Detection of Membrane Cholesterol by Filipin in Isolated Rat Liver Coated Vesicles Is Dependent upon Removal of the Clathrin Coat

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ABSTRACT We investigated the cholesterol content of highly purified populations of coated vesicles from rat liver by biochemical quantitation and by cytochemical electron microscopy using the polyene antibiotic filipin. Failure of this reagent to elicit its typical response for a cholesterol-containing membrane, i.e., a characteristically corrugated or rippled appearance by thin section analysis, had led to the hypothesis (Montesano, R., A. Perrelet, P. Vassalli, and L. Orci, 1979, *Proc. Natl. Acad. Sci. USA.*, 76:6391–6395) that cholesterol is specifically excluded from the plasma membrane domains associated with coated pit regions. The present electron microscopic results showed that although the response of coated vesicle membranes to filipin was also negative, uncoated vesicles whose clathrin coats had been removed in vitro exhibited a strong filipin-positive response. Quantitated biochemically, the cholesterol-to-phospholipid ratio of the coated vesicles was found to be indistinguishable from that of control preparations of plasma membranes isolated from rat liver. Taken together, the results indicate that the filipin-negative response of coated vesicles (and probably also that of coated pits) is due not to abnormally low cholesterol content, but rather to the stabilizing influence of their enveloping clathrin coats which inhibit the characteristic structural expression of the filipin-cholesterol complexes.

Coated pits are specialized regions of the plasma membrane which play a central role in the pathway whereby eucaryotic cells internalize molecules by receptor-mediated endocytosis (1–8). As observed by thin section electron microscopy, coated pits appear as invaginations underlined by the characteristic bristle coat of clathrin protein (9–13). In an attempt to elucidate the distinctive qualities of coated pit regions, Montesano et al. (14) examined the cholesterol content of these areas by freeze-fracture and thin-section electron microscopy. Using the polyene antibiotic filipin, a cytochemical marker that specifically binds to cholesterol and related 3-β-hydroxysterols, they found no visible reaction to the probe in coated pit regions. This was in contrast to a positive response in surrounding tracts of uncoated membrane. On the basis of this observation, they concluded that coated pits are deficient in cholesterol, and as such might represent specialized domains of membrane which act as molecular filters in removing the sterol from internalizing membrane (15). Their results agreed with earlier biochemical (16) as well as cytochemical studies (17) which suggested that depletion of cholesterol in the coated pit region might be important in attaining the degree of fluidity required for membrane curvature and in creating a membrane environment conducive to receptor clustering (14, 18).

In a recent report, McGookey et al. (19) used ferritin-labeled probes to identify endocytic vesicles involved in cellular uptake of low-density lipoprotein in cultured human fibroblasts. They found that although the membranes of coated pits and coated vesicles were largely insensitive to filipin, uncoated vesicles, derived from coated vesicles during endocytosis of the ferritin-conjugated ligand, exhibited the characteristic morphologic response of a filipin-sensitive membrane. Although they could not rule out the possibility that cholesterol moves rapidly into the endocytic vesicles subsequent to dissociation of the clathrin coat, they also considered that the negative response of coated pits and coated vesicles to filipin could well be due to causes other than the absence of cholesterol. Specifically, attention was drawn to the possibility that interaction between the clathrin coat and the underlying membrane somehow inhibits either the binding of filipin to cholesterol or inhibits the characteristic membrane changes associated with the formation of such complexes (20).

To ascertain whether coated membranes do, in fact, contain detectable amounts of cholesterol, we examined the effect of

filipin on coated vesicles isolated from rat liver. By using highly purified preparations, we minimized contamination by extraneous sources of cholesterol and provided material suitable for biochemical quantitation of the lipid content. We found that coated vesicle membranes contain cholesterol in amounts similar to those found in isolated plasma membranes and that upon removal of the clathrin coat, they become susceptible to the perturbing effects of filipin. In view of these findings, we offer a reinterpretation of the filipin-negative reponse of both coated pit and coated vesicle membranes.

