Filipin-Cholesterol Complexes Form in Uncoated Vesicle Membrane Derived from Coated Vesicles during Receptor-mediated Endocytosis of Low Density Lipoprotein

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ABSTRACT Filipin has been widely used as an electron microscopic probe to detect 3-β-hydroxysterols, principally cholesterol, in cellular membranes. When it complexes with sterol, it forms globular deposits that disrupt the planar organization of the membrane. Previous studies have shown that coated pits and coated vesicles, specialized membranes involved in receptor-mediated endocytosis, do not appear to bind filipin. This has led to the suggestion that these membranes are low in cholesterol compared with the remainder of the plasma membrane. Since coated endocytic vesicles become uncoated vesicles during the transport of internalized ligands to the lysosome, we have carried out studies to determine whether or not the membranes that surround these transport vesicles are unable to bind filipin and therefore, are also low in cholesterol. Cells were incubated with ferritin-conjugated ligands that bind to low density lipoprotein (LDL) receptors in coated pits. After allowing internalization of the conjugates, we fixed the cells in either the presence or absence of filipin. This permitted us to identify all of the vesicles involved in the transport of LDL to the lysosome and to determine whether the membranes of these vesicles were able to bind filipin. We found that, coordinate with the dissociation of the clathrin coat from the endocytic vesicles, the membranes became sensitive to the formation of filipin-sterol complexes. Furthermore, all of the uncoated endocytic vesicle membranes, as well as the lysosomal membranes, bound filipin. This suggests either that coated membrane contains normal cholesterol levels, which is not easily detected with filipin, or that cholesterol rapidly moves into endocytic vesicles after the clathrin coat dissociates from the membrane.

Cholesterol is important for the proper biological function of most eucaryotic cells. On the basis of biochemical measurements of isolated cellular membranes, plasma membranes contain a higher cholesterol-to-phospholipid molar ratio (0.3–1.2) than intracellular membranes from mitochondria (0.03–0.09) and endoplasmic reticulum (ER) (0.03–0.08) (1). This difference in cholesterol content has also been detected by electron microscopy, using either digitonin or filipin, which binds with specificity to 3-β-hydroxysterols (2–5). Experimental studies with phospholipid-cholesterol liposomes have shown that these agents form complexes with cholesterol that can be detected by either freeze-fracture or thin-section electron microscopy (2–4). When applied to cells during fixation, filipin-cholesterol complexes are prominent in the plasma membrane; however, they are absent from ER and mitochondria membranes (5–9). A correlation between free cholesterol content and the presence of digitonin-cholesterol complexes has also been found in lipid-laden aortic cells (10).

This cytochemical technique also permits the detection of microheterogeneity in cholesterol distribution in membranes (2). Gap junctions, tight junctions, and coated pits are not disrupted by either filipin or digitonin (4, 7), even though the membrane adjacent to these membrane specializations readily binds these agents. These observations suggest that continuous portions of membrane can become differentiated in cholesterol content, which may be important for the proper function of the membrane specialization.

The absence of filipin-detectable cholesterol in coated pits has been of special interest to investigators studying receptor-mediated endocytosis. Coated pits contain cell surface receptors for various extracellular macromolecules, including LDL (reviewed in reference 11). When bound to receptors in these

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 MAY 1983 1273-1278

membrane regions, the ligands are internalized by the conversion of coated pits to coated vesicles. Eventually, the molecules are delivered to various intracellular sites by uncoated vesicles.

If coated pits are deficient in cholesterol, as suggested by the absence of filipin binding, then endocytic vesicle membrane derived from coated vesicles should have similar amounts of cholesterol. To answer this question, we have used filipin to detect cholesterol in endocytic membranes that originated from coated pits. When either LDL (12–14) or monoclonal antibodies against the LDL receptor (15) are conjugated to ferritin, they can be used as specific electron microscopic probes to identify all of the intermediate endocytic vesicles involved in the normal delivery of LDL to the lysosome. Therefore, we have incubated cells at 37°C with either LDL-ferritin or monoclonal anti-LDL receptor antibody conjugated to ferritin and then fixed them in the presence or absence of filipin. With filipin as a probe for membrane cholesterol, and receptor-specific ferritin conjugates as markers for the membrane-bound compartments that transport LDL to the lysosome, we have been able to survey all of the endocytic and lysosomal membranes to see whether filipin-cholesterol complexes are present.

