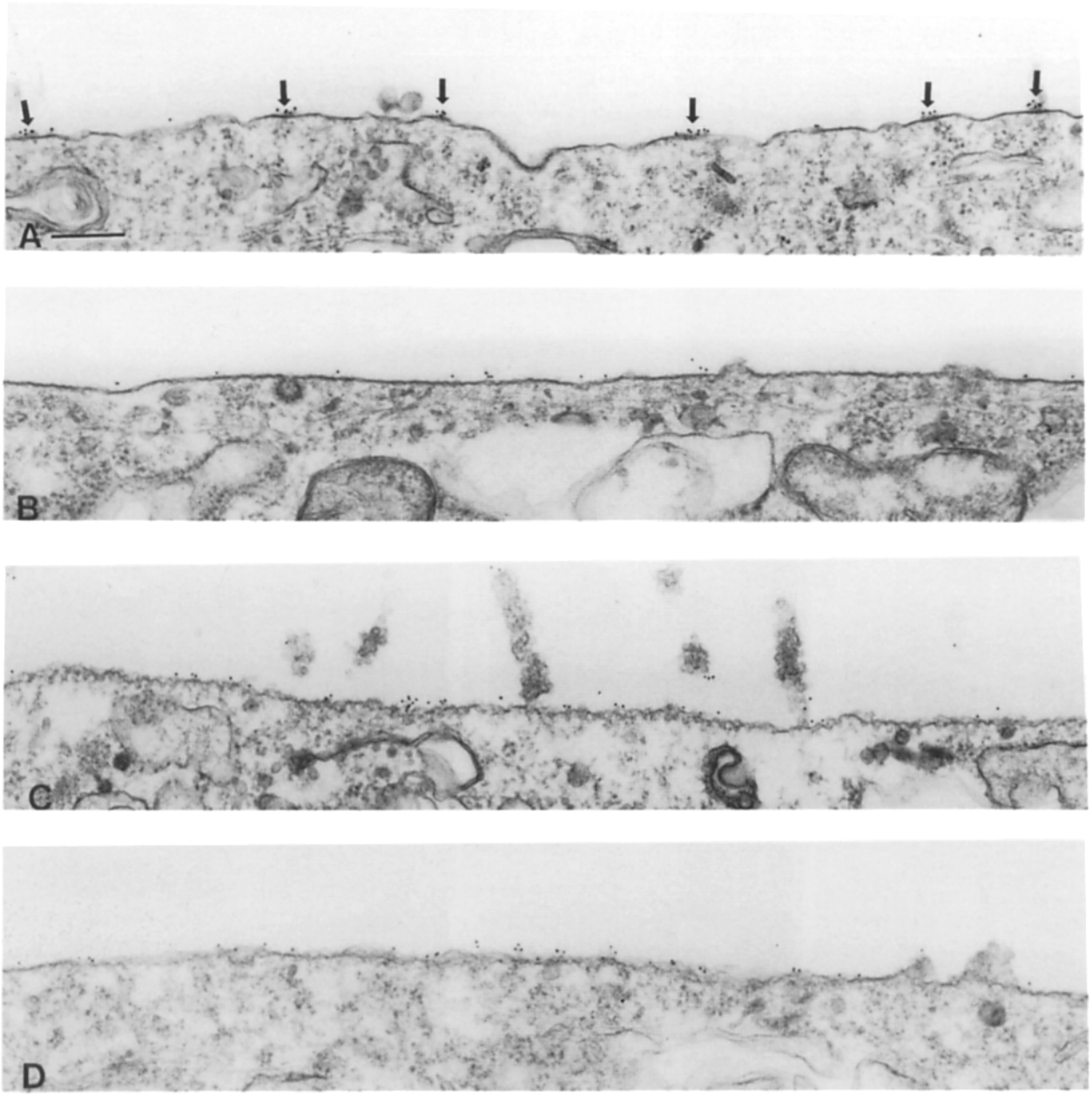

Figure 4. Immunogold localization of anti-folate receptor IgG binding sites in fixed cells after control treatment at 4°C (*A*), treatment with filipin at 4°C (*B*), or treatment with filipin at 37°C (*C*), and localization after filipin treatment of live cells at 4°C (*D*). Cells for *A–C* were processed as described in Fig. 1. After treatment with 7.5 μg/ml of filipin at the indicated temperature, cells were incubated with goat anti-rabbit IgG (25 μg/ml) for 1 h at 4°C, followed by a 1-h incubation at 4°C with rabbit anti-goat IgG conjugated to gold. Cells were then fixed, dehydrated, and embedded in Epon. For live cells (*D*), cells were chilled to 4°C, incubated with 5 μg/ml of filipin for 15 min, then fixed and processed as described in Materials and Methods. Bar, 0.3 μm.

