A Role for Caveolin in Transport of Cholesterol from Endoplasmic Reticulum to Plasma Membrane*

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Caveolin is a 22-kDa membrane protein found associated with a coat material decorating the inner membrane surface of caveolae. A remarkable feature of this protein is its ability to migrate from caveolae directly to the endoplasmic reticulum (ER) when membrane cholesterol is oxidized. We now present evidence caveolin is involved in transporting newly synthesized cholesterol from the ER directly to caveolae. MA104 cells and normal human fibroblasts transported new cholesterol to caveolae with a half-time of 10 min. The cholesterol then rapidly flowed from caveolae to non-caveolae membrane. Cholesterol moved out of caveolae even when the supply of fresh cholesterol from the ER was interrupted. Treatment of cells with 10 μg/ml progesterone blocked cholesterol movement from ER to caveolae. Simultaneously, caveolin accumulated in the lumen of the ER, suggesting cholesterol transport is linked to caveolin movement. Caveola fractions from cells expressing caveolin were enriched in cholesterol 3-4-fold, while the same fractions from cells lacking caveolin were not enriched. Cholesterol transport to the cell surface was nearly 4 times more rapid in cells expressing caveolin than in matched cells lacking caveolin.

EXPERIMENTAL PROCEDURES

Materials—Medium 199 with Earle’s salts minus folate acid was prepared in the laboratory by standard methods. Dulbecco’s modified Eagle’s medium (DMEM), glutamine, trypsin-EDTA, and penicillin/streptomycin were from Life Technologies, Inc. Fetal calf serum was from Haeleron Research Products, Inc. (Lenexa, KS). The analytical silica gel thin-layer chromatography plates, heptane, petroleum ether, ethyl ether, acetic acid, and 2-propanol were from J.T. Baker, Inc. [3H]Acetate (specific activity 4.13 Ci/mmol) was from DuPont. [3H]Folic acid (specific activity 27 Ci/mmol) was purchased from Moravek Biochemicals (City of Industry, CA). The sulfuric-dichromate spray was from Supelco (Bellevante, PA). The Bradford assay kit was from Bio-Rad. Calcium salts (City of Industry, CA). The sulfuric-dichromate spray was from Supelco (Bellevante, PA). The Bradford assay kit was from Bio-Rad. Percoll was from Pharmacia Biotech Inc. OptiPrep was from Life Technologies, Inc. The anti-caveolin IgG was from Transduction Labs (Lexington, KY), and anti-folate receptor IgG (Mov19) was from Centocor, Inc. (Malvern, PA). The caveolin expression construct was developed in the laboratory. Human lipoprotein-deficient serum was prepared as described (23).

Buffers—Buffer A consisted of 0.25 M sucrose, 1 mM EDTA, 20 mM Tricine, pH 7.8. Buffer B consisted of 0.25 M sucrose, 6 mM EDTA, 120 mM Tricine, pH 7.8. Buffer C was 50% OptiPrep in Buffer B. Buffer D consisted of 20 mM Tris, pH 7.6, 137 mM NaCl, 0.5% Tween 20. Buffer E consisted of 25 mM MES, pH 6.5, NaCl, 0.15 mM, 1% Triton X-100, and 60 mM octylglucoside. Buffer F (5 × sample buffer) was 0.3 M Tris-HCl, pH 6.8, 2.5% SDS, 50% glycerol, 0.125% bromphenol blue.

Cell Culture—Normal human fibroblasts were obtained by skin biopsy, cultured in a monolayer, and set up according to a standard format (23). On day zero, 2.5 × 10^5 cells were seeded into 100-mm dishes with 5 ml of DMEM supplemented with 100 units/ml penicillin/streptomycin and 10% (v/v) fetal calf serum. The medium was changed

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1 The abbreviations used are: ER, endoplasmic reticulum; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; GPI, glycosphosphatidylinositol; MES, 4-morpholineethanesulfonic acid; Tricine, N-(2-hydroxy-1,1-bis-hydroxy-methyl)glycine.

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on day 3 and day 5. The day 5 medium contained 10% (v/v) human lipoprotein-deficient serum. The cholesterol pool was radiolabeled by changing the medium on day 6 or day 7 to DMEM plus 20 µM Hepes, pH 7.4, adding [3H]acetate (50 µCi/dish) to the dish, and was incubated for the indicated times. All experiments were carried out on day 7. MA104 cells were also grown according to a standard format (24). On day zero, 3.0 × 10^6 cells were seeded into a T-75 culture flask and cultured for 5 days in folate-free medium supplemented with 5% (v/v) fetal calf serum and 100 units/ml penicillin/streptomycin. The cholesterol pool was labeled as described for human fibroblasts. For [3H]follate uptake assays, the cells were used directly on day 5 as described previously (25). L1210-JF cells (kindly provided by Dr. Bart Kamen) are a murine lymphoma cell line expressing the folate receptor (26). On day zero, 5 × 10^6 cells were seeded into T-75 flasks in RPMI medium 1640 plus 10% fetal (Life Technologies, Inc.) and 20 µM Hepes with 10 µCi/ml serum. Transfected cell medium also contained 300 µg/ml Geneticin. Cells were grown for 5 days. On day 5 the medium was changed to the same medium containing 10% (v/v) human lipoprotein-deficient serum, and on day 6 or day 7 the cholesterol pool was labeled as for the human fibroblasts.