MATERIALS AND METHODS

Materials: Sucrose (ultrapure) was purchased from Bethesda Research Laboratories, Rockville, MD; 2-(*N*-morpholino)ethane sulfonic acid (MES) from Calbiochem Behring Corp., La Jolla, CA; and deuterium oxide (D_2O) from the Aldrich Chemical Co., Milwaukee, WI. Glutaraldehyde and osmium tetroxide (OsO_4) were obtained from Electron Microscopy Sciences, Fort Washington, PA; and Poly Bed 812 and Araldite 502 from Polysciences, Inc., Warrington, PA. Filipin (U-5956) was generously provided by the Upjohn Co., Kalamazoo, MI. All other chemicals were reagent grade. Male Sprague-Dawley rats (200–250 g) were obtained from Taconic Farms, Germantown, NY.

Isolation of Coated Vesicles: Coated vesicles were isolated from rat liver according to the procedure of Nandi et al. (21) as modified by Steer et al. (22). Final purification of the coated vesicles was achieved by Sephacryl S-1000 gel filtration column chromatography (Pharmacia, Inc., Piscataway, NJ). Approximately 8 ml of the coated vesicle suspension (4–5 mg protein/ml) was applied to a 2 × 85 cm column prewashed in Buffer A (100 mM MES, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$ and 0.02% [wt/vol] NaN_3), eluted in this buffer at 45 ml/h and collected in 2-ml fractions (Fig. 1). The coated vesicles eluted as a well-defined peak (peak II) (cf. Results) of which fractions 69–95 were pooled and then pelleted by centrifugation at 100,000 *g* for 60 min. The supernatant was aspirated and the clear pellet of coated vesicles was overlaid with 10 ml of Buffer A and allowed to resuspend overnight to a protein concentration of ~2.5 mg/ml.

Preparation of Uncoated Vesicles and Plasma Membranes: A 5-ml aliquot of purified coated vesicles in Buffer A was dialyzed overnight against 100 mM Tris-Cl, pH 8.2, and then centrifuged at 100,000 *g* for 60 min in a Beckman 65 rotor (Beckman Instruments, Palo Alto, CA). The supernatant containing the dissociated clathrin coats was removed. The pellet of uncoated vesicles was overlaid with 1 ml of 100 mM Tris-Cl, pH 8.2, and allowed to resuspend for several hours at 4°C. Rat liver plasma membranes were prepared according to the procedure of Hubbard et al. (23).

Protein and Lipid Analysis: Protein was determined according to the method of Lowry et al. (24) with BSA as standard. Total cholesterol was determined using a colormetric assay (Sigma Chemical Co., St. Louis, MO; kit No. 350). Sample was added directly to the assay mix and the absorbance at 500 nm was recorded. Turbidity from the sample (<3% of the total absorbance) was subtracted after remeasuring the absorbance in the presence of ascorbic acid. Phospholipid was determined by the method of Fiske and Subbarow (25) after extraction of the membrane lipid by the procedure of Bligh and Dyer (26).

Electron Microscopy: Approximately 300 μl of sample was brought to a total volume of 3 ml with ice-cold 0.1 M cacodylate buffer, pH 7.4, containing 2.5% glutaraldehyde and 5% sucrose, and allowed to incubate on ice for 45 min. One set of samples then received 1 ml of the same fixative buffer containing 300 μM filipin and 0.5% DMSO in final concentration, and the other set received a similar volume of buffer containing DMSO but no filipin (27, 28). The samples were incubated in the dark at room temperature for 2 h, after which they were centrifuged at 150,000 *g* for 45 min at 4°C in a Beckman SW 50.1 rotor. The supernatants were aspirated and the pellets were washed twice for 20 min each with 0.1 M cacodylate buffer. The pellets were then prepared for electron microscopy according to Wall and Hubbard (7) except that Poly/Bed 812/Araldite 502 was used as embedding resin. Silver sections were cut using a Sorvall microtome MT-2 (E. I. DuPont de Nemours & Co., Inc., Newton, CT) and stained with lead citrate and uranyl acetate. The samples were observed using a Philips EM300 electron microscope.

RESULTS

Coated vesicles were purified according to a recently reported method (21, 29), modified to include an additional step of gel filtration (30). The elution profile of the Sephacryl S-1000 column, shown in Fig. 1, reveals two peaks. The minor leading peak (*I*) contained primarily large smooth membrane contaminants; whereas peak *II* contained almost exclusively coated vesicles. Their purity was assessed by particle counting of negatively stained preparations. Under such conditions, coated vesicles constituted 97.1% of all particles counted (n = 1,842). The remaining 3% of structures appeared to be noncoated vesicles exhibiting a size range (50–150 nm diam) similar to that of the coated vesicles. An independent quantitation of purity, assessed by counting particles in thin section, revealed an almost identical degree of coated vesicle purity (96.3%; n = 1,651).

