Potassium-dependent Assembly of Coated Pits: New Coated Pits Form as Planar Clathrin Lattices

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Abstract. Previous studies have shown that when human fibroblasts are depleted of intracellular K⁺, coated pits disappear from the cell surface and the receptor-mediated endocytosis of low density lipoprotein (LDL) is inhibited. We have now used the K⁺ depletion protocol to study several aspects of coated pit function. First, since coated pits rapidly form when K⁺-depleted fibroblasts are incubated in the presence of 10 mM KCl, we studied the sequence of assembly of coated pits as visualized in carbon-platinum replicas of inner membrane surfaces from cells that had been incubated in the presence of K⁺ for various times. New coated pits initially appeared as planar clathrin lattices that increased in size by the formation of polygons at the margin of the lattice. Once the lattice reached a critical size it invaginated to form coated vesicles. Second, we determined that LDL–ferritin can induce clustering of LDL receptors over noncoated membrane on the surface of K⁺-depleted fibroblasts; however, when these cells are subsequently incubated in the presence of K⁺, these clusters become associated with newly formed coated pits and are internalized. Finally, we determined that K⁺ depletion inhibits the assembly of coated pits, but that existing coated pits in K⁺-depleted cells are able to internalize LDL. These results suggest that the clathrin lattice of coated pits is actively involved in membrane shape change during endocytosis and that the structural proteins of the lattice are cyclically assembled and disassembled in the process.

Coated pits are the ubiquitous cell surface specialization that mediate the receptor-dependent endocytosis of a wide variety of macromolecules (7, 9). In many tissue culture cells, coated pits occupy ~2% of the cell surface (4) and can contain as much as 70% of the cell surface receptors for ligands such as low density lipoprotein (LDL) (3). Two important functions have been ascribed to these regions of plasma membrane. (a) Their molecular architecture specifies the clustering of certain membrane receptors; and (b) they are directly or indirectly responsible for controlling the invagination of plasma membrane to form endocytic vesicles (4).

The cytoplasmic portion of the transmembrane receptor for LDL appears to contain information that causes the receptor to cluster over coated pits. This segment of the molecule contains the 50-amino acid COOH-terminal sequence (18). Four different mutant LDL receptors that are unable to cluster in coated pits all have defects in this region of the molecule (6, 14, 15). The most revealing of these mutants is one where the tyrosine at positions 807 is changed to a cysteine (6). The aberrant behavior of a receptor with a single amino acid substitution suggests that clustering depends upon the precise interaction of the cytoplasmic tail with some molecular component(s) of the coated pit.

For a ligand to be internalized during receptor-mediated endocytosis, portions of plasma membrane that contain the ligand–receptor complex must invaginate to form endocytic vesicles (7). Numerous morphologic studies have provided evidence that the coated pit is responsible for invagination (4). Heuser and Evans (11) proposed that the polygonal clathrin lattice of the coated pit undergoes a molecular rearrangement that converts planar coated pits into invaginated coated pits, which subsequently bud from the membrane. However, there is not any direct evidence that a coated pit can undergo this shape transformation; moreover, candidate molecules that might be responsible for this reaction have yet to be identified.

Recently we reported that when human fibroblasts were depleted of intracellular K⁺ the number of coated pits declined by 80–90% and there was a corresponding inhibition of LDL internalization (12, 13). Although these results implied that cells without coated pits are unable to internalize receptor-bound macromolecules, the K⁺ depletion protocol may have nonspecifically inhibited endocytosis. However, Moya et al. (17) have recently shown that when Hep2 cells are depleted of intracellular K⁺ the endocytosis of [¹²⁵]transferrin by coated pits is blocked, but [¹²⁵]–ricin toxin continues to be internalized. Therefore, cells that lack K⁺ can internalize selected molecules even though coated pits are absent.