**Radio-labeled Cholesterol Determination—** Thin-layer chromatography was used, as described previously (27), to measure the amount of [3H]cholesterol in membrane fractions from cells incubated in the presence of [3H]acetate. Fractions (0.65 ml) were adjusted to 1 ml (final volume) by adding 0.33 ml of 30% taurodeoxycholate and 0.02 ml Buffer A before mixing with 2 ml of Dole reagent (78:20:2; 2-propanol:heptane:water) and 1 ml of heptane. The samples were vortexed and spun in a table top centrifuge for 30 min at 9000 × g. The supernatant (0.65 ml) was diluted with heptane and the supernatant fractions containing lipids was saved for the thin-layer chromatography plates. The heptane phase was dried under N2 and suspended in 50 µl of the solvent system (80:20:1; petroleum ether:ethyl ether:acetic acid). Pure cholesterol and pure cholestanone were dissolved in the solvent system and used as standards (5 µg/spot). Lipids were visualized by charring with sulfuric acid-dichromate and heating at 180 °C for 10 min. Unla-beled cholesterol and cholestanone were added to each fraction to facilitate visualization. The appropriate spots were scraped and the amount of radiation quantified by liquid scintillation counting.

**Purification of Caveolae—** The caveolae were isolated as described previously (28) and modified (29). All steps were carried out at 4 °C. A plasma membrane fraction was first prepared from 10-100 mm dishes of T-75 flasks of confluent tissue culture cells (8–10 mg of total protein). A post-nuclear supernatant fraction (~4 mg of total protein) was layered on the top of 23 ml of 30% Percoll in Buffer A, and centrifuged at 84,000 × g for 30 min in a Beckman Ti 60 rotor. The cytosol corresponded to the top two fractions of the Percoll gradient, the plasma membrane was a 1-ml fraction (a visible band) taken ~5.7 cm from the bottom of the tube (the bottom plasma membrane fraction), and the bottom 21 1-ml fractions in the gradient. The plasma membrane, which contained ~0.6 mg of protein, was collected with a Pasteur pipette, adjusted to 2.0 ml with Buffer A and placed in a Sorvall TH841 centrifuge tube on ice. The membrane was sonicated before mixing with 1.84 ml of Buffer C and 0.16 ml of Buffer A (final OptiPrep concentra-tion, 23%) in the bottom of the same TH841 tube. A linear 20–100 OptiPrep gradient (prepared by diluting Buffer C with Buffer A) was poured on top of the sample and then centrifuged at 52,000 × g, 90 min in a Sorvall TH841 swinging bucket rotor. Fourteen fractions were collected from this first OptiPrep gradient. The top seven fractions contain the bulk of the caveolae membrane markers but very little protein, while the bottom seven fractions contain low concentrations of caveolae markers but the bulk of the membrane protein (29). Caveolae were prepared from the top seven fractions of the first OptiPrep gradi-ent as described previously (29).

**Protease Protection—** Cells that had been preincubated for 3 h in the presence of 50 µg/ml cycloheximide were washed extensively in PBS and subjected to the indicated treatments, all in the presence of cycloheximide. At the end of the treatments, cells were fractionated as described above. The appropriate samples were incubated with 300 µg of trypsin or trypsin plus 0.5% SDS for 30 min on ice. Soybean trypsin inhibitor (300 µg) was then added before caveolin was immunoprecipitated.

**Immunoprecipitation of Caveolin—** Protein A-Sepharose beads were first blocked by incubating them for 4 h at 4 °C with human fibroblast cell lysate (200 µl of a 30 mg/ml BSA in Buffer E). Blocked beads were used to preclear each experimental fraction after it had been adjusted to 1% Triton X-100 and 60 µM molybdate. Precleared fractions were then incubated for 19 h at 4 °C with a 1:400 dilution of anti-caveolin monoclonal antibody before adding blocked, Protein A-Sepharose beads and incubating an additional 2 h at 4 °C. Beads were removed by centrifugation, dissolved in Buffer F (30), and proteins separated by electrophoresis. The immunoprecipitated caveolin was detected by immunoblots with a polyclonal anti-caveolin IgG.
Table I

Subcellular cholesterol profile

On day 6 the indicated cell types grown for 24 h in the absence of lipoproteins were washed and placed in DMEM plus 20 mM Hepes, pH 7.4, containing 50 μCi of [3H]acetate and 10 μCi cold acetate for 24 h at 37 °C. After the incubation the cells were washed before the various fractions were prepared as described. PNS, postnuclear supernatant; Cyto, cytosol (fractions 1 and 2, Percoll gradient); IM, internal membranes (bottom 21 fractions, Percoll gradient); PM, plasma membrane (2-ml fraction 5.7 cm from the bottom of the centrifuge bottle, Percoll gradient); NCM, non-caveolae membrane (fraction 2, Optiprep 1 gradient); CM, caveolae membrane (fraction 2, Optiprep 2 gradient).

| Cell type        | Fractions | Protein  | Cholesterol × 10⁻⁵ | Chol/mg protein | Membrane cholesterol | Enrichment |
|------------------|-----------|----------|-------------------|----------------|---------------------|------------|
| Normal Human     |           |          |                   |                |                     |            |
| Fibroblast       | PNS       | 3.2      | 1490              | 466            |                     |            |
|                  | Cyto      | 2.6      | 17.8              | 6.8            |                     |            |
|                  | IM        | 0.51     | 219               | 706            |                     |            |
|                  | PM        | 0.27     | 1200              | 4444           |                     |            |
|                  | NCM       | 0.23     | 1060              | 4608           |                     |            |
|                  | CM        | 0.014    | 198               | 14142          | 16.5                | 3.18       |
| MA104 cell       | PNS       | 4.2      | 1800              | 428            |                     |            |
|                  | Cyto      | 3.4      | 6.6               | 1.9            |                     |            |
|                  | IM        | 0.36     | 82                | 277            |                     |            |
|                  | PM        | 0.35     | 1670              | 4771           |                     |            |
|                  | NCM       | 0.33     | 1330              | 4030           |                     |            |
|                  | CM        | 0.018    | 357               | 19833          | 21                  |             |