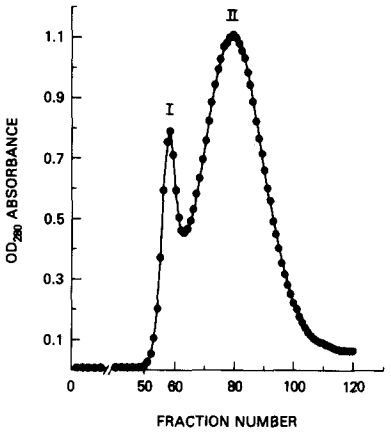

FIGURE 1 Elution profile of a suspension of rat liver coated vesicles purified as described (21, 22) and applied to a Sephacryl S-1000 gel filtration column. Peak *I* contains contaminating membrane sheets as well as smooth membrane vesicles; Peak *II* contains highly purified coated vesicles.

Previous studies have shown that the role of filipin as an effective cytochemical probe of membrane structure is best realized by filipin treatment subsequent to glutaraldehyde fixation (27, 28). Under these conditions and by thin section analysis, the positive response of a cholesterol-containing membrane to filipin is characterized by a corrugated, wavy, and at times disrupted appearance (19, 20, 27). Figs. 2 and 3 illustrate the results of incubation of the various samples with filipin. In each of the thin sections, there is a lack of conspicuous perturbation by filipin of membranes that are clathrin coated. Close examination of the filipin-treated coated vesicles (Figs. 2*b* and 3*b*) reveals that certain of the membranes do not exhibit an absolutely smooth, vesicular contour. Similar appearances are also noted in coated vesicles not exposed to filipin (Figs. 2*a* and 3*a*) although the incidence may be marginally higher in the filipin-treated samples (19). These irregularities, some of which presumably result from fixation and sectioning, are on quite a different scale than the characteristic and pronounced perturbation conventionally associated with the formation of filipin-cholesterol complexes. In this regard, a rare smooth membrane contaminant (arrows in 3*b*) exhibits the typical filipin-positive response of a cholesterol-containing membrane.

For comparison, we also examined the effect of filipin on isolated rat liver plasma membranes (Fig. 3, *c* and *d*). The response of the membranes and, in particular, the coated pit regions to incubation with filipin revealed changes similar to those reported previously in intact cells (14, 19). Whereas the surrounding noncoated membranes exhibited a pronounced filipin-positive response (Fig. 3*d*), those membranes within the coated pit regions were unaffected.

Ideally, removal of the clathrin coat from the coated pit regions would have allowed us to address the issue of filipin sensitivity directly to determine whether the coated pit membranes are in fact devoid of cholesterol. However, because of

