Serial-section Analysis of Coated Pits and Vesicles Involved in Adsorptive Pinocytosis in Cultured Fibroblasts

O. W. PETERSEN and B. VAN DEURS
Institute of Anatomy, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark

ABSTRACT We have examined, by analyzing thin (15-20 nm) serial sections, whether coated pits involved in adsorptive pinocytosis in cultured fibroblasts give rise to free coated vesicles or represent permanently surface-associated structures from the neck of which uncoated receptosomes pinch off and carry ligand into the cell. Human skin fibroblasts and mouse L-929 fibroblasts were incubated with cationized ferritin (CF), a ligand known to bind to coated pit regions, at 37°C before fixation. In thin sections, CF was found in coated vesicular profiles within the cytoplasm. Serial sections revealed that whereas many of these coated profiles communicated with the cell surface, thus representing pits, about 10% in L-cells and 36% in skin fibroblasts were actually free coated vesicles. Moreover, evidence for uncoated vesicular structures (receptosomes) budding off from the coated pits was not obtained. We therefore conclude that coated pits do pinch off from the plasma membrane to form free, coated vesicles (pinosomes).

Coated pits at the cell surface represent membrane specializations (3, 13, 17, 18, 21) somehow involved in the binding and internalization of several specific ligands and certain viruses (receptor-mediated endocytosis) (5, 10-12, 16, 19, 20, 24, 28, 31). The mechanism of this internalization has lately become a controversial issue. It has been suggested repeatedly that coated pits pinch off from the plasma membrane to form free, coated vesicles (although such "vesicles," as shown in numerous published micrographs, may communicate with the cell surface at another plane of sectioning; see reference 3) carrying their load into the cell (see, for instance, references 1, 7, 12, 16, 24, 26-28). Alternatively, it has been suggested that coated vesicles never exist as free entities but always remain surface associated, and that the vehicle responsible for the internalization of receptor-ligand complexes is not a coated vesicle, but a structure named the receptosome, a relatively large (250-400 nm) smooth-surfaced vesicle budding off from the coated pits, about 10% in L-cells and 36% in skin fibroblasts were actually free coated vesicles. Moreover, evidence for uncoated vesicular structures (receptosomes) budding off from the coated pits was not obtained. We therefore conclude that coated pits do pinch off from the plasma membrane to form free, coated vesicles (pinosomes).

MATERIALS AND METHODS

Mouse L-929 fibroblasts (L-cells) and human skin fibroblasts were grown in monolayers at 37°C under standard conditions in Dulbecco’s modified medium supplemented with calf serum.

In some experiments L-cells in monolayers were rinsed with PBS and fixed with 1% formaldehyde, 1.25% glutaraldehyde and 0.1 M sodium cacodylate buffer, pH 7.2, for 60 min at room temperature. After a rinse with PBS, the fixed cells were labeled for 5 min at 37°C with CF (Miles Yeda; Miles Laboratories, Inc., Elkhart, IN), 0.1 mg/ml PBS, before a final PBS rinse. In other experiments L-cells or skin fibroblasts in monolayers were rinsed with PBS and thereafter incubated for 5 or 15 min at 37°C with CF (0.1 mg/ml PBS), before final rinse and fixation.

After fixation the cells were scraped off the plates and centrifuged in cacodylate buffer for 25 min at 1,600 g. Thereafter the pellets were postfixed with 2% OsO₄ in cacodylate buffer, pH 7.2, for 1 h at 4°C, block-stained for 1 h at room temperature with 1% uranyl acetate in distilled H₂O₂, dehydrated in ethanol, and embedded in Epon.

Thin sections were cut at 15-20 nm with a diamond knife (Jemdi; Juniper Ultra Micro, Stockholm, Sweden) on a LKB Ultrotome III. Evaluation of section thickness was performed by measuring the maximal diameter of small circular vesicular profiles and counting the number of consecutive sections in which such profiles were included. Series with 10-20 consecutive sections were collected on single slot grids (3.01 mm, LR 2 × 1 mm; Ernest F. Fullam, Inc., Schenectady, NY) covered with 1% Formvar and supported by a carbon film. The sections were photographed in a JEOL 100 CX electron microscope operated at 60 kV without further contrasting. Grid staining with lead and uranyl was avoided.
because this procedure often damaged the Formvar film or made it unstable in the microscope. Moreover, grid contrasting mostly caused some unwanted contamination of the specimens. In the thin, low-contrast sections the incorporation of electron-dense ferritin molecules facilitated identification of structures and, in particular, focusing. In a given series of sections, CF-labeled coated vesicular profiles within the cells were found in the middle section and then followed to either side of the series. The primary magnification was ×24,200. To enhance the contrast, prints were made on Ilfochrome 5.1 M paper (Ilford).