1. Abbreviation used in this paper: LDL, low density lipoprotein.
Although we do not know why low intracellular K+ has such a dramatic effect on coated pits (13), we have used this technique to obtain new information about the function of coated pits in receptor clustering and plasma membrane invagination. First, since coated pits rapidly reform when K+-depleted cells are incubated in the presence of K+, we have mapped the sequence of events in coated pit assembly as visualized in carbon–platinum replicas of the inner membrane surface. Second, we have used 125I-LDL and LDL-ferritin as probes for the LDL receptor to determine both the distribution of the ligand–receptor complex on the surface of cells that lack coated pits and how the complex is internalized when new coated pits form. Finally, we have used this protocol to show that coated pit structural proteins recycle during endocytosis.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DME; No. 320-1885) and Dulbecco's phosphate-buffered saline (No. 310-4190) were purchased from Gibco (Grand Island, NY). A mixture of insulin, transferrin, and selenium (ITS Premix) was purchased from Collaborative Research, Inc. (Lexington, MA). Human LDL (d = 1.019-1.063 g/ml) and lipoprotein-deficient serum (d > 1.215 g/ml) were prepared by ultracentrifugation of plasma (8). LDL was radiolabeled with 35S as previously described (8). LDL was covalently coupled to ferritin as previously described (2). Epon was obtained from Ladd Research Industries, Inc. (Burlington, VT). Poly-l-lysine (No. P1524) was from Sigma Chemical Co. (St. Louis, MO), amyl acetate (No. 45950) from Fluka (Hauppauge, NY), and bone dry CO2 (No. 131303) from Liquid Air Corp. (San Francisco, CA). All fixatives were from Electron Microscopy Sciences (Fort Washington, PA). Other supplies were obtained from sources as previously reported (12).

The buffers used were as follows: buffer A, 50 mM Hepes, 100 mM NaCl, pH 7.4; buffer B, 50 mM Hepes, 100 mM NaCl, 1 mM CaCl2, 10% lipoprotein-deficient serum, pH 7.4; and buffer C, 20 mM Hepes, 100 mM KCl, 5 mM MgCl2, 3 mM EGTA, pH 6.8.

Cell Culture

Cultured fibroblasts were derived from a skin biopsy obtained from a normal subject. Cells were grown in monolayer and set up for experiments according to a standard format. On day 0, 3-4 x 10⁶ cells were seeded into each petri dish (60 x 15 mm) containing 3 ml DME supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% vol/vol fetal calf serum. Fresh medium of the same composition was added on day 3. On day 5 of cell growth, each monolayer received 2 ml DME supplemented with penicillin, streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, 5 mg/ml selenium (ITS Premix), and 10% vol/vol human lipoprotein-deficient serum. Experiments were performed on day 7 after 48 h of incubation in lipoprotein-deficient serum.

Protocols for Preparing K+-depleted Fibroblasts

Cells were depleted of K+ at 37°C using the hypotonic shock procedure (12). On day 7 of cell growth, the medium was discarded and each monolayer was washed in 3 ml of buffer A. Each monolayer was then incubated for 5 min in 2 ml of hypotonic medium (DME/water, 1:1), followed by a 10-min incubation in buffer A. The medium was discarded and the cells were incubated for the indicated time in 2 ml of buffer B.

To deplete cells of K+ at 4°C, each monolayer of fibroblasts was subjected to the following sequential incubations on day 7 of cell growth: (a) 5 min of incubation in hypotonic medium at 37°C; (b) 10 min of incubation in buffer A at 37°C; and (c) 180 min of incubation in buffer B at 4°C.

Labeling of Cells with 125I-LDL

Each monolayer received a direct addition of 125I-LDL (12 μg/ml) in the indicated buffer in the absence or presence of 500 μg/ml of unlabeled LDL. After incubation for the indicated times, receptor-bound or internalized 125I-LDL was measured by the dextran sulfate release assay (8). Values for specific binding or internalization were calculated by subtracting the amount of 3H-LDL bound or internalized in the presence of unlabeled LDL (nonspecific) from that bound or internalized in the absence of unlabeled LDL (total). The values for nonspecific binding or internalization in control incubations were <5% of the total values. Each value shown in the figures represents the average of duplicate incubations.

Labeling of Cells with LDL–Ferritin

Each monolayer received a direct addition of LDL–ferritin (50-65 μg/ml LDL protein), in the presence or absence of 500 μg/ml of unlabeled LDL, in the indicated buffer. After incubation for the indicated times at the appropriate temperature, the monolayers were washed at 4°C to remove nonspecifically bound LDL–ferritin (2). Cells were fixed and processed for electron microscopy (see below). The distribution of LDL–ferritin on the cell surface or within the cell was quantified as previously described (2, 3).