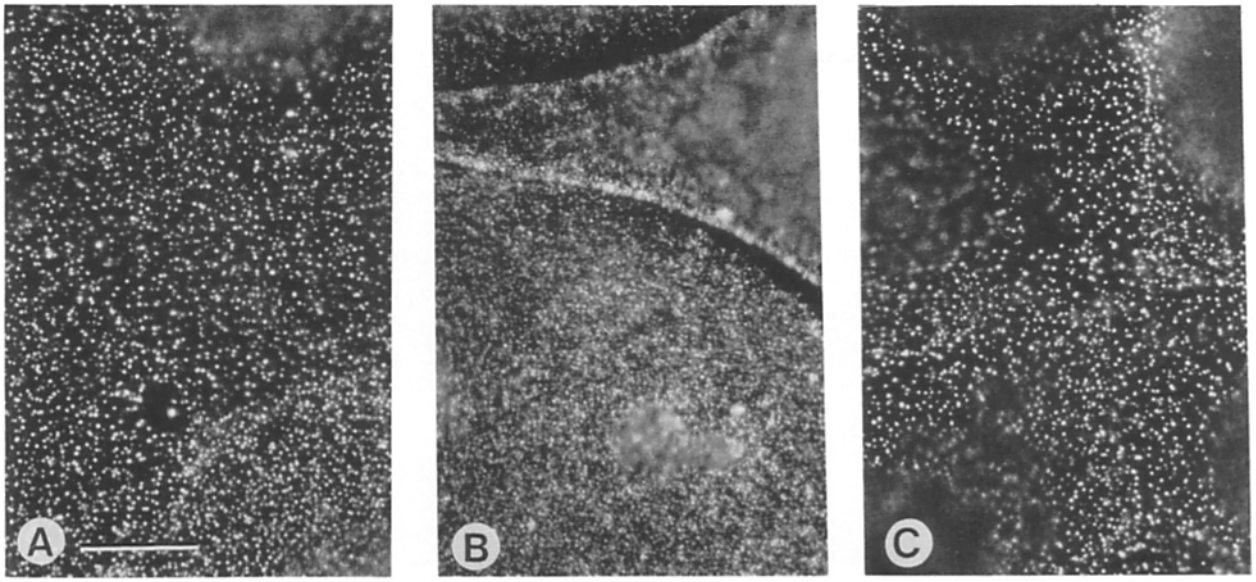

Figure 5. Distribution of anti-folate IgG binding sites in cells grown in the absence of compactin (*A*), in the presence of compactin (*B*), or in the presence of compactin plus LDL (*C*). Cells were grown as described in Fig. 3. After placement in lipoprotein-deficient medium, either 200 μM mevalonate (*A*) or 25 μM compactin and 200 μM mevalonate was added to the dishes (*B* and *C*). After 36 h in this medium, 75 μg/ml of LDL was added to dishes *A* and *C*. On day 5, all cells were chilled and processed for indirect immunofluorescence localization of anti-folate receptor IgG binding sites as described in Materials and Methods. Bar, 10 μm.

ment normal receptor clusters were observed but that incubation with filipin either at 4°C (Fig. 4 *B*) or 37°C (Fig. 4 *C*) caused a reduction in the size of the clusters and the appearance of numerous single gold particles dispersed on the cell surface. Likewise, when live cells were treated briefly with filipin at 4°C, the gold label was unclustered (Fig. 4 *D*).