MATERIALS AND METHODS

Materials: Glutaraldehyde was purchased from Electron Microscopy Sciences (Fort Washington, PA). Epon and Araldite were obtained from Ladd Research Industries, Inc. (Burlington, VT). Filipin was generously provided by Dr. Joseph Grady of the Upjohn Co. (Kalamazoo, MI).

Cultured Cells: Cultured human fibroblasts were derived from the foreskin of a healthy newborn (16). Cells were grown in monolayer and maintained in a humidified incubator (5% CO_2) at 37°C in 250-ml flasks containing 10 ml of growth medium consisting of Dulbecco's modified Eagle's medium (KC Biological, Inc., Lenexa, KS) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% (vol/vol) fetal calf serum (FCS). All experiments were performed with a similar format. On day 0, confluent monolayers of cells from stock flasks were dissociated with a 0.05% trypsin, 0.02% EDTA solution, and 5×10^4 cells were seeded into each 60 × 15-mm petri dish (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) containing 3 ml of growth medium with 10% FCS. On day 3, the medium was replaced with 3 ml of fresh growth medium containing 10% FCS. On day 5, each monolayer was washed with 3 ml of phosphate-buffered saline, after which 2 ml of fresh medium containing 10% (vol/vol) human lipoprotein-deficient serum (17) was added. (The final protein concentration was 5 mg/ml.) Experiments were initiated on day 7 after the cells had been incubated for 48 h in the presence of lipoprotein-deficient serum.

Preparation of LDL-Ferritin and IgG-C7-Ferritin: Human LDL (d = 1.019–1.063 gm/ml) and lipoprotein-deficient serum (d > 1.215 gm/ml) were obtained from the plasma of individual healthy subjects and prepared by ultracentrifugation (17). LDL was coupled to ferritin as previously described (12). The concentration of LDL-ferritin is expressed in terms of its content of LDL protein. Monoclonal antibodies against bovine LDL receptors (designated IgG-C7 [18]) were covalently conjugated to ferritin according to the method of Kishida et al. (19) and purified by column chromatography (15). The concentration of IgG-C7-ferritin is expressed in terms of its antibody content.

Binding and Uptake of LDL-Ferritin in Intact Fibroblasts: Monolayers of fibroblasts were placed in a 4°C cold room for 30 min after which the medium was removed and replaced with 2 ml of ice cold medium A (Eagle's minimum essential medium without bicarbonate supplemented with 20 mM HEPES buffer, pH 7.4, and 5% human lipoprotein-deficient serum) that contained 50 μg/ml LDL-ferritin. After incubation at 4°C for 2 h, each monolayer was washed to remove nonspecifically bound LDL-ferritin (12). The dishes then received 2 ml of medium A at 37°C. Duplicate samples of dishes were warmed to 37°C for the following periods: 0, 2, 5, 10, and 20 min. At the end of each time period, the monolayers were fixed for 15 min with ice cold, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). One set of dishes then received 2 ml of fixative containing 200 μg/ml filipin dissolved in 10 μl of dimethyl sulfoxide (DMSO) and one set received fixative containing an equivalent amount of DMSO (3, 4). The cells were incubated for 30 min at room temperature and then washed with 0.1 M cacodylate (pH 7.4) and prepared for electron microscopy.