Preparation of Carbon–Platinum Replicas of the Fibroblast Inner Cell Surface

Carbon–platinum replicas were prepared according to the method of Aggeler et al. (1). On day 7 of cell growth, fibroblasts grown on coverslips were subjected to the sequential incubations indicated in the figure legends. At the end of the incubation, the monolayers were washed twice with ice-cold Dulbecco's PBS and once with ice-cold buffer C at 4°C. The coverslips containing the monolayer was then overlayed with a second coverslip that had been coated with poly-l-lysine (1 mg/ml >300,000 mol wt, in distilled water for 1 h). The overlayed coverslip was tapped gently to bind the cells to the poly-l-lysine and then the two coverslips were separated in ice-cold buffer C. The poly-l-lysine-coated coverslip, which contained membranes from the upper surface of the cell, was fixed with 1% vol/vol glutaraldehyde plus 1% vol/vol formaldehyde in buffer C for 30 min. The coverslips were then fixed for 30 min with 0.5% OsO4 in buffer C, dehydrated into amyl acetate, and critically point dried in a critical point dryer (model CPD020; Balzers, Hudson, NH) using bone dry CO2. 7.5-nm thick carbon–platinum replicas of the membranes were made in a freeze fracture machine (model 301; Balzers, Hudson, NH) using bone dry CO2. The replicas were then floated free of the coverslip in a distilled water bath and digested with Chlorox for 2 min before mounting on Formvar-coated grids.

Electron Microscopy

Fibroblast monolayers were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3) for 30 min at room temperature. The fixed cells were postfixed with 2% OsO4 in 0.1 M sodium cacodylate, pH 7.2, and embedded directly on the dish in Epon (2). Sections were prepared for quantification as previously described (2). Sections were cut with an ultramicrotome (MT-2B; DuPont Co., Sorvall Instruments Div., Newton, CT) and viewed with a JEOL 100 CX electron microscope.

Other Assays

The content of intracellular K+ was determined by flame photometry (12). The content of cellular protein was measured by the Lowry method (16).

Results

New Coated Pits Form as Planar Clathrin Lattices

The three-dimensional organization of coated pits is revealed in carbon–platinum replicas of the inner membrane surface (1, 11). The electron microscopic images of these replicas allow the unambiguous identification of clathrin lattices and give information about the relationship between the polygon arrangement within the lattice and the curvature of the coated pit. Therefore, we used this technique to study the initial stages of coated pit formation that take place when K+-depleted human fibroblasts are incubated in the presence of K+ for different lengths of time.

We restricted our analysis to the upper plasma membrane of the cultured human fibroblast where coated pit–mediated...
Figure 1. Carbon–platinum replica of the inner cell surface of normal human fibroblasts. Fibroblasts were grown on coverslips, and on
day 7 the upper cell surface was attached to a poly-l-lysine-coated coverslip and processed as described. (A) A low magnification view
of the inner cell surface: open arrowheads, small planar coated pits; solid arrowheads, breaks in lattice of large planar coated pits; curved
arrow, irregular shaped, invaginated coated pit. (B, C, and D) High magnification view of irregularly shaped, invaginated coated pits. (E)
A low magnification view of the inner cell surface: open arrowheads, small planar coated pits; solid arrowheads, breaks in lattice of large
planar coated pits; circles, uniformly curved, invaginated coated pits; brackets, one coated pit with two domains that have different degrees
of curvature. Bars: (A and E) 0.5 μm; (B, C, and D) 0.2 μm.
Figure 2. Carbon–platinum replica of the inner cell surface of a K⁺-depleted human fibroblast. Fibroblasts grown on coverslips were subjected to the following sequential incubations at 37°C: (a) a 5-min hypotonic shock followed by a 10-min incubation in isotonic K⁺-free buffer A; and (b) a 30-min incubation in K⁺-free buffer B. The upper plasma membrane was removed and replicas prepared as described. (A) A region of the surface between two stress fibers. Numerous microcoated pits are present (arrows). (B) A high magnification view of several microcoated pits showing the presence of a coat material that has the appearance of a polygonal lattice. Bars: (A and B) 0.2 µm.