RESULTS

Previous measurements of caveolae cholesterol were made on detergent-resistant caveolae separated from soluble plasma membrane by sucrose gradient centrifugation (12). In the current study we used a new detergent-free method of purification that retains resident molecules removed by detergents (28). In addition we made measurements on both MA104 cells and normal human fibroblasts (Table I). Cells were incubated overnight in the presence of [3H]acetate before the [3H]cholesterol content of different fractions was measured. Most of the labeled cholesterol was in the plasma membrane (~80% for fibroblasts and ~92% for MA104 cells). A substantial portion of this cholesterol was in the caveolae fraction (16% for fibroblasts and 21% for MA104 cells). When normalized for the amount of protein, caveolae were significantly enriched in cholesterol relative to the remainder of the plasma membrane (3.18-fold for fibroblasts and 4.16-fold for MA104 cells).

Transport of Cholesterol to Caveolae—Cholesterol synthesized at 14 °C is largely retained in the ER (16). Shifting the temperature to 37 °C allows this cholesterol to migrate to the cell surface. To see if caveolae in MA104 cells were involved in transport of newly synthesized cholesterol, we incubated cells in the presence of [3H]acetate for 1 h at 14 °C before adding excess unlabeled acetate and shifting the temperature to 37 °C for various times. We measured the amount of radiolabeled cholesterol in the plasma membrane, non-caveolae, and caveolae fractions (Fig. 1A). The pattern of transport was similar in human fibroblasts (data not shown). A small amount of labeled cholesterol was in the plasma membrane (△) at the end of the pulse but all of this cholesterol was in the caveolae fraction (□). During the first 10 min at 37 °C, most of the labeled membrane cholesterol was in the caveolae fraction (compare △ with □). The level of label in the caveolae fraction peaked at 10–20 min and then declined while the amount in the non-caveolae fraction steadily increased after 10 min (○). Membrane and non-caveolae membrane cholesterol reached a plateau as the amount in the caveolae fraction declined to zero. Therefore, newly synthesized cholesterol appears at the cell surface first in caveolae and then in non-caveolae membrane.

Another way of looking at the dynamics of caveolae cholesterol is to uniformly label the plasma membrane cholesterol pool before removing the label and measuring the amount of labeled cholesterol in the various fractions during a chase period (Fig. 1B). MA104 cells were incubated for 24 h in the presence of [3H]acetate at 37 °C, washed, and then chased for the indicated times. Initially we found ~400 × 10⁵ dpm of [3H]cholesterol in the caveolae fraction (□). During the chase period, the amount of label declined to zero and a corresponding amount disappeared from the plasma membrane (△). The labeled cholesterol pool in non-caveolae membrane remained...
Caveolin Transports Cholesterol

Progesterone blocks movement of [3H]cholesterol to caveolae fraction of MA104 cells. A, cells were washed and placed in DMEM plus 20 mM Hepes, pH 7.4, containing 50 μCi of [3H]acetate and 10 μM cold acetate for 24 h at 37 °C. At the end of the labeling period, progesterone (10 μg/ml) plus 0.5 mg/ml fatty acid-free BSA was added directly to the medium containing [3H]cholesterol and the cells further incubated for the indicated time before plasma membrane (□), non-caveolae membrane (○), and caveolae membrane (●) fractions were prepared. B, MA104 cells were washed and placed in DMEM containing 50 μCi of [3H]acetate and 10 μM cold acetate for 24 h at 37 °C. After the labeling period, progesterone (10 μg/ml) plus 0.5 mg/ml fatty acid-free BSA was added directly to the radioactive medium for 1 h at 37 °C. The cells were then extensively washed to remove progesterone and incubated in fresh medium containing 50 μCi of [3H]acetate and 10 μM cold acetate for 0 or 60 min in the presence (striped bar) or absence (solid bar) of 100 μM compactin. The caveolae fractions were prepared as described and the quantity of [3H]cholesterol determined.

unchanged. The loss of [3H]cholesterol from the caveolae fraction, either during a pulse-chase (A) or a chase (B), suggests cholesterol flows from the ER through caveolae on its way to the surrounding membrane.

Progesterone interrupts two-way traffic of cholesterol between the plasma membrane and internal membranes (17). It also inhibits cholesterol synthesis. If cholesterol movement to the plasma membrane involves caveolae, then progesterone should block the appearance of radiolabeled cholesterol in this fraction. We labeled the cholesterol pool by culturing MA104 cells for 24 h in the presence of [3H]acetate (Fig. 2A). Progesterone was added to the dish without removing the [3H]acetate and the cells further incubated for various times before preparing cell fractions. The presence of progesterone caused a rapid decline in the level of [3H]cholesterol in the caveolae fraction (□). The plasma membrane fraction (○) also lost [3H]cholesterol during the incubation, while the non-caveolae membrane (●) did not change. The effect of progesterone further indicates cholesterol first appears in caveolae after leaving the ER.