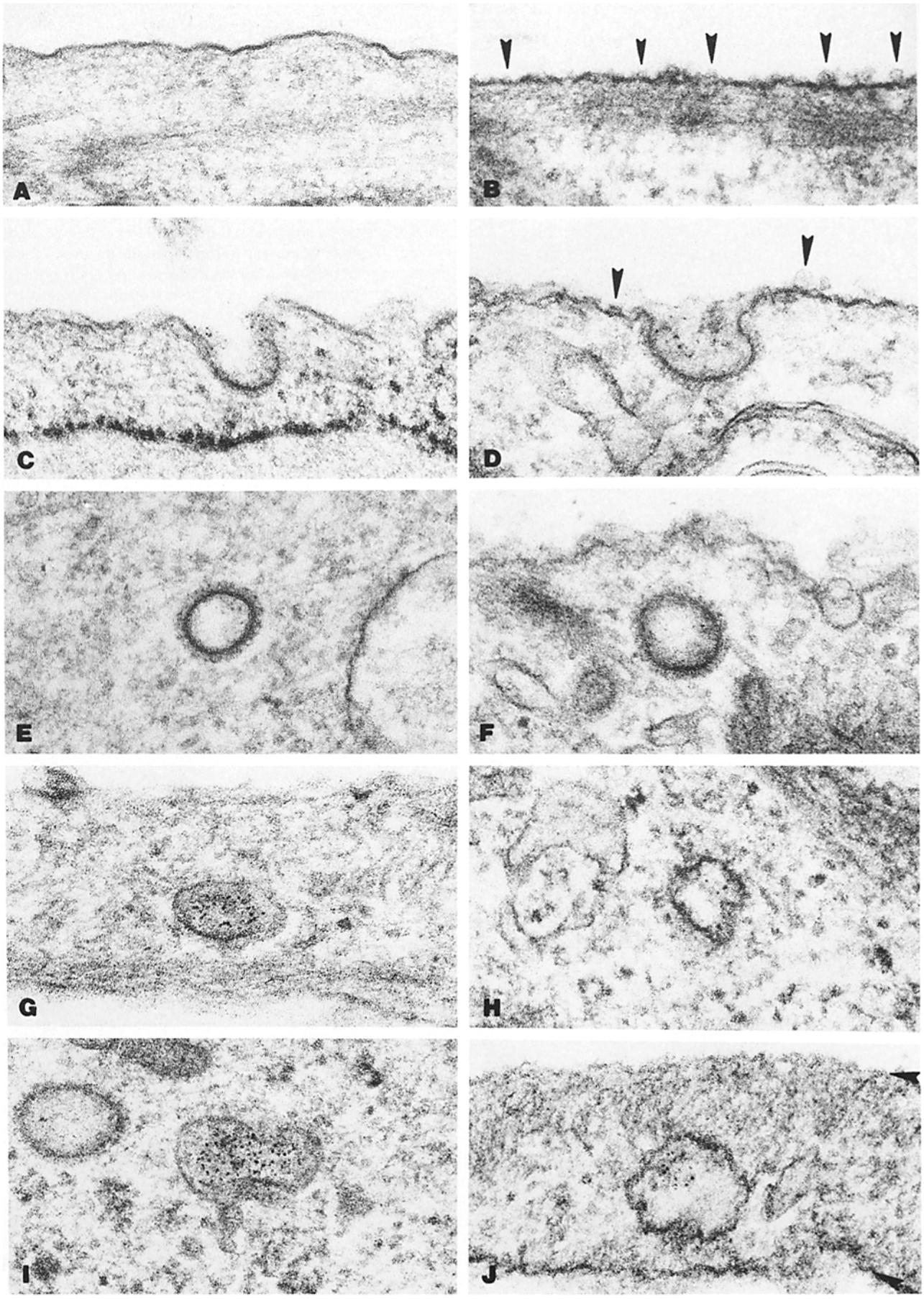
Uptake of IgG-C7-Ferritin by Intact Fibroblasts: Monolayers of fibroblasts were incubated at 37°C in medium A that contained 50 μg/ml IgG-C7-ferritin for 30 min. Dishes were washed at 4°C to remove nonspecifically bound IgG-C7-ferritin (12). The cells were fixed at 4° for 15 min with cold, 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7.4) and then fixed for an additional 30 min at room temperature in either fixative with DMSO or fixative containing 200 μg/ml filipin dissolved in DMSO. The cells were processed for electron microscopy.