Endocytosis is an active process (3, 7). Fig. 1 shows several replica images of the cytoplasmic surface of membrane from untreated fibroblasts. Usually there were several bundles of filaments that had the appearance of actin-containing stress fibers spaced along the membrane (Fig. 1, A and E). Between the stress fibers were numerous coated pits that varied in size (200–850 nm in diameter) and degree of curvature. The majority of these coated pits consisted of planar (flat) or slightly curved clathrin lattices (open arrowheads, Fig. 1, A and E). The larger planar lattices usually were subdivided into irregularly sized domains by breaks in the lattice (solid arrowheads, Fig. 1, A and E). Each domain appeared to function independently of the adjacent domain within the same coated region because they often had different degrees of curvature (brackets, Fig. 1 E). Another striking feature of the coated pits on the surface of untreated fibroblasts was the pleomorphic shape of the invaginated clathrin lattices. Some of the different shapes encountered are illustrated in Fig. 1 (curved arrow, Fig. 1 A and Fig. 1, B–D). In addition, uniformly curved invaginated areas were also seen (circles, Fig. 1 E).

As judged by indirect immunofluorescence using anti-clathrin IgG or by thin-section electron microscopy, K⁺-depleted human fibroblasts have few coated pits (12). This was also found to be the case in carbon–platinum replicas of membranes from K⁺-depleted cells (Fig. 2 A). The stress fiber arrangement was normal in these membranes, but the portions of membrane between the fibers were virtually devoid of coated pits. A unique feature of the K⁺-depleted membrane, however, was the presence of numerous small membrane blebs (arrows, Fig. 2 A) that measured 60–70 nm in diameter, which at high magnification appeared to be decorated with a polygonal lattice (Fig. 2 B). Due to the extreme curvature of these blebs, often only the outline of a polygonal network could be resolved. Although we can not be sure that these blebs are coated with clathrin, for future discussion (see below) we refer to these structures as microcoated pits because they were 3 to 14 times smaller than the coated pits that were present in untreated membranes.

The first evidence of coated pit formation was seen in replicas of membranes from K⁺-depleted cells that had been incubated in the presence of 10 mM KCl for 2 to 5 min. At this time, small planar clathrin lattices, consisting of five or more distinct polygons, were found randomly distributed across the membrane (circles, Fig. 3 A). The size of these lattices was quite variable but usually contained <20 polygons. Several structural features of coated pit assembly are illustrated in Fig. 3, B, C, and D. Assembly appeared to begin with the formation of several polygons at focal sites on the membrane (Fig. 3 B). At the margins of these completed lattices, partially assembled polygons were found to emanate from fully formed polygons, which suggests that growth in the size of the lattice involves the progressive assembly of new polygons from the sides of existing polygons. In Fig. 3 B, forming polygons with two sides (arrow 1), four sides (arrow 2) and five sides (arrow 3) are seen. In some cases, two lattices began to form adjacent to each other (Fig. 3 C and D). These two lattices either merged into one lattice by the formation of new polygons in the region between each completed lattice (arrows, Fig. 3 C) or the two lattices did not merge with each other (arrows, Fig. 3 D), which most likely accounts for the presence of the discontinuous lattices seen in untreated cells (solid arrowheads, Fig. 1 A). In all cases, forming lattices were composed predominantly of hexagons.

The images in Fig. 3 also show that usually one or more microcoated pits were associated with the forming coated pits. Usually these structures were near the margin of the coated pit, but occasionally they were seen in the middle of
a partially assembled lattice. However, on the same segment of membrane there were also many microcoated pits that were not associated with forming coated regions. The coated pits in K⁺-depleted cells that had been incubated for longer times in the presence of K⁺ were larger and appeared to be more completely assembled (arrows, Fig. 4). In addition, by 5 to 10 min of K⁺ repletion, deeply invaginated coated pits were evident (circles, Fig. 4). After 15-30 min of K⁺ repletion, the distribution and appearance of coated pits was similar to control cells (data not shown). The number of microcoated pits steadily declined over this period of time so that by 15-30 min they were rarely seen.