Cholesterol returned to caveolae after progesterone was removed (Fig. 2B). Uniformly labeled MA104 cells were incubated in the presence of progesterone for 1 h. The progesterone was removed, and either caveolae fractions were prepared immediately (0 min) or the cells were incubated an additional 1 h (60 min) in the presence (striped bar) or absence (solid bar) of 100 μM compactin before caveolae were isolated. The amount of [3H]cholesterol in the caveolae fraction (solid bar) rose from 0 to 311 × 10^5 dpm within 60 min after progesterone was removed. The presence of compactin completely blocked the appearance of radiolabeled cholesterol (60 min, striped bar), indicating a requirement for new cholesterol biosynthesis. This suggests that [3H]cholesterol does not accumulate in internal membranes during the exposure to progesterone, nor does non-caveolae [3H]cholesterol from contiguous plasma membrane migrate back into caveolae when transport to the cell surface is blocked.

Progesterone Inhibits Caveolae Internalization—Previously we found that internalization of folate by caveolae is reduced in cells starved of cholesterol (7). This treatment, however, has too many side effects to ever be a useful experimental tool for studying caveolae function. Progesterone should have a similar effect to cholesterol depletion with the advantage of being rapid and reversible (Fig. 3). MA104 cells were incubated in the presence of different concentrations of progesterone for 1 h at 37 °C before [3H]folic acid was added to the dish and the cells further incubated for 1 h (A). At the end of the incubation, the amount of internal (□) and external (○) bound [3H]folic acid was measured (24). In the absence of any progesterone, the cells had equal amounts of internal and external bound [3H]folic acid, indicating normal caveolae function. As little as 10 μg/ml progesterone caused a dramatic relocation of the
In the presence of [3H]folic acid at 37°C for 1 h to label both internalization. Progesterone, therefore, appears to prevent folate receptor internalization. Higher concentrations of progesterone had similar effects.

Inhibition by progesterone occurred coordinately with the loss of cholesterol from caveolae (Fig. 3B). Cells were incubated in the presence of [3H]folic acid at 37°C for 1 h to label both internal and external receptors (B). Progesterone (10 μg/ml) was then added to the dish and the cells further incubated for the indicated time. Within 20 min, most of the internal receptors became exposed at the cell surface, which matches closely the time it takes for cholesterol to leave the caveolae fraction (Fig. 1B). In other experiments (data not shown), we found that it took 60–90 min for the receptor ratio to return to normal after progesterone was removed.

Lowering the cholesterol content of caveolae can have two effects: (a) prevention of caveolae internalization along with reducing the number of invaginated caveolae (7) and (b) unclustering of folate receptors (6, 29). We used fibroblasts to determine if progesterone changed the number of invaginated caveolae because these cells respond to progesterone exactly the same as MA104 cells (data not shown). Cells were prepared for electron microscopy after they had been incubated in the presence or absence of progesterone for 1 h (Table II). Progesterone caused a 32–36% decline in the number of invaginated caveolae. We then used the caveolae isolation procedure to assess how progesterone affected the surface distribution of the folate receptor (Fig. 4). Plasma membranes were isolated from untreated cells, sonicated, and separated on the first OptiPrep gradient (Control). Immunoblots of total protein in each fraction were either immunoprecipitated directly with anti-caveolin IgG or first incubated in the presence of 50 μg/ml cycloheximide for 3 h and subsequently washed and incubated an additional 1 h at 37°C in the absence of progesterone (Wash-out). Equal amounts of protein from each fraction were either immunoprecipitated directly with anti-caveolin IgG or first incubated in the presence of 300 μg/ml trypsin (Control+T) or of 10 μg/ml progestrone plus 0.5 mg/ml fatty acid-free BSA for 1 h at 37°C. One set of cells treated with the progesterone was incubated an additional hour at 37°C in the absence of progesterone (Wash-out). Cells were processed to isolate caveolae up to the first OptiPrep gradient step. Total amount of protein in each fraction from this gradient was separated on polyacrylamide and immunoblotted with anti-caveolin IgG. For the [3H]folic uptake experiment, each point is the average of three separate measurements where the standard deviation was less than 1%

In human fibroblasts, caveolin can move directly between the plasma membrane and the ER (12, 13). Caveolin also binds both cholesterol (19) and fatty acids (21). This raises the possibility that caveolin might shuttle newly made cholesterol to caveolae.

**Table II**

| Exp. no. | Treatment | Membrane evaluated | No. of caveolae | Decrease |
|---------|-----------|-------------------|----------------|---------|
| 1       | None      | 0.25              | 2197           | 32      |
| 1       | Progesterone | 0.27              | 1434           |         |
| 2       | None      | 0.17              | 1572           |         |
| 2       | Progesterone | 0.16              | 940            |         |

**Fig. 4.** Progesterone unclusters the folate receptor. MA104 cells were incubated in the presence (Progesterone and Wash-out) or absence (Control) of progesterone plus 0.5 mg/ml fatty acid-free BSA for 1 h at 37°C. One set of cells treated with progesterone was incubated an additional hour at 37°C in the absence of progesterone (Wash-out). Cells were processed to isolate caveolae up to the first OptiPrep gradient step. Total amount of protein in each fraction from this gradient was separated on polyacrylamide and immunoblotted with an anti-folate receptor IgG. For the [3H]folic uptake experiment, each point is the average of three separate measurements where the standard deviation was less than 1%.