Reduction in Folate Receptor Clusters in Cholesterol-depleted Cells

The effects of filipin as well as other sterol binding reagents on the distribution of the folate receptor suggests that cholesterol may mediate receptor clustering. If this sterol is directly involved, then cells depleted of cholesterol should have an alteration in the receptor clustering pattern. To test this hypothesis, cells were starved of cholesterol by depriving them of both endogenous and exogenous sources of the sterol. By inhibiting the endogenous synthesis of cholesterol with compactin, adjusting the concentration of mevalonate in the media to provide substrate for nonsterol biosynthetic processes, and depleting the serum of lipoproteins, we were able to routinely reduce the total cellular cholesterol from 160 μg/mg of protein to 70.2 μg/mg without changing the viability of the cells. These cells were morphologically indistinguishable from cells grown in the presence of lipoprotein deficient serum alone; however, by indirect immunofluorescence the receptor distribution was much more dispersed (Fig. 5 *B*) than in cells that were grown in the absence of compactin (Fig. 5 *A*). The number of clusters was increased and on average each cluster appeared smaller, which contributed to the dispersed appearance of the receptor. Addition of LDL to the media of compactin grown cells for 24 h before the immunofluorescence assay restored the staining pattern (Fig. 5 *C*) to normal (compare with Fig. 5 *A*).

Electron microscopic analysis of cholesterol-depleted cells also showed that receptor clustering was affected (Fig. 6). Untreated cells had normal appearing receptor clusters, many of which were associated with caveolae (Fig. 6 *A*). Cholesterol-depleted cells, however, had fewer numbers of gold particles in each cluster (Fig. 6 *B*) and there also appeared to be an increase in the number of single gold particles. Moreover, there were many fewer caveolae in these cells. Addition of LDL to the media of compactin-treated cells appeared to restore both the normal clustering pattern and the number of caveolae (Fig. 6 *C*). These observations were confirmed by quantitative analysis of gold particles and caveolae. As seen in Table I, the average cluster in untreated cells contained 5.64 gold particles, whereas in cholesterol-depleted cells each cluster averaged 4.89 gold particles ($P < .005$). Moreover, there was a 15% decrease in the amount of gold that was in clusters. By comparison, either the addition of LDL to compactin-grown cells or growing the cells in the presence of fetal calf serum resulted in the cells having normal size clusters. There also was a dramatic decrease in the number of caveolae in cholesterol-depleted cells. These cells had 10-fold fewer caveolae as compared either to control cells or compactin-treated cells that received LDL. This reduction in the number of caveolae contributed to the decrease in the association of clusters with this membrane specialization (Table I).

Discussion

Among receptors that ordinarily are clustered on the cell surface, the folate receptor is remarkable for its high packing density. Because the receptor lacks a transmembrane domain and cytoplasmic tail, we originally thought that clustering would be mediated through protein-protein interactions be-