Electron Microscopy Procedures: Fixed cells were scraped from the culture dish and pelleted in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, in a Coleman microfuge (Coleman Instruments Inc., Maywood, IL). The cell pellets were postfixed for 1 h with 2% OsO_4 in a buffer composed of 0.1 M cacodylate, pH 7.4, and 4.5% sucrose. Following postfixation, some cells were en bloc stained with 0.5% uranyl acetate in veronal acetate buffer, pH 6.0, for 30 min before dehydration and embedding in Araldite (procedure A). Other samples were embedded in Epon without en bloc staining (procedure B). The sections were cut on a Sorvall MT-2B microtome (E. I. Du Pont de Nemours & Co., Inc., Sorvall Instruments Div., Newtown, CT), stained with uranyl acetate and lead citrate, and viewed with a JEOL 100 CX.

RESULTS

All of the cells used in these studies were grown for 2 d in the presence of lipoprotein-deficient serum to induce a maximum expression of LDL receptors (20). Under these conditions, lipoprotein digestion in the lysosome was not taking place and, therefore, could not be a cholesterol source for membranes that surround the lysosomes. To delineate the intermediates in the uptake process, we incubated monolayers of fibroblasts with LDL-ferritin at 4°C and then warmed them to 37°C for various periods of time before fixation. To detect filipin-sterol complexes, we fixed one set of cells with glutaraldehyde and then exposed the cells to glutaraldehyde plus filipin for 30 min at 23°C. For comparison, another set of cells was processed in the absence of filipin. As shown by other investigators (2–4), under these conditions filipin-sterol complexes could be detected in thin sections by virtue of the corrugated or wavy appearance of the membrane (compare Fig. 1 *A* with Fig. 1 *B*). Often, 20–25-nm globules were visible in the plane of the

FIGURE 1 Electron micrographs showing the sequence of LDL-ferritin internalization from coated pits to intermediate endocytic vesicles. Monolayers of human fibroblasts were incubated with medium A containing 50 μg/ml of LDL-ferritin at 4°C for 2 h and then washed. The cells were then incubated in medium A for various times at 37°C. After incubation, the monolayers were fixed either without filipin present (*A, C, E, G, I*) or with filipin present (*B, D, F, H, J*). The cells were pelleted and processed for electron microscopy by either procedure A (*A, B, D, E–J*) or procedure B (*C*). (*A*) Surface membrane of a cell not exposed to filipin. (*B*) Surface membrane of a cell fixed in the presence of filipin. Arrows indicate filipin-cholesterol complexes. (*C*) Coated pit of a cell that was not exposed to filipin that contains LDL-ferritin. (*D*) Coated pit of a cell that was exposed to filipin that contains LDL-ferritin. Arrows indicate cholesterol-filipin complexes. (*E*) Coated endocytic vesicle in a cell that was not exposed to filipin. (*F*) Coated endocytic vesicle in a cell that was exposed to filipin. (*G*) Uncoated endocytic vesicle in a cell that was not exposed to filipin. (*H*) Uncoated endocytic vesicle in a cell that was exposed to filipin, which has a corrugated membrane. (*I*) Intermediate endocytic vesicle in a cell that was not exposed to filipin. (*J*) Intermediate endocytic vesicle in a cell that was exposed to filipin. The arrows indicate the plane of the plasma membrane of this cell. × 93,000.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membrane (arrows, Fig. 1 *B*).

Fig. 1 is a series of photographs that illustrates the sequence of LDL-ferritin internalization in human fibroblasts. The left-hand panel (*A*, *C*, *E*, *G*, *I*) shows cells fixed in the absence of filipin and the right-hand panel (*B*, *D*, *F*, *H*, *J*) shows cells that were fixed in the presence of filipin. LDL-ferritin was easily distinguished by the characteristic electron-dense core of the ferritin molecule. During internalization, ferritin progressively appeared in coated pits (*C*, *D*), coated vesicles (*E*, *F*), uncoated vesicles (*G*, *H*), and intermediate vesicles (*I*, *J*).