**Fig. 5.** Progesterone causes redistribution of caveolin to an internal membrane compartment. A, human fibroblasts were incubated in the presence (Progesterone and Wash-out) or absence (Control) of progesterone plus 0.5 mg/ml fatty acid-free BSA for 1 h at 37°C. One set of cells treated with the progesterone was incubated an additional hour at 37°C in the absence of progesterone (Wash-out). Cells were processed to isolate caveolae up to the first OptiPrep gradient step. Total amount of protein in each fraction from this gradient was separated on polyacrylamide and immunoblotted with an anti-caveolin IgG as described. B, human fibroblasts preincubated in the presence of 50 μg/ml cycloheximide for 3 h were incubated in the presence (Progesterone, Wash-out, Progesterone+T, Wash-out+T, Progesterone+T+SDS) or absence (Control, Control+T) of 10 μg/ml progesterone plus 0.5 mg/ml fatty acid-free BSA for 1 h. One set of progesterone-treated cells was subsequently washed and incubated an additional 1 h at 37°C in the absence of progesterone (Wash-out, Wash-out+T). Cells were fractionated into caveolae membrane (CM), non-caveolae membrane (NCM), plasma membrane (PM), internal membranes (IM), cytosol (Cytool) and postnuclear supernatant fraction (PNS). Equal amounts of protein from each fraction were either immunoprecipitated directly (Control, Progesterone, Wash-out) with anti-caveolin IgG or first incubated in the presence of 300 μg/ml trypsin (Control+T, Progesterone+T, Wash-out+T, and Progesterone+T+SDS) for 30 min on ice before immunoprecipitation. Each immunoprecipitate was separated on polyacrylamide gels and immunoblotted with anti-caveolin IgG.
We first used immunoblotting to determine the location of caveolin in progesterone-treated cells (Fig. 5A). Fibroblasts were either not treated (Control) or incubated in the presence of progesterone for 1 h at 37 °C (Progesterone and Wash-out). One set was analyzed immediately (Progesterone), while the other was incubated further in the absence of progesterone (Wash-out). The plasma membrane from each set was isolated and fractionated on OptiPrep 1 gradients in order to evaluate the distribution of caveolin in the whole membrane (28). In control cells, most of the caveolin was in the top seven fractions, which is the normal distribution of the protein (28). There was markedly less caveolin in the same fractions of progesterone-treated cells, although all of the protein that remained was in the caveolae fractions. The level of caveolin returned to normal once the progesterone was removed from the medium. Progesterone, therefore, causes a reversible loss of caveolin from the plasma membrane.

We used an immunoprecipitation assay to see if progesterone caused caveolin to accumulate in the ER (Fig. 5B). All treatments were carried out on cells cultured in the presence of protein synthesis inhibitors. Most of the caveolin was in the caveola fraction (CM) of control human fibroblasts (Control) and very little in the internal membrane fractions containing ER and Golgi apparatus (IM). There was a dramatic reduction in the amount of caveolin in caveolae (CM) after progesterone treatment (Progesterone) and a corresponding increase in the amount precipitated from internal membranes (IM). The distribution of caveolin returned to normal after progesterone was removed (Wash-out). The caveolin in the internal membrane fraction of progesterone-treated cells (Progesterone + T) was resistant to trypsin treatment, while the caveolin at the cell surface (Control + T and Wash-out + T) was completely digested by the protease. If the internal membrane fraction from progesterone-treated cells was permeabilized with ionic detergents, however, the caveolin was degraded by the trypsin (progesterone + T + SDS). Therefore, progesterone causes caveolin to reversibly accumulate in ER and Golgi membranes. This suggests that caveolin movement is linked to the flow of cholesterol between ER and caveolae.

Caveolin might function as a shuttle protein that moves cholesterol from the ER to the cell surface. In this case, the expression of caveolin should effect the rate and direction of cholesterol transport to the cell surface. We measured the cholesterol level of caveolae fractions prepared from lymphocytes expressing or not expressing caveolin. A lymphocyte variant of L1210 cells that expresses the folate receptor (designated L1210-JF cells; Ref. 26) was transfected either with vector alone or a cDNA for caveolin (Fig. 6). Permanent transformants were selected and maintained as stable cell lines. Anti-caveolin immunoblots of the whole cell lysate from parent cells (L1210-JF) and mock-transfected cells (Mock) did not detect any caveolin. By contrast, cells transfected with the cDNA to caveolin (Caveolin) had a strong reactive band, comparable in intensity to the band obtained from MA104 cell lysates (MA104). Caveolae isolated from either parent cells or mock-transfected cells were not enriched in [3H]cholesterol compared to non-caveolae membrane (Table III) after cells were grown in the presence of [3H]acetate for 24 h. The caveolae fraction from cells expressing caveolin, however, contained 15% of the plasma membrane [3H]cholesterol and were significantly enriched in the sterol (4.4-fold relative to plasma membrane). The total protein in the caveolae fractions from each set of cells was about the same.