Formation of Coated Pits Is Coupled to the Internalization of LDL

An important test of whether this assembly sequence represents a normal pathway for coated pit formation is to determine if these coated pits are capable of internalizing macromolecules. We have addressed this question by comparing the kinetics of coated pit formation with the kinetics of LDL internalization during K⁺ repletion.

The kinetics of coated pit disappearance and reappearance in response to K⁺ was determined by quantitative thin-section electron microscopy. Human fibroblasts were treated with hypertonic medium and then incubated in K⁺-free buffer B for 45 min. At this time, buffer B was replaced with DME and the cells were incubated for an additional 45 min. Cells were fixed at regular time intervals during the incubations and processed for quantitative electron microscopic analysis. During the 45-min incubation in the absence of K⁺ (Fig. 5), there was a steady decline in the number of coated pits, reaching zero per millimeter of cell surface at 45 min. The half-time for loss of coated pits was 22.5 min. When the K⁺-free buffer was replaced with DME, there was a rapid increase in the number of coated pits. The half-time for coated pit recovery was 5 min. The kinetics of coated pit recovery determined with this technique was in agreement with the time it took for new coated pits to appear when
visualized by the carbon–platinum replica technique (see Figs. 3 and 4).

To assess the K⁺-dependent return of coated pit function, K⁺-depleted fibroblasts were labeled with 125I-LDL at 37°C. The cells were washed to remove nonspecifically bound 125I-LDL, one set was incubated for various times in the presence of 10 mM KCl and a companion set was incubated in the absence of KCl. As seen in Fig. 6, K⁺ caused a rapid loss of surface bound 125I-LDL (Fig. 6 A) and a corresponding increase in internalized 125I-LDL (Fig. 6 B). Cells that were incubated in the absence of K⁺ did not internalize 125I-LDL. The half-time for K⁺-dependent internalization was 7 min. Therefore, the kinetics of LDL internalization was nearly the same as the kinetics of coated pit formation (Fig. 5).

The close correspondence between the return of coated pits and the internalization of pre-bound 125I-LDL in response to K⁺ suggested that newly formed coated pits were functional. Therefore, we used electron microscopy to visualize the initial phases of LDL internalization when K⁺-depleted fibroblasts that had been labeled with LDL-ferritin were incubated in the presence of K⁺ for various times. Immediately after the incubation of K⁺-depleted cells in the presence of LDL–ferritin, ferritin was found to be clustered in groups of 5 to 10 cores and the clusters were randomly distributed on the cell surface (Fig. 7 A). Although the few remaining coated pits were labeled (Table I), most of the LDL–ferritin clusters were over noncoated membrane. After 5 min in the presence of K⁺, however, large clusters of ferritin were found over coated pits (Fig. 7 B) but only a modest amount of LDL–ferritin had been internalized (Table I). After further time in the presence of K⁺, there was an increase in the number of coated pits, a decline in surface-bound LDL–ferritin, and an increase in internalized LDL–ferritin (Table I).

Figure 5. Kinetics of coated pit disappearance and reappearance in response to intracellular K⁺. Fibroblast monolayers were subjected to the following sequential incubations at 37°C: (a) a 5-min hypotonic shock followed by a 10-min incubation in isotonic K⁺-free buffer A (shock + wash); (b) a 30-min incubation in K⁺-free buffer B followed by a 40-min incubation in DME. At the indicated times, samples were fixed with 3% glutaraldehyde in 0.1 M Na cacodylate, pH 7.3, and processed for electron microscopy. The number of coated pits per millimeter of cell surface were tabulated as previously described (2).