After 4°C binding, coated pits contained most of the LDL-ferritin found on the cell surface (Fig. 1, *C* and *D*). As seen in Fig. 1 *D*, whereas the noncoated plasma membrane had a corrugated appearance and often contained filipin-sterol complexes (arrows), the coated pits appeared smooth and had an undisrupted trilaminar membrane (compare Fig. 1 *C* and 1 *D*). Even with bound LDL-ferritin, coated pits did not bind filipin, which is in agreement with previous freeze-fracture and thin-section electron microscopic studies (4, 7). Following a brief warming to 37°C, coated endocytic vesicles that contained LDL-ferritin were seen (Fig. 1, *E* and *F*). Regardless of whether or not filipin was present during fixation, in most cases the membrane of these vesicles was uniform and not disrupted (see below for further analysis). As internalization proceeded at 37°C, ferritin-labeled, uncoated vesicles of approximately the same size as the coated vesicles were found in many cells (Fig. 1, *G* and *H*). The membranes surrounding these newly formed, uncoated vesicles appeared corrugated, an indication that filipin-sterol complexes had formed (Fig. 1 *H*). This was in contrast with the undisrupted membrane profiles of uncoated endocytic vesicles seen in cells that were fixed in the absence of filipin (Fig. 1 *G*). Larger, intermediate vesicles that contained LDL-ferritin also displayed a corrugated membrane in filipin-treated cells (Fig. 1 *J*) but not in control cells (Fig. 1 *I*). In Fig. 1 *J*, it can be seen that the degree of membrane corrugation in the intermediate vesicle is similar to that of the plasma membrane (arrows).

This analysis of the internalization sequence shows that the membrane of even the earliest uncoated vesicle (as judged from size) binds filipin (Fig. 1 *H*). We also found evidence that some coated, as well as partially coated, vesicles were sensitive to filipin. Fig. 2 *A* shows a coated vesicle from a filipin-treated cell that showed evidence of filipin binding. The membrane of this vesicle had definite regions that were scalloped (outlined by dark lines), and within the vesicle there appeared to be filipin-sterol complexes (arrows). In partially coated vesicles such as the one shown in Fig. 2 *B*, filipin-sterol complexes were evident in the portions of the vesicle membrane that were not coated (arrows).

Lysosomal membranes were also disrupted by filipin. Lysosomes were identified by the characteristic matrix material that is found in these organelles. Whereas lysosomal membranes in untreated cells had uniform membranes with trilaminar staining, in filipin-treated cells these membranes had a marked scalloped appearance and filipin-sterol complexes could be identified (Fig. 2 *C*). According to this criterion, these membranes appeared to contain cholesterol.

A few of the intermediate endocytic vesicles and lysosomes had a region of the membrane that appeared dense owing to the presence of a cytoplasmic coat. In filipin-treated cells, this portion of membrane was not disrupted, indicating that filipin-sterol complexes had not formed (Fig. 2 *C*). However, the membrane continuous with these dense regions had the characteristic corrugated appearance of a membrane that bound filipin.