The rate of new cholesterol transport to the cell surface was significantly faster in cells expressing caveolin (Fig. 7). We used a 1-h pulse of [3H]acetate at 14 °C, followed by a chase at 37 °C in the absence of label to measure the rate of [3H]cholesterol appearance at the cell surface. The initial rate of transport to plasma membrane (Δ) in both parental cells (A) and mock-transfected cells (B) was ~9 × 10^5 dpm/min. Cells expressing caveolin, on the other hand, had initial transport rates

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**Table III**

Effect of caveolin on cholesterol location

| Cell Line   | Fractions | Protein | Cholesterol × 10^-5 | Chol/mg protein | Membrane cholesterol | Enrichment |
|-------------|-----------|---------|---------------------|-----------------|----------------------|------------|
| L1210-JF    | PNS       | 4.3     | 1040                | 241.8           | 0.17                 | 4.0        |
|             | Cyto      | 3.5     | 0.6                 | 0.17            | 0.17                 | 1.0        |
|             | IM        | 0.42    | 160                 | 380             | 380                  | 1.0        |
|             | PM        | 0.3     | 866                 | 2886            | 2886                 | 1.0        |
|             | NCM       | 0.28    | 788                 | 2814            | 2814                 | 1.0        |
|             | CM        | 0.012   | 34.9                | 2908            | 2908                 | 1.0        |
| L1210-JF (Mock) | PNS       | 4.9     | 955                 | 195             | 195                  | 1.0        |
|             | Cyto      | 4.0     | 0.7                 | 0.17            | 0.17                 | 1.0        |
|             | IM        | 0.44    | 155                 | 352             | 352                  | 1.0        |
|             | PM        | 0.32    | 844                 | 2637            | 2637                 | 1.0        |
|             | NCM       | 0.3     | 793                 | 2643            | 2643                 | 1.0        |
|             | CM        | 0.010   | 26.6                | 2660            | 2660                 | 1.0        |
| L1210-JF (caveolin) | PNS | 4.3 | 1000 | 232 | 232 | 1.0 |
|             | Cyto      | 3.6     | 0.4                 | 0.11            | 0.11                 | 1.0        |
|             | IM        | 0.42    | 135                 | 321             | 321                  | 1.0        |
|             | PM        | 0.28    | 821                 | 2932            | 2932                 | 1.0        |
|             | NCM       | 0.27    | 684                 | 2533            | 2533                 | 1.0        |
|             | CM        | 0.0096  | 124                 | 12916           | 12916                | 4.42       |

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**FIG. 6.** L1210-JF cells permanently transfected with a cDNA to caveolin express the protein. Whole cell lysates (50 μg) from control L1210-JF cells (L1210-JF), mock-transfected L1210-JF cells (Mock), caveolin-transfected L1210-JF cells (Caveolin), and MA104 cells were immunoblotted with anti-caveolin IgG using standard conditions.
found that cholesterol reaches the cell surface within 10–20 min after synthesis in the ER (14, 16) and appears to move directly to the plasma membrane without passing through the Golgi apparatus (16). We have now determined that caveolae are the initial site on the cell surface where this new cholesterol appears. A population of cholesterol-rich membranes with the properties of a membrane intermediate carrying cholesterol from the ER have been detected in several studies (16, 33). This membrane, like caveolae membrane, has a light buoyant density on sucrose gradients. Therefore, new cholesterol may reach the plasma membrane in vesicles targeted to caveolae or through contact sites between cholesterol-rich regions of ER membrane and either caveolae or caveolae-derived vesicles (e.g. plasmalemmal vesicles; Ref. 34).

Several studies have implicated caveolin in intracellular cholesterol traffic (12, 19, 22). Two observations in the current study suggest caveolin functions in rapid cholesterol transport to the cell surface. First, progesterone lowered the cholesterol content of caveolae and at the same time caused caveolin to accumulate in internal membranes. The internal caveolin was protease-resistant, indicating it had moved to the luminal side of the ER-Golgi membrane, as it does when plasma membrane cholesterol is oxidized by cholesterol oxidase (12). New cholesterol synthesis was required for the cholesterol levels in caveolae to return to normal after the removal of progesterone, so cholesterol did not accumulate in the presence of the steroid. This is curious because progesterone blocks cholesterol synthesis by preventing the conversion of cholesterol precursors (35, 36), as well as several oxidized sterols (35), into cholesterol while stimulating 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity (35). Most likely cholesterol was synthesized from these intermediates (35) after progesterone was removed, yet none of it appeared in caveolae. This suggests progesterone directly causes the mislocation of both cholesterol intermediates and cholesterol in the cell. On the other hand, we do not know if inhibiting cholesterol synthesis with compactin might also cause caveolin accumulation in internal membranes and lower caveolae cholesterol. A second and more direct indication of caveolin involvement in cholesterol transport is that lymphocytes expressing caveolin have a 4.4-fold higher concentration of cholesterol in the caveolae fraction than control cells. Furthermore, they transport sterol to the plasma membrane ~ 4 times more rapidly.

One of the remarkable findings in this study was that newly synthesized cholesterol appears to flow through caveolae membrane. When we allowed the labeled cholesterol pool to accumulate in the ER at 14 °C, for example, a bolus of [3H]cholesterol first appeared in the caveolae fraction after the cells were shifted to 37 °C and then immediately migrated into the surrounding membrane. Likewise, the level of [3H]cholesterol in caveolae rapidly declined when the supply of [3H]acetate was interrupted or when the flow of cholesterol into the caveolae was blocked by progesterone. There even appears to be a mechanism for exporting cholesterol from caveolae to the surrounding membrane in the absence of new cholesterol synthesis. We also did not detect any movement of cholesterol back into caveolae from the surrounding membrane. Vectorial movement of cholesterol through caveolae may contribute to the overall organization and function of this membrane domain.