K⁺-depletion Inhibits Coated Pit Assembly

Our results suggest that in the potassium-depleted cell, coated pits are disassembled and the clathrin subunits are poised for re-assembly once potassium levels are restored. Coated pits could be disassembled in these cells either because low concentrations of intracellular K⁺ promotes the dissociation of the clathrin lattice from the cell membrane or because K⁺ is required for coated pit assembly. The first explanation predicts that coated pits in K⁺-depleted cells would not internalize macromolecules because they disassemble before internalization. However, if the second explanation is correct, then existing coated pits would internalize, but the number of coated pits would decline due to the lack of new coated pit formation. To distinguish between these two possibilities, conditions were established where coated pit function could be measured in K⁺-depleted cells. This was accomplished by depleting cells of K⁺ at 4°C, labeling them with LDL–ferritin, and assessing internalization of LDL–ferritin when the cells were shifted to 37°C in the absence of K⁺.

Cells that were K⁺-depleted at 4°C and then labeled with LDL–ferritin had normal numbers of coated pits and 50% of the ferritin was clustered over these regions of membrane (Table II). When these cells were incubated for 15 min at 37°C in the absence of K⁺, however, there was a dramatic decrease in both the number of coated pits and the amount of surface-bound LDL–ferritin. Coated pits and surface-bound LDL–ferritin declined further by 30 min at 37°C; moreover, these cells had internalized ~50% of the LDL–ferritin that was initially bound to the cell surface at 4°C (Table II), which corresponds to the amount of LDL–ferritin
Figure 6. Kinetics of 125I-LDL internalization during K+ recovery. Fibroblast monolayers were subjected to the following sequential incubations at 37°C: (a) a 5-min hypotonic shock followed by a 10-min incubation in isotonic K+–free buffer A; (b) a 30-min incubation in K+–free buffer B; (c) a 30-min incubation in K+–free buffer B that contained 15 μg/ml 125I-LDL in the presence and absence of 500 μg/ml LDL; and (d) incubation for the indicated times with buffer B that either contained no K+ (solid circle) or 10 mM KCl (open circle), after which receptor-bound 125I-LDL (A) and intracellular 125I-LDL (B) were measured as described.

Table I. Internalization of LDL–Ferritin in Response to the Return of Coated Pits to the Cell Surface

<table>
<thead>
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<th>Time after addition of 10 mM KCl</th>
<th>Coated pits</th>
<th>Surface LDL–ferritin</th>
<th>Ferritin in coated pits</th>
<th>Internalized LDL–ferritin</th>
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<td>No./mm</td>
<td>No./mm %</td>
<td>No./mm</td>
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<td>144</td>
<td>30</td>
<td>764</td>
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<tr>
<td>30</td>
<td>10</td>
<td>24</td>
<td>42</td>
<td>1,255</td>
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Fibroblast monolayers were subjected to the following incubations at 37°C: (a) a 5-min hypotonic shock followed by a 10-min incubation in K+–free buffer A; (b) a 30-min incubation in K+–free buffer B; (c) a 60-min incubation in K+–free buffer B plus 60 μg/ml LDL–ferritin; and (d) incubation with buffer B that contained 10 mM KCl for the indicated times. Cells were processed for electron microscopy and the quantification was performed as previously described (3). Values were normalized to 1 mm of cell surface.

Discussion

The K+ depletion technique continues to provide important information about the structure and function of coated pits. From the current work, we draw three major conclusions about the physiology of this specialized membrane. (a) The polygonal lattice of the coated pit, by actively changing shape, is involved in the conversion of planar segments of plasma membrane into endocytic vesicles. (b) LDL can induce receptor clustering in the absence of coated pits but these clusters rapidly associate with newly formed coated pits when endocytosis resumes after the restoration of normal K+ levels. (c) The clathrin subunits (triskelions) and associated proteins recycle during endocytosis and K+ is required for the assembly phase of the cycle.

Coated Pit Assembly

Heuser and Evans (11) and Aggeler et al. (1) introduced the use of carbon–platinum replicas to study the three-dimensional organization of coated pits in cultured cells. Our use of this technique revealed that coated pits in human fibroblasts assume a variety of shapes and sizes. A visual inspection of these images suggests that at any time during eng-
coated pits are dynamic structures capable of assuming a variety of shapes in response to specific stimuli, they do not reveal the shape of an invaginated area may be determined by where in the lattice the hexagons are converted to pentagons. If pentagons are introduced into the lattice unevenly, puckers or bulges in the lattice may form, giving rise to the various shapes that were seen.

Although the images in Fig. 1 imply that the clathrin lattice is a dynamic structure capable of assuming a variety of shapes in response to specific stimuli, they do not reveal the sequence of events during endocytosis. On the other hand, the K+-dependent assembly experiments indicate that planar coated pits correspond to newly formed lattices and that the invaginated pits are formed from these lattices. Since coated pits that assembled in response to K+ were able to internalize LDL, it is most likely that the clathrin lattice plays an active role in the conversion of plasma membrane into endocytic vesicles.

When new coated pits formed in response to K+, there was not any obvious preferential site of assembly; moreover, we could not detect any morphological specializations of the membrane at sites where the lattices were forming. The first visible sign of a new coated pit was the presence of two or three polygons. The coated pit appeared to grow in size by the ordered formation of new polygons at the margin of the planar lattice, and once it reached a critical size, invagination began. This assembly sequence suggests that there are (a) sites on the plasma membrane that initiate lattice assembly; (b) factors that control the size of the lattice; and (c) factors that rearrange the lattice to cause the invagination of membrane.

A completely unexpected finding was the appearance of microcoated pits on the surface of both K+-depleted cells and cells that were actively engaged in coated pit assembly. These highly curved regions of membrane appeared to be covered with a polygonal lattice, which suggests that they are coated with clathrin. However, future immunocytochemical studies with clathrin-specific antibodies will be needed to identify the molecular composition of this material. Their close association with forming coated pits suggests that they may be an assembly precursor, possibly a form of clathrin that appears resistant to the absence of K+ in these cells.

**LDL Receptors Can Cluster in the Absence of Coated Pits**

Although the cytoplasmic tail of the LDL receptor directs the receptor to the coated pit (9), there has not been a way to determine how LDL influences receptor clustering independently of the coated pit. With the K+-depleted cell, however, we found that LDL-ferritin was capable of inducing receptor clustering in the absence of coated pits. Clustering was temperature-dependent because when binding was carried out at 4°C, LDL-ferritin was not clustered on the surface of the K+-depleted cell (data not shown). Therefore, LDL can influence the surface dynamics of its receptor, which may contribute to a more efficient internalization when cells are grown in the presence of LDL.

**Recycling of Coated Pit Structural Proteins**

Just as the experimental disruption of intracellular pH gradients proved to be a useful tool for studying receptor recycling during receptor-mediated endocytosis (5), the K+-depletion method has provided direct evidence that the structural proteins of coated pits also recycle. K+ appears to be required for this recycling because in its absence existing coated pits can internalize normally but coated pit assembly stops.

This new information provides a more complete understanding of the receptor-mediated endocytosis cycle. A coated pit begins life as a small planar clathrin lattice that consists of several polygons. These planar lattices increase in size as more polygons assemble. Eventually the planar lattices become curved, by either the introduction of pentagons into the hexagonal lattice or the formation of pentagons at the edge of the lattice, and the subtotating plasma membrane invaginates. The LDL receptor, as well as other receptors involved in receptor-mediated endocytosis, are trapped in the invaginating coated pits and carried into the cell by the transformation of the coated pit into a coated vesicle. The structural proteins of the coated vesicle lattice dissociate from the membrane and are competent to re-assemble into coated pits.
at the cell surface. The LDL receptor, on the other hand, is sorted from LDL and returns to the cell surface in a recycling vesicle.

Whereas receptor recycling depends upon the presence of a low pH in the endosome, coated pit structural proteins recycle independently of endosomal pH (5). In contrast, coated pit assembly is K+-dependent, but LDL receptor recycling to the cell surface proceeds in the absence of intracellular K+ (12). Therefore, the recycling of receptors and coated pit proteins are sensitive to different intracellular ionic conditions. Moreover, the movement of receptors from the endosome to the cell surface may not involve a coated vesicle intermediate.

In conclusion, even though we have identified the K+-dependent step during endocytosis, these studies do not reveal how K+ acts on the assembly processes. Nevertheless, we have uncovered a potential site for cellular regulation of coated pit-mediated endocytosis. There is considerable evidence that certain cells can regulate the formation of coated pits and coated vesicles (4); maybe the K+-dependent step is a regulatory site that cells normally use to control endocytosis.

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References