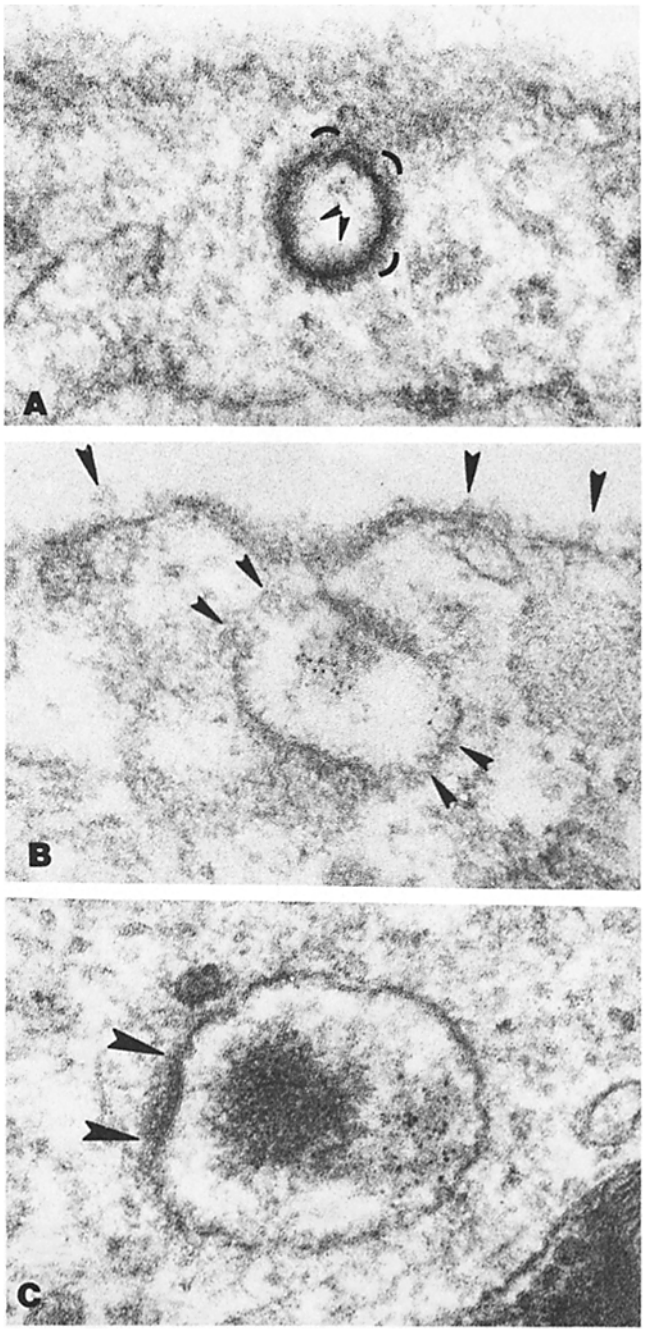

FIGURE 2 Characteristics of filipin binding to coated vesicles (*A*), partially coated vesicles (*B*), and lysosomes that contain dense segments of membrane (*C*). Human fibroblasts were prepared as described in Fig. 1. (*A*) A coated vesicle that shows evidence of filipin binding. The presence of corrugated portions of membrane (dark lines) and the presence of filipin-cholesterol complexes (solid arrows) indicate that filipin bound to portions of the membrane surrounding this coated vesicle; embedded according to procedure A. × 115,000. (*B*) A coated vesicle that appears to have lost the clathrin coat from two regions of the membrane. Filipin-cholesterol complexes in these portions of the membrane are indicated by the arrows; embedded according to procedure B. × 100,000. (*C*) A lysosome containing a dense segment of membrane (between arrows). Unlike the remainder of the membrane, the dense segment was not disrupted by filipin; embedded according to procedure A. × 93,000.

Since LDL contains a small amount of free cholesterol (21, 22) it is possible that the appearance of filipin-sterol complexes in the vesicle membranes was due to an exchange of free cholesterol between LDL-ferritin and the membrane. Furthermore, in the lysosomal compartment, the digestion of a small amount of LDL-ferritin could have liberated free cholesterol that intercalated into the lysosomal membrane and rendered it sensitive to filipin. Therefore, we carried out similar experiments using a monoclonal anti-LDL receptor antibody conjugated to ferritin (IgG-C7-ferritin) to follow the endocytic pathway. In these experiments, cells were exposed continuously at 37°C for 30 min to 50 μg/ml of IgG-C7-ferritin and fixed in the presence or absence of filipin. As with LDL-ferritin, the coated pits and coated vesicles were not disrupted by filipin; however, the ferritin-containing endocytic vesicles were found to have a corrugated membrane (Fig. 3*A*). Furthermore, the lysosomal membrane in filipin-treated cells also had the characteristic scalloped appearance of cholesterol-containing membrane (Fig. 3*B*). Cells fixed in the absence of filipin had normal-appearing membranes (data not shown). Therefore, even when endocytic vesicles and lysosomes did not contain LDL, they were sensitive to filipin.