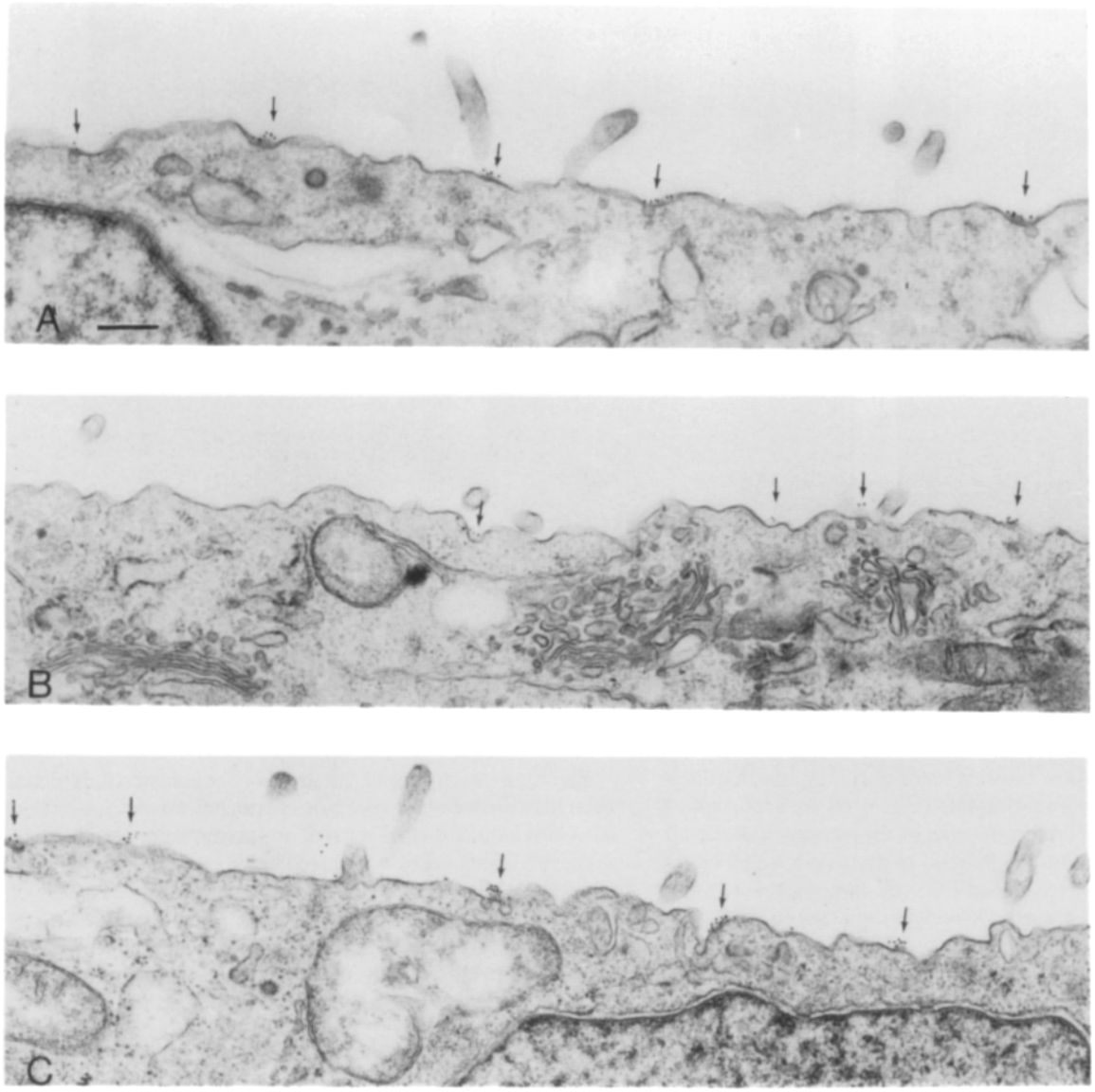

Figure 6. Immunogold localization of anti-folate receptor IgG binding sites in cells that were grown in the absence of compactin (*A*), in the presence of compactin (*B*), or in the presence of compactin plus LDL (*C*). Cells were grown exactly as described in Fig. 5. On day 5, all cells were chilled and processed for indirect immunogold localization of anti-folate receptor binding sites as described in Materials and Methods. Bar, 0.4 μm.

tween the ectodomains. The results of the current experiments, however, implies that clustering is controlled by lipid–lipid interactions between the fatty alkyl/acyl chains of the GPI anchor and the surrounding lipids in the bilayer.

Two lines of evidence have implicated cholesterol as the lipid that modulates folate receptor clustering: (*a*) sterol binding agents disperse the receptor in the plane of the membrane even after formaldehyde fixation, and (*b*) cholesterol-depleted cells have fewer receptors per cluster as well as a reduced number of receptors that are clustered.

Many sterol binding reagents form precipitates with cholesterol in the bilayer, a phenomenon that is best characterized for filipin (38). These precipitates could have physically deranged the lipid bilayer and thereby caused the dispersion of the receptors. Yet polymyxin B, which also forms precipitates in the membrane bilayer (6, 7), did not disperse the receptors whereas nystatin, a sterol binding reagent that does not form recognizable precipitates, did uncluster the receptor. Therefore, most likely the sterol binding reagents have their effect by sequestering cholesterol, which effectively depletes the membrane of sterol.

When cells were grown in the presence of compactin, as judged by immunofluorescence, receptor clustering was reduced. By immunogold analysis, however, these conditions did not uncluster the receptors as dramatically as did the sterol binding agents. We assayed for receptor unclustering by measuring the average density of gold particles in receptor clusters as a function of the treatment. The immunogold method involves the addition of three different antibodies; therefore, the number of gold particles per cluster is only an approximation of the number of receptors at that site. Nevertheless, there was a significant decrease in the size of

Table I. Effects of Cholesterol Depletion on Anti-Folate Receptor IgG Clusters

Treatment	Gold/cluster	Gold clustered	Gold in caveolae	Caveolae/10 μm	Caveolae labeled
	n	%	%		%
Lipoprotein-deficient serum	5.64 ± .17	82.4	18.3	2.72	66.4
Compactin	4.89 ± .18	67.2	1.7	0.27	28.6
Compactin plus LDL	5.86 ± .25	76.7	13.4	1.78	51.9
Fetal calf serum	6.18 ± .22	84.6	18.3	2.82	50