RESULTS

L-cells and human skin fibroblasts were chosen for this study because they have been used in previous studies on pinocytosis of CF and show distinct differences. Whereas L-cells exhibit a very irregularly shaped surface with numerous projections (microvilli) and invaginations and appear to internalize CF predominantly by means of 100–400-nm smooth-surfaced pinosomes (25), skin fibroblasts show a rather smooth surface and apparently internalize CF exclusively via coated pits (26).

When prefixed L-cells were labeled by CF, the surface binding including that of coated pits was rather even. Coated vesicular profiles with CF as revealed in a given section were found to communicate with the cell surface in one or more other sections. Coated vesicular profiles without CF were also present close to the cell surface. This may indicate that there is no communication between the profile and the cell surface, although the possibility must be considered that a narrow neck may not allow passage of the large, charged CF molecules (nor of smaller molecules; the "cryptic" type of coated pits, see reference 33). Such necks are expected to measure at least 15–20 nm in width (two membranes plus surface coats) and should easily be observed in the thin sections used here. Analysis of the serial sections revealed that some of the unlabeled, coated vesicular profiles found in a given section were actually freely located in the cytoplasm. Other unlabeled coated profiles turned out to be surface connected via necks apparently too narrow to allow passage of CF. Such narrow necks were on occasion revealed in only one of the 6–8 consecutive sections of a coated profile.

However, because coated vesicles are formed from intracellular compartments (ER, Golgi) (7, 22, 23) it was, for the present approach, necessary to label incoming pinosomes. This was done in the experiments with fibroblasts incubated in a CF-medium at 37°C before fixation. Here the surface labeling was uneven and patchy, but coated pits often showed distinct CF labeling. Many coated vesicular profiles also contained CF.

Figure 1 Serial sections of a free, coated vesicle (arrow) containing CF. L-cell. Bar, 100 nm. ×76,800.
Such profiles were typically located close to the cell surface, whereas they were rarely observed in central regions of the cells with lysosomal elements. Examination of CF-labeled coated profiles in serial sections of L-cells revealed that they were sometimes (~10%) truly free vesicles (Fig. 1 and Table I) although most of them communicated with the cell surface. In skin fibroblasts a higher percentage (~36%) of the CF-labeled, coated profiles represented truly free vesicles (Table I). Actual spherical diameters (as opposed to sectioned diameters revealed from single section quantification) of coated vesicles with CF varied considerably, in the range of 70–140 nm.

The existence of smooth-surfaced invaginations (receptosomes) from coated pits or their necks "budding" into the cytoplasm could not be established by serial sectioning. In skin fibroblasts and particularly in L-cells (because of their very

### Table I

|                | A  | B  |
|----------------|----|----|
| L-Cells        | 97 | 10 |
| Human skin fibroblasts | 81 | 29 |

A, No. of “free” coated vesicular profiles with CF found in the midsections of the series.
B, No. of profiles in A that turned out to be free (no surface connection) when consecutive sections were examined on either side of the midsection.

![Serial sections of L-cells. a-d show sections no. 1, 6, 8, and 11 of a series. b might be interpreted as a coated pit (Cp) associated with a larger, uncoated vesicular profile (a receptosome, asterisk) and communicating with the cell surface via an elongated neck (arrow). However, analysis of serial sections clearly shows that the “uncoated profile” as well as the “neck” are simply invaginations of the irregularly-shaped cell surface. Also note that if a section were to be cut perpendicular to the plane in b (along the indicated line), an image would arise suggesting a coated profile connected with a larger, uncoated profile (a receptosome), both freely in the cytoplasm. Mv, microvilli. In the other series (e-g), a smooth vesicular structure (*) is apparently budding off from a coated pit (Cp) in f. However, already the following section (g) suggests that the coated pit is facing the exterior directly (arrow). Bar, 100 nm. × 67,200.](image)
irregular surface contour) it turned out that coated pits often communicated with the cell surface via invaginations of the surface, sometimes clearly formed by microvilli or processes (Fig. 2). Similarly, coated vesicular profiles apparently communicating with the cell surface via elongated necks (as evaluated from single sections) turned out to represent pits at the base of adjacent microvilli.

In addition to coated vesicular profiles with CF, labeling of vesicles and vacuoles of various size, sometimes appearing as multivesicular bodies (MVBs; particularly in skin fibroblasts, cf. reference 26), was observed.

DISCUSSION

It has recently been suggested that coated pits represent stable structures at the cell surface (19, 29-33). However, the present observations show that coated pits involved in adsorptive pinocytosis in cultured fibroblasts give rise to free vesicles (pinosomes) which initially are coated.1 Because such free, coated vesicles are relatively rare and in general are seen only close to the cell surface, it is indicated that the clathrin coat is shed rapidly after internalization, before fusion with elements of the lysosomal (vacuolar) apparatus (26, 27).