An additional consideration in these studies was that filipin nonspecifically disrupted all of the membranes in these cells. Therefore, we examined mitochondria and ER membranes because other investigators have shown that these membranes are not disrupted by filipin (5–9). In our preparations, the integrity of the mitochondria membrane was the same in filipin-treated and in nontreated cells. Likewise, the ER membranes were not disrupted in filipin-treated cells (data not shown).

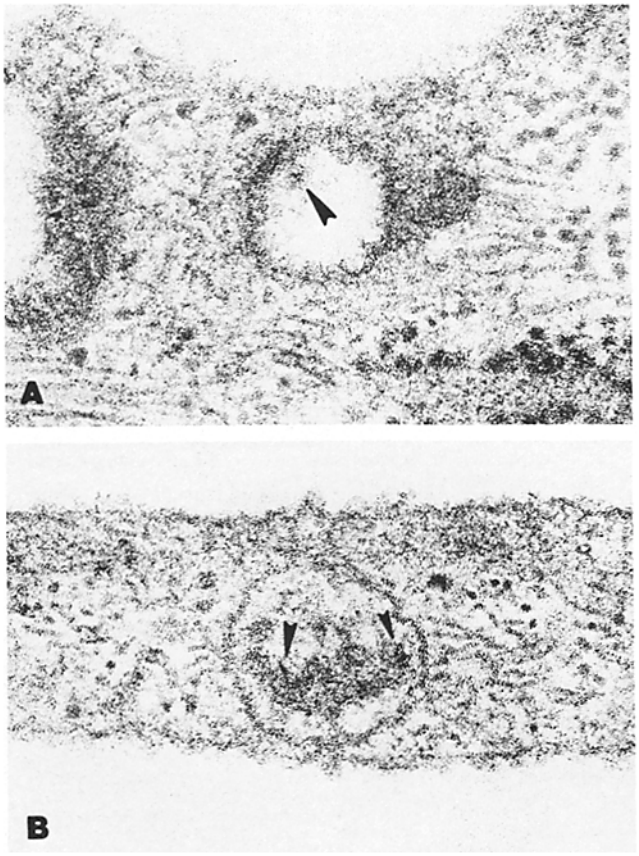

FIGURE 3 Uncoated endocytic vesicle (*A*) and lysosome (*B*) in filipin-treated cells following the uptake of IgG-C7-ferritin. Monolayers of fibroblasts were incubated at 37°C in medium A containing 50 μg/ml IgG-C7-ferritin for 30 min. Following a wash at 4°C, the cells were fixed and embedded according to procedure B. Arrows indicate ferritin. × 99,000.

DISCUSSION

Using ferritin-labeled probes that are specific for the LDL receptor, we have been able to identify all of the endocytic vesicles that form during LDL endocytosis and to follow the membrane surrounding these vesicles to the lysosomal compartment. Whereas coated membrane was relatively insensitive to filipin exposure, as soon as the endocytic vesicles became uncoated, the membranes were disrupted by filipin. The endocytic vesicle membranes remained sensitive to filipin as the vesicle contents were delivered to the lysosomal compartment. Finally, the lysosomal membranes bound filipin even under conditions where the cells had been grown in the absence of lipoproteins for 2 d to deprive them of an exogenous source of cholesterol.

The current understanding of how filipin disrupts cellular membranes is that it binds to cholesterol and forms filipin-cholesterol complexes (2–5). For this reason, this polyene antibiotic is thought to be a specific cytochemical marker for membranes that contain cholesterol. Previous studies with filipin indicate that there is a direct relationship between the susceptibility of a membrane to filipin disruption (2–9) and the biochemical levels of cholesterol that have been measured in that membrane (1). Therefore, the best interpretation of the present studies is that uncoated endocytic vesicle membranes as well as lysosomal membranes contain cholesterol in amounts greater than that found in ER membrane or mitochondria membrane.