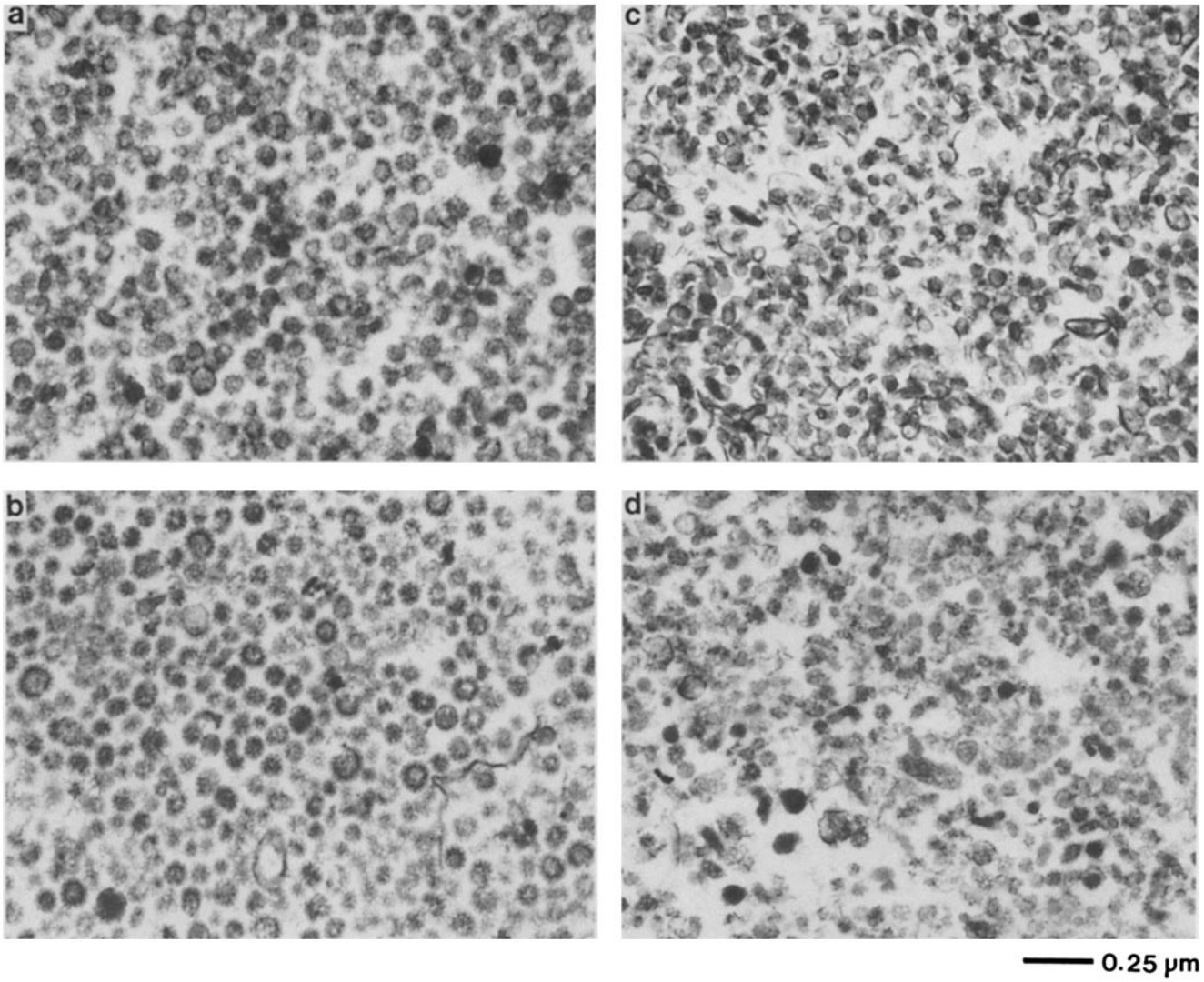

FIGURE 2 Large field, low magnification thin section electron micrographs of coated vesicles (*a* and *b*) and uncoated vesicles (*c* and *d*) prepared according to Materials and Methods. Specimens *b* and *d* were treated with the cytochemical reagent filipin (cf. Materials and Methods), whereas *a* and *c* represent untreated samples. × 40,000.

the difficulty in recognizing those areas after coat removal, we elected to examine the filipin response of isolated coated vesicles whose clathrin coats had been removed.

Thin section micrographs of uncoated vesicles fixed in the absence of filipin (Figs. 2*c* and 3*e*) revealed smooth trilaminar staining membranes. In contrast, incubation with filipin resulted in marked disruption of the membranes affecting virtually all the uncoated vesicle structures (Figs. 2*d* and 3, *f–h*). Although there are areas in which the contour of the uncoated vesicle membranes is still discernible (Fig. 3*i*), many vesicle membranes were so disrupted as to show little evidence of membrane integrity and continuity. Whether this was a result of differences in membrane cholesterol content or of variations in the plane of detection on thin sectioning is unknown. A few uncoated vesicles were noted which retained a portion of their clathrin coat. In those cases, filipin treatment resulted in a characteristically corrugated or partially disrupted membrane except in that portion underlying the clathrin coat, which like the coated pit membrane was apparently unaffected.

To correlate the electron microscopic results with quantitative determinations of cholesterol, we assayed coated vesicles, plasma membranes, and material from Peak I of the Sephacryl column. The molar ratio of cholesterol to phospholipid in coated vesicles (0.36 ± 0.04; $n = 4$) was found to be essentially identical to that of plasma membranes (0.37 ± 0.02; $n = 4$). The cholesterol content of the Peak I material from the Sephacryl S-1000 column (56 μg/mg protein) was very similar to the values obtained for isolated plasma membranes (59 μg/mg protein) and for clathrin-stripped uncoated vesicles (50 μg/mg protein).