The concept that coated structures are permanently surface connected (reviewed in reference 19) has in part been based on studies with cell surface labels such as ruthenium red (33) and on immunocytochemistry (29, 30). Thus, at 4°C virtually all labeled with ruthenium red. After warming to 37°C, many observations show that coated pits involved in adsorptive pinocytosis in cultured fibroblasts give rise to free vesicles (pinosomes) which initially are coated. Because such free, coated vesicles are relatively rare and in general are seen only close to the cell surface, it is indicated that the clathrin coat is shed rapidly after internalization, before fusion with elements of the lysosomal (vacuolar) apparatus (26, 27).

The concept that coated structures are permanently surface connected (reviewed in reference 19) has in part been based on studies with cell surface labels such as ruthenium red (33) and on immunocytochemistry (29, 30). Thus, at 4°C virtually all coated structures close to the cell surface were found to be labeled with ruthenium red. After warming to 37°C, many coated structures became "cryptic," that is, not accessible to surface markers but still assumed to be surface connected (33). In agreement with this, our observations on prefixed cells indicated that some coated profiles are connected with the cell surface via necks too narrow to allow passage of marker molecules. However, in our interpretation this may represent the situation immediately before the pit is pinched off to form a free vesicle. Moreover, according to our serial-section analysis a fraction of unlabeled profiles in prefixed cells represent truly free, coated vesicles. In studies with micro-injected anticalarin antibodies, clathrin was found to be localized (in addition to the Golgi-GERL apparatus) exclusively to coated regions (pits) of the plasma membrane, and excess of antibody in living cells apparently had no effect on receptor-mediated endocytosis (29, 30).

Nevertheless, some of the antibody-labeled coated "pits" in these studies may actually represent free, coated vesicles.

The existence of free, coated vesicles shedding the coat rapidly after internalization strongly suggests that the coat material is recycled back to the plasma membrane, possibly in a nonlattice (soluble) form rather than as empty "baskets" (13). This view appears inconsistent with the above-mentioned observations (29, 30) as well as other immunocytochemical findings (2, 14) that clathrin cannot be detected in the cytoplasmic matrix. However, released coat material may reassociate very rapidly with the plasma membrane and/or become antigenetically undetectable within the cytoplasm (31). Moreover, results of Cheng et al. (6) on synapses suggest that clathrin may exist both in a lattice form associated with vesicles and in a soluble form in the cytoplasmic matrix.

Two models (at least) have been suggested for the recycling of coat material after internalization of coated pits in human skin fibroblasts: a two-compartment and a three-compartment model (9). In the first one, the recycling time (T) corresponds to the lifetime of a coated vesicle, whereas in the second one the recycling time is the lifetime of a coated vesicle plus the lifetime of the nonmembrane-associated coat. In spite of the qualitative differences in the two definitions, the T value calculated on experimental data is independent of, and applicable to, both models. On the basis of the assumption that all coated vesicular profiles represent truly free vesicles, Goldstein and Wofsy (9) calculated that the recirculation time was about 3.5 min. However, assuming that <40% of all coated profiles represent free coated vesicles, and using all other data and formula of Goldstein and Wofsy (9), we calculated the T value to be <1 min. This value concerns only the skin fibroblasts and holds only when the internalization rate constant is 0.25 min⁻¹ (9).

The significance of our observation, that considerably fewer coated vesicular profiles containing CF represent free vesicles in L-cells than in skin fibroblasts, remains obscure. It may be that the time spent at the cell surface as a coated pit and/or the recycling time varies from cell type to cell type. In the rat choroid plexus epithelium and the guinea pig yolk sac epithelium some coated pinosomes do not shed the coat at all, but instead carry membrane and ligand (CF) across the cells (transcellular transport) (15, 26).

Based on the present serial-section analysis, it appears conceivable that many peculiar configurations of coated vesicular profiles, for instance coated pits with elongated necks or associated with uncoated profiles (see Fig. 3 in reference 31; Fig. 4 in reference 33), represent coated pits at the base of adjacent microvilli or pits localized in invaginations of the plasma membrane. Thus, a section cut perpendicular to the plane pictured in Fig. 2 b would suggest that a large, smooth vesicle (a receptosome) is connected to, or is even budding from, the coated profile. Our observations suggest that the more irregular the cell surface (such as in L-cells) the higher the probability for finding receptosomalike structures in a given section.

The receptosome has been characterized, among other features, by its content of small (60 nm) vesicular profiles within the electron-lucent matrix (19, 31). This is, however, also a characteristic feature of MVBs. In conclusion, therefore, the present observations suggest that ligand, rather than being internalized by receptosomes budding off from coated pits, is carried into the cytoplasm by coated vesicles which, after the coat has been shed, eventually deliver their load (and membrane) to vacuoles which may be of the MVB-type. Whether such vacuoles are different from those reached by (smooth-surfaced) pinosomes involved in nonspecific (fluid-phase) pinocytosis remains to be elucidated.

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