During the time course of these experiments, LDL-ferritin and IgG-C7-ferritin appeared sequentially in coated pits, coated vesicles, uncoated vesicles, intermediate vesicles, and lysosomes. Although it has been suggested that uncoated endocytic vesicles are not derived from coated pits and coated vesicles (23, 24), other investigators have shown that coated vesicles represent an intermediate in the endocytic pathway (25–27). Furthermore, coated vesicles have been identified in serial sections of cells internalizing ferritin (28). This agrees with previous studies from this laboratory on the endocytic pathway in human fibroblasts (14). Therefore, the uncoated vesicles most likely come from coated vesicles that have lost their clathrin coats. Since all of the uncoated membranes surrounding these vesicles bound filipin, cholesterol is not excluded from these endocytic membranes during endocytosis. What remains to be established is how cholesterol gets into these membranes.

There are several possible ways that cholesterol could reach the uncoated, endocytic vesicle membrane compartment. Possibly, normal amounts of cholesterol are present in coated pits and coated vesicles, but the coat protein or some other feature of the coated membrane interferes with the formation of filipin-cholesterol complexes. If this was the case, this cholesterol would only complex with filipin after the coat protein comes off of the membrane. Another possibility is that cholesterol rapidly moves into endocytic vesicles after the coat is removed. This could be accomplished either by free cholesterol intercalating into the membrane or by a cholesterol-rich portion of membrane, for example a vesicle, fusing with the uncoated membrane. Finally, during endocytosis it is possible that a portion of the uncoated plasma membrane becomes incorpo-

rated into the coated vesicle. The cholesterol from this membrane equilibrates with the remainder of the vesicle membrane once the coat protein dissociates, making it sensitive to filipin. We cannot unequivocally distinguish between these possibilities now. However, since we were able to find some coated vesicles that showed evidence of filipin binding (Fig. 2*A*), it is possible that coated vesicles and coated pits are not deficient in cholesterol.

If coated vesicle membrane has the same amount of cholesterol as the plasma membrane, this should be biochemically detectable in preparations of isolated coated vesicles. Pearse (29) measured the cholesterol-to-phospholipid molar ratio in isolated brain coated vesicles and found it to be 0.1 to 0.3. This is a lower molar ratio than that measured in many plasma membrane fractions (1), but higher than that measured for ER and mitochondria membranes (1). It is quite possible that Pearse's coated vesicle membranes were contaminated with intracellular membranes, which artifactually lowered the measured cholesterol content. Recent measurements of coated vesicles isolated from brain and liver suggest that they have a cholesterol content similar to that found in the plasma membrane (30).

Bretscher (31) has suggested that coated pits may function to deliver low cholesterol membrane to various intracellular membrane compartments. In the present study, we showed that in filipin-treated cells, ER membranes were dramatically different from uncoated, endocytic vesicle membranes or lysosomal membranes in their sensitivity to this sterol-binding antibiotic. Most likely, the two membranes have quite different amounts of cholesterol. If this is the case, neither endocytic nor lysosomal membrane contributes low cholesterol membrane to the ER. This conclusion is consistent with recent studies on the mechanism of membrane recycling.

Farquhar and collaborators (32, 33) used cationized ferritin (CF), an electron-dense marker for anionic sites on the plasma membrane, to show that surface membrane undergoes continuous recycling in secretory cells. Surface membrane was carried in endocytic vesicles to the lysosome and to the mature face of the Golgi apparatus. From the Golgi apparatus, membrane moved back to the cell surface in secretory granules. The present studies, together with the studies from Orci's laboratory (9, 34), suggest that each of these membrane compartments is able to bind filipin. Therefore, it is possible that during membrane recycling, membrane cholesterol is conserved. In addition to the anionic membrane proteins (marked by CF) flowing through these compartments, membrane cholesterol may follow the same route.

Despite the limitations of using filipin as an electron microscopic probe for membrane cholesterol, the present studies raise some interesting new questions about the distribution of cholesterol in intracellular membranes. We do not know whether coated membrane is truly low in cholesterol compared with the rest of the plasma membrane. However, our observations raise the possibility that the amount of cholesterol in recycling membrane remains relatively constant. More detailed studies using these various markers on the same cell system need to be carried out to verify this observation.

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