DISCUSSION

The results of the present study show that in contrast to isolated liver coated vesicles, uncoated vesicles derived from them are sensitive to filipin binding. Thin section electron microscopy reveals the characteristic corrugated and disrupted appearance associated with perturbation of cholesterol-containing membranes. These observations are consistent with the biochemical determination that the ratio of cholesterol to phospholipid in the isolated coated vesicles is indistinguishable from that of isolated plasma membranes. In this regard, it is not possible for the 3% of contaminating structures to

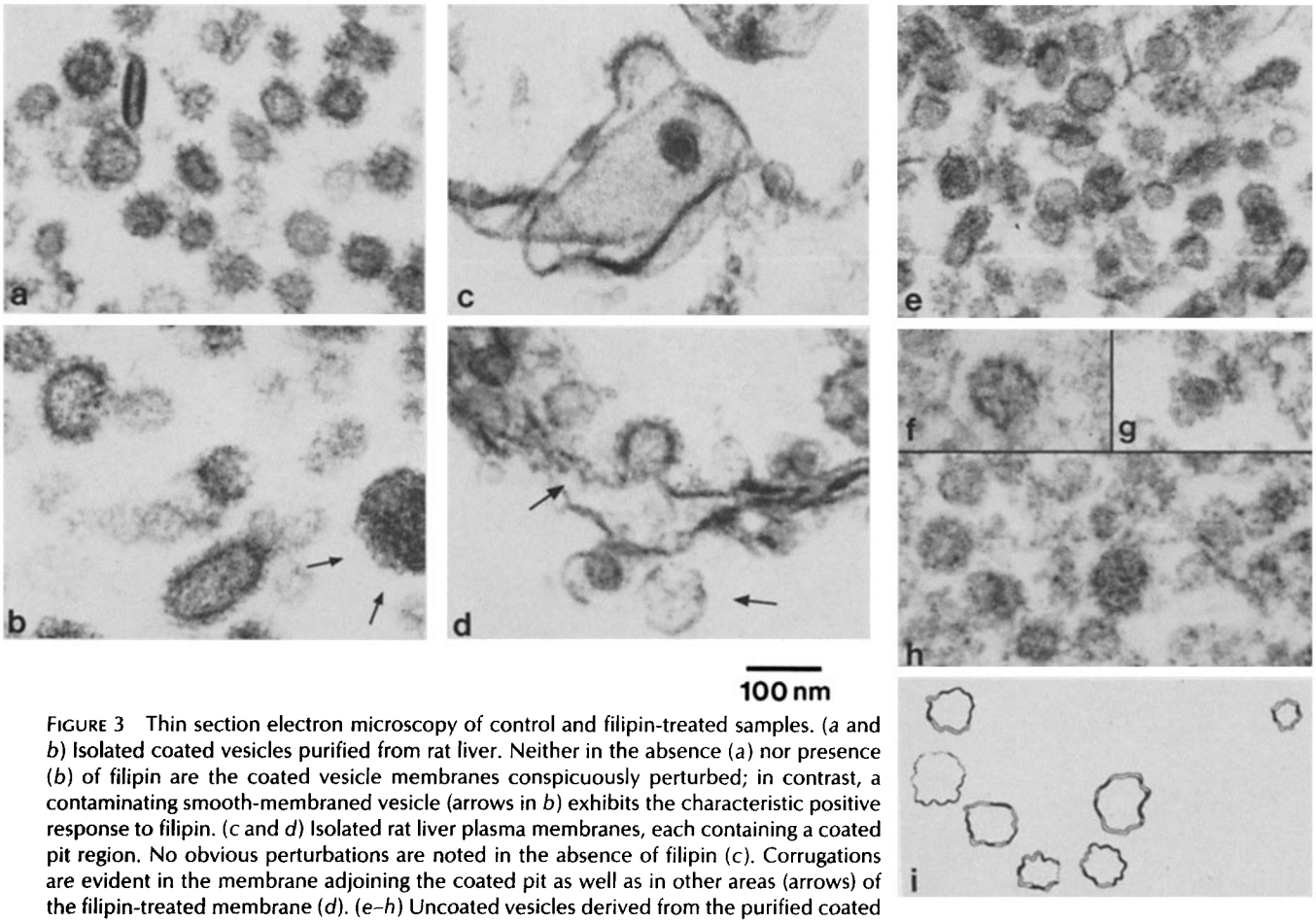

FIGURE 3 Thin section electron microscopy of control and filipin-treated samples. (*a* and *b*) Isolated coated vesicles purified from rat liver. Neither in the absence (*a*) nor presence (*b*) of filipin are the coated vesicle membranes conspicuously perturbed; in contrast, a contaminating smooth-membraned vesicle (arrows in *b*) exhibits the characteristic positive response to filipin. (*c* and *d*) Isolated rat liver plasma membranes, each containing a coated pit region. No obvious perturbations are noted in the absence of filipin (*c*). Corrugations are evident in the membrane adjoining the coated pit as well as in other areas (arrows) of the filipin-treated membrane (*d*). (*e–h*) Uncoated vesicles derived from the purified coated vesicles by removal of the clathrin coats, as described in Materials and Methods. Intact, smooth uncoated vesicular membranes are noted in the absence of filipin (*e*). The membranes associated with the filipin-treated samples are corrugated and partially disrupted (*f–h*). (*i*) A schematic representation of the uncoated vesicles present in field (*h*) for which the corrugated membrane contour is discernible. × 106,000.

account for the observed amounts of cholesterol. Even in the most extreme case, i.e., one in which the lipid content of the contaminants consists exclusively of cholesterol, the 97% population of coated vesicles would still yield an average cholesterol/phospholipid ratio of 0.34 as compared with a ratio of 0.36 for all particles counted. More plausibly, the 3% of contaminants represents a trailing component of the Peak I material which has essentially the same cholesterol content as plasma membranes and uncoated vesicles.