The cholesterol oxidase-induced movement of caveolin to the Golgi apparatus provided the first clue that molecular traffic occurs directly between caveolae and ER (12). We have now detected molecular movement in the opposite direction, and it appears to involve caveolin. Two-way transfer of molecules between the plasma membrane and the ER may be an important route of molecular exchange between the cell and its environment. The endoplasmic reticulum in most cells is orga-

Fig. 7. Transport of newly synthesized cholesterol to plasma membrane (©) and caveolae fractions (©) of L1210-JF cells (A), mock-transfected L1210-JF cells (B), and caveolin cDNA transfected L1210-JF cells (C). Cells were washed and incubated in DMEM plus 20 mM Hepes, pH 7.4, containing 50 µCi of [3H]acetate and 10 µM cold acetate for 1 h at 14 °C. At the end of the incubation, cells were washed before further incubating at 37 °C in the absence of label for the indicated times. Cells were then fractionated and the amount of [3H] cholesterol measured.

of ~33 × 10^3 dpm/min. Moreover, only the caveolae fraction (©) from transfected cells acquired significant cholesterol during the chase.

In addition to restoring the cholesterol level of caveolae to near normal, we found that expression of caveolin had other effects. Although the number of invaginated caveolae per cell was low compared to fibroblasts and MA104 cells (Table IV), 36% of caveolin-expressing L1210-JF cells had typical omega-shaped membrane structures, while non-expressing cells had none. Caveolin-expressing cells also had a higher density of folate receptors in the caveolae fractions (Fig. 8). Less than 5% of the receptors were in the caveolae fraction of parent (control) and mock-transfected (mock) cells compared to 35% in cells expressing caveolin (caveolin). Expression of caveolin, therefore, appears to have a direct effect on caveolae structure and organization.

DISCUSSION

Cholesterol Flows through Caveolae—Previous studies have found that cholesterol reaches the cell surface within 10–20
nized into a network that extends beneath the entire plasma membrane (37), so the travel distance between caveole and ER compartments is quite short. The ER houses a number of enzymes involved in various aspects of lipid metabolism and storage and is the major site of membrane bilayer synthesis. It makes sense, therefore, that there would be a mechanism for direct delivery of lipid intermediates to the ER. From this perspective, lipids such as fatty acids and cholesterol would be among the molecules expected to be internalized by caveole. Caveolin may be one member of a class of caveole molecules that facilitate uptake and excretion of lipids, as well as other hydrophobic molecules, by the cell.

Cholesterol and Caveole Function—The caveole membrane fraction in lymphocytes not expressing caveolin behaved exactly the same during cell fractionation as caveole that contain caveolin. Although we have not carried out a detailed molecular analysis, we know this fraction was low in both cholesterol and the GPI-anchored folate receptor compared to caveole fractions from cells expressing caveolin. Nevertheless, the membrane must contain sorting information for caveolin because this was the exclusive location for the molecule in cells transfected with caveolin cDNA. An important next step will be to determine what effect caveolin and cholesterol have on the presence in the caveole fraction of signal-transducing molecules such as tyrosine kinase receptors (38, 39), heterotrimeric GTP-binding proteins (1, 40) and non-receptor tyrosine kinases (1, 38, 41) and whether or not these molecules function properly in signal transduction. Non-receptor tyrosine kinases have been found in Triton X-100-insoluble membrane fractions from lymphocytes lacking detectable caveolin, and kinase activity is stimulated in these cells by antibodies against GPI-anchored proteins (42). Still, caveolin as well as cholesterol may have important modulatory influences on signal integration in caveole.

We confirmed previous observations (43) that cells transfected with the caveolin cDNA tend to have invaginated caveole similar in overall morphology to those found in fibroblasts and endothelial cells. We also found that the caveole fraction from these cells had considerably more folate receptor than control cells. This is the third set of experimental evidence (6, 7, 29) that cholesterol is important for organizing GPI-anchored proteins in caveole. Nevertheless, we were unable to detect folate internalization in these cells (data not shown). Either cholesterol is necessary but not sufficient for caveole internalization or the cells did not express high enough levels of caveolin to support receptor internalization. These cells express many more receptors than an MA104 cell, the standard cell for these assays, so at best, internalization would be expected to be inefficient.

Progesterone is a new reagent for inhibiting potocytosis. The mechanism of action is quite different from other inhibitors that have been identified (20, 27). Progesterone appears to inhibit by reducing the cholesterol level of caveole. In part this causes the dispersal of GPI-anchored proteins in the plane of the membrane, but it also reduces the number of invaginated caveole. If the number of invaginated caveole is a measure of how many caveole are cycling (27), then both folate receptor unclustering and loss of internalization contribute to the inhibition of folate uptake. Progesterone could be a useful tool for assessing caveole function in other cell types.

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TABLE IV
Effect of caveolin on invaginated caveole

| Exp. no. | cDNA transfected | No. of cells counted | No. of caveole | Cells with caveole |
|----------|------------------|---------------------|---------------|-------------------|
| 1        | None             | 100                 | 0             | 0                 |
|          | Caveolin         | 70                  | 25            | 36                |
| 2        | None             | 100                 | 0             | 0                 |
|          | Vector alone     | 100                 | 0             | 0                 |
|          | Caveolin         | 136                 | 50            | 37                |

FIG. 8. Effect of caveolin expression on clustering of folate receptor in L1210-JF cells. L1210-JF cells (Control), mock-transfected L1210-JF cells (Mock), caveolin cDNA-transfected L1210-JF cells (Caveolin), and MA104 cells (MA104) were incubated in the presence of 5 nM [3H]folate for 1 h at 37 °C. The cells were washed and the caveole fractions prepared. The amount of bound [3H]folate associated with caveole fraction is expressed as a percent of the total [3H]folate in the post-nuclear supernatant.