Cells were grown in the presence and absence of 25 μM compactin plus 200 μM mevalonate as described in Fig. 5. Cells were processed for immunoelectron microscopy as described in Fig. 4. The data were analyzed using a Bessler Lantern Slide Projector, which magnified negatives (n = 42–49 per treatment) to 90,720×. Total membrane measured for each group was >425 μm. Each cluster of gold was defined as containing three or more particles. Values for the density of gold per cluster are the mean ± the standard error. Based on a Student's t test, the cluster size for the cells grown in the presence of compactin was significantly smaller ($P < .005$) than for the cells grown under the other conditions.

the clusters in compactin grown cells. When LDL was added to compactin-treated cells the density of gold in each cluster returned to control levels, which is strong evidence that the observed reduction in cluster size was due to a deficiency in membrane cholesterol.

As shown previously (37), we found that receptor clusters were enriched over caveolae, an association that may be important for receptor internalization. Whereas 18% of the gold clusters were associated with these uncoated pits, >66% of the pits where labeled with one or more particles. Conceivably this association is also modulated by cholesterol. The sterol-depleted cells had 10-fold fewer caveolae than did either control cells or cells grown in the presence of fetal calf serum and the addition of LDL to compactin treated cells restored the density of caveolae to normal (Table I). Moreover, the percentage of caveolae that were labeled in cholesterol-depleted cells was reduced by about one-half compared to control cells. Although we can not be sure of the accuracy of these measurements because of the tenuous microscopic criteria used to identify this type of uncoated pit, this is not the first instance where cholesterol has been implicated in the structure/function of caveolae. Simionescu et al. (39) found that in capillary endothelial cells sterol-filipin precipitates are enriched around the opening of caveolae, which suggests that they are a cholesterol-rich membrane specialization. Within these domains cholesterol may function to cause certain membrane proteins to congregate. Other GPI-anchored membrane proteins might have a similar fate (23).

Cholesterol may cause receptor clustering by modulating the phase separation of the fatty acid and fatty alcohol chains attached to the glycerol of the inositol phospholipid (16). Alternatively, this sterol may maintain the clustered organization through hydrogen bonding between the fatty chains in the GPI anchor (36). Recently Luhrs and Slomiany (28) reported that the folate receptor GPI linkage contains a 1-*O*-alkyl-2-*O*-acylglycerol that has mixed fatty alcohols at the C1 and predominantly docosanoic acid (22:0) at the C2 position. Although the exact behavior of these types of fatty chains in the plane of the membrane is not known, there is good evidence that lipid domains exist in biological membranes (6, 35, 38). Quite possibly cholesterol can modulate the formation of these domains (44). Since both the cholera toxin and the tetanus toxin binding gangliosides also appear to be enriched in caveolae (30, 41), the interactions between certain fatty chains and cholesterol may lead to the formation of specific lipid clusters in caveolae.

A surprising recent discovery is that in polarized epithelial cells GPI-anchored membrane proteins predominantly reside on the apical surface (26). The exact function of the GPI anchor in the proper targeting to this surface has not been determined; however, Lisanti et al. (27) recently showed that when the GPI-attachment signal was transferred to Herpes simplex glycoprotein D, a membrane protein that ordinarily is located on the basal surface, the chimeric protein traveled to the apical surface. Similarly, Brown et al. (9) also found that the GPI anchor controls targeting of membrane proteins to the apical surface. Our results raise the possibility that clustering could play a role in the proper targeting of GPI-linked proteins to the apical cell surface. GPI-anchored proteins synthesized in the ER would travel to the Golgi apparatus in a dispersed state due to the low cholesterol in these membranes (4). Once arriving at the cholesterol-rich *trans*-Golgi region (33), the proteins become clustered and this step directs the movement of the protein to the apical cell surface. Since certain glycolipids seem also to be sequestered into domains and reside at the apical surface of polarized cells (42), possibly the clustering reaction is part of a general mechanism for controlling the lipid and protein composition of membranes in polarized cells (25). Cholesterol might have a central function in managing the traffic of certain lipids as well as lipid-linked proteins to the cell surface.

In conclusion, the folate receptor has revealed that GPI-anchored proteins can be ordered in the plane of the membrane through lipid–lipid interactions within the bilayer. In the future this class of membrane proteins may serve as convenient markers that will divulge new insights into the behavior of lipids in biological membranes.

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ments on the effects of Nystatin on caveolae substructure, which led us to carry out this investigation.

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