We conclude that cholesterol is present in the coated vesicles in amounts very similar to that of plasma membranes. However, filipin cannot exert its characteristic morphological perturbation until the clathrin coats are removed. Because it seems unlikely that the clathrin coat prevents filipin from penetrating the membrane and binding to cholesterol, the coat lattice may act to physically restrain perturbation of the membrane subsequent to filipin exposure. For example, one cannot rule out the possibility that glutaraldehyde cross-links the clathrin molecules rendering the cholesterol-containing membrane of the coated vesicle unable to respond to filipin binding because of regional restraints. Several recent studies examining the influence of peripheral membrane proteins on filipin binding have concluded that a negative response is frequently observed in membranes exhibiting dense peripheral protein structures, areas that when analyzed by other cytochemical probes reveal membrane rich in cholesterol (31–34).

Examples of systems for which biochemical studies have confirmed a high cholesterol content in contrast to a negative filipin response include gap junctions (35, 36), subunit arrays of mammalian bladder luminal plasma membrane (37), and acetylcholine-rich membrane regions of the *Torpedo* electric organ (38). In each case, lack of interaction between cholesterol and filipin was noted in areas of dense protein packing.

Coated pits are specialized areas of membrane that unlike contiguous membrane regions are enriched in receptor sites for various macromolecules (10) and possess a higher density of large intramembranous particles (18). They have also been reported to contain low levels of cholesterol (14), which was felt to be a reflection of the postulated molecular filtering capacity of the coated pit (15) and to be important in achieving the membrane fluidity required to facilitate the membrane deformation involved in coated vesicle formation (14). However, recent studies (30, 39) have shown that isolated coated vesicles do not have the low cholesterol levels originally reported by Pearse (16). Furthermore, the present study demonstrates that the clathrin coat inhibits the characteristically disruptive membrane changes associated with filipin, in line with other recent reports that focal absence of filipin binding in expansive membrane domains must be interpreted with

caution.

Although it is difficult to estimate the fraction of isolated coated vesicles derived from plasma membrane coated pits, the apparent sensitivity to filipin of essentially all the uncoated vesicles in this study suggests that plasma membrane coated pits are not deficient in cholesterol but are insensitive to the sterol probe because of the clathrin coat protein. Aside from reconsideration of the putative lipid-filtering capacity of the surface membrane coated pit, factors responsible for membrane invagination and coated vesicle formation must also be reevaluated in light of the present findings.

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