REFERENCES
1. Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M. P. (1993) J. Cell Biol. 122, 789–808
2. Liu, P., and Anderson, R. G. W. (1995) J. Biol. Chem. 270, 27179–27185
3. Palade, G. E. (1955) J. Appl. Phys. 24, 1424
4. Yamada, E. (1955) J. Biophys. Biochem. Cytol. 1, 445–458
5. Rothberg, K. G., Ying, Y.-S., Kolhouse, J. F., Kamen, B. A., and Anderson, R. G. W. (1990) J. Cell Biol. 110, 637–646
6. Rothberg, K. G., Ying, Y.-S., Kamen, B. A., and Anderson, R. G. W. (1990) J. Cell Biol. 111, 2931–2938
7. Chang, W.-J., Rothberg, K. G., Kamen, B. A., and Anderson, R. G. W. (1992) J. Cell Biol. 118, 63–69
8. Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y.-S., Glenney, J. R., and Anderson, R. G. W. (1992) Cell 68, 673–682
9. Anderson, R. G. W., Kamen, B. A., Rothberg, K. G., and Lacey, S. W. (1992) Science 255, 410–411
10. Anderson, R. G. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10909–10913
11. Dietzen, D. J., Hastings, W. R., and Lublin, D. M. (1995) J. Biol. Chem. 270, 6640–6642
12. Smart, E. J., Ying, Y.-U., Conrad, P. A., and Anderson, R. G. W. (1994) J. Cell Biol. 127, 1185–1197
13. Conrad, P. A., Smart, E. J., Ying, Y.-S., Anderson, R. G. W., and Bloom, G. S. (1995) J. Cell Biol. 131, 1424–1433
14. DeGrella, R. F., and Simoni, R. D. (1982) J. Biol. Chem. 257, 14256–14262
15. Kaplan, M. R., and Simoni, R. D. (1986) J. Cell Biol. 101, 446–453
16. Urbani, L., and Simoni, R. D. (1990) J. Biol. Chem. 265, 1919–1923
17. Lange, Y. (1994) J. Biol. Chem. 269, 3431–3414
18. Fielding, P. E., and Fielding, C. J. (1995) Biochemistry 34, 14268–14292
19. Murata, M., Peranen, J., Schreiner, B., Wieland, F., Karrbach, T. V., and Simons, K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10339–10343
20. Smart, E. J., Estes, K., and Anderson, R. G. W. (1995) Cold Spring Harbor Symp. Quant. Biol. 60, 245–248
21. Trigatti, B. L., Mangroo, D., and Gerber, G. E. (1991) *J. Biol. Chem.* **266**, 22621–22625
22. Li, S., Song, K. S., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271**, 568–573
23. Goldstein, J. L., Basu, S. K., and Brown, M. S. (1983) *Methods Enzymol.* **98**, 241–260
24. Kamen, B. A., Wang, M.-T., Streckfuss, A. J., Peryea, X., and Anderson, R. G. W. (1988) *J. Biol. Chem.* **263**, 13602–13609
25. Kamen, B. A., and Capdevila, A. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5983–5987
26. Fan, J., Vitols, K. S., and Huennekens, F. M. (1991) *J. Biol. Chem.* **266**, 14862–14865
27. Smart, E. J., Foster, D. C., Ying, Y.-S., Kamen, B. A., and Anderson, R. G. W. (1994) *J. Cell Biol.* **124**, 307–313
28. Smart, E. J., Ying, Y.-S., Mineo, C., and Anderson, R. G. W. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10104–10108
29. Smart, E. J., Ying, Y.-S., Donzell, W. C., and Anderson, R. G. W. (1996) *J. Cell Biol.* **134**, 1169–1177
30. Laemmli, U. K. (1970) *Nature* **227**, 680–685
31. Smart, E. J., Ying, Y.-S., and Anderson, R. G. W. (1995) *J. Cell Biol.* **131**, 929–938
32. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
33. Lange, Y., and Stock, T. L. (1985) *J. Biol. Chem.* **260**, 15592–15597
34. Palade, G. E., and Bruns, R. R. (1968) *J Cell Biol.* **37**, 633–649
35. Panini, S. R., Gupta, A., Sexton, R. C., Parish, E. J., and Rudney, H. (1987) *J. Biol. Chem.* **262**, 14435–14440
36. Metherall, J. E., Waugh, K., and Li, H. (1996) *J. Biol. Chem.* **271**, 2627–2633
37. Bergeron, M., Thiery, G., Lenoir, F., Giocondi, M.-C., and Grimmellec, C. L. (1994) *Cell Tissue Res.* **277**, 297–307
38. Liu, P., Ying, Y., Ko, Y.-G., and Anderson, R. G. W. (1996) *J. Biol. Chem.* **271**, 10299–10303
39. Mineo, C., James, G. L., Smart, E. J., and Anderson, R. G. W. (1996) *J. Biol. Chem.* **271**, 11930–11935
40. Chang, W.-J., Ying, Y.-S., Rothberg, K. G., Hooper, N. M., Turner, A. J., Gambriel, H. A., De Ganzburg, J., Mumby, S. M., Gilman, A. G., and Anderson, R. G. W. (1994) *J. Cell Biol.* **126**, 127–138
41. Shenoy-Scaria, A. M., Dietzen, D. J., Kwong, J., Link, D. C., and Lublin, D. M. (1994) *J. Cell Biol.* **126**, 353–363
42. Van den Berg, C. W., Cinek, T., Hallett, M. B., Horejsi, V., and Morgan, B. P. (1995) *J. Cell Biol.* **131**, 669–677
43. Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8655–8659