Coat Proteins Isolated from Clathrin Coated Vesicles Can Assemble into Coated Pits

David T. Mahaffey,* Mary Shannon Moore,* Frances M. Brodsky, and Richard G. W. Anderson*

*Department of Cell Biology and Anatomy, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235; and †Department of Pharmacy, University of California at San Francisco, San Francisco, California 74143

Abstract. Isolated human fibroblast plasma membranes that were attached by their extracellular surface to a solid substratum contained numerous clathrin coated pits that could be removed with a high pH buffer (Moore, M. S., D. T. Mahaffey, F. M. Brodsky, and R. G. W. Anderson. 1987. Science [Wash. DC]. 236:558–563). When these membranes were incubated with coat proteins extracted from purified bovine coated vesicles, new coated pits formed that were indistinguishable from native coated pits. Assembly was dependent on the concentration of coat protein with half maximal assembly occurring at 7 µg/ml. Assembly was only slightly affected by the presence of divalent cations. Whereas normal appearing lattices formed in a low ionic strength buffer, when assembly was carried out in a low pH buffer, few coated pits were evident but numerous small clathrin cages decorated the membrane. Coated pits did not form randomly on the surface; instead, they assembled at differentiated regions of membrane that could be distinguished in carbon/platinum replicas of frozen and etched membranes by the presence of numerous particles clustered into patches the size and shape of a coated pit.

Once clathrin coated vesicles were isolated from pig brain (31), there was a rapid development of methods for disassembling and reassembling the clathrin coat in vitro (36, 39, 42, 43). The relative ease with which the polygonal lattice can be taken apart and put back together has spawned the many studies that contribute to our current understanding of this interesting organelle (5, 16, 25, 33, 37). We know, for example, that (a) the three-legged triskelion, composed of three 180-kD and three 33–36-kD molecules, is the basic building block of the lattice (17, 38); (b) an assembly protein complex consisting of proteins of approximate mol. wt. 100,000, 50,000, and 16,000 facilitates lattice assembly at physiologic pH (1, 16, 32, 44); (c) certain of the coat proteins are substrates for protein kinases (2, 3, 14, 28, 29, 30); (d) the triskelion subunits are linked to the membrane of coated vesicles in part by a class of peripheral membrane proteins with mol. wt. 100,000–116,000 (16, 37, 41).

Coated vesicles originate from coated pits at either the cell surface (11, 20) or the trans-Golgi region (8, 27). Coated pits originally gained prominence because of their involvement in the endocytosis of membrane bound macromolecules such as yolk proteins (34) and ferritin (7). Subsequent work has established that these regions of membrane are the site for receptor-mediated endocytosis of a wide variety of proteins, hormones, and macromolecular complexes (10). To internalize these molecules, the coated pit must control receptor clustering (6, 10) as well as modulate membrane shape during the transformation of a planar segment of membrane into an endosomal compartment (10). Receptor-mediated endocytosis is sustained by the rapid recycling of receptors (10) and by the continuous replenishment of coated pits (10, 20).

In contrast to coated vesicles, there is little information about the requirements for coated pit assembly. Presumably many of the same proteins are involved; however, the requirements for constructing a relatively flat, open lattice may be quite different than for building a closed, vesicle lattice. The ability to control coated pit assembly in vivo by regulating the level of intracellular K+ (19, 20) has provided some important clues about the assembly process: (a) assembly is rapid ($t_{1/2} \sim \sim 5$ min); (b) new coated pits initially have a planar configuration and are composed of 5–10 polygons (principally hexagons); (c) the initially formed lattices grow in size by the addition of polygons at the margin of the new lattice; and (d) curvature of the lattice appears to occur independently of assembly.

Since coated pits only occupy 2–3% of the cell surface, to understand coated pit assembly requires the identification of the membrane factors that control where the pit will assemble. Unanue et al. (37) identified the 100-kD component of the assembly protein complex as being important for triskelion binding and assembly on isolated coated vesicle membranes. More recently, Virshup and Bennett (41) demonstrated a high affinity interaction between assembly proteins and isolated coated vesicles as well as brain membranes after treatment to remove endogenous coat proteins. Therefore, there may be a high affinity coated pit "receptor" exposed on the inner membrane surface that initiates pit formation.

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Recently we developed a method for reconstituting coated pit formation in vitro using a cytoplasmic extract together with isolated membranes attached to a substrate (23). Under these conditions, the coat proteins in the cytoplasm are recruited to a limited number of assembly sites where new lattices form. Assembly mimics many of the features observed in vivo and, in addition, does not require the addition of ATP.

Despite the successful assembly of coated pits using cytoplasm as a source of clathrin, it will be difficult to use this method to discover the basic mechanisms that operate during assembly. To identify the membrane components that regulate assembly as well as the molecular components that form a coated pit, assembly must be studied using more purified components. Since coated pits have never been isolated and their molecular compositions have not been determined, we could not assume that knowledge gained from studying coated vesicle assembly would apply. For this reason, we have begun the dissection of the assembly process by identifying the conditions that will support coated pit formation when coat proteins isolated from purified vesicles are used as the source of clathrin.

Materials and Methods

Materials

Immuno I Removewell 96-well plates (01-010-6301) were purchased from Dynatech Laboratories, Inc. (Alexandria, VA). Human plasma fibronectin was obtained from the New York Blood Center: MEM (330-4,345), Dulbecco's PBS (310-4,190), DME (320-1885), FCS (200-6,140 AG), and NaN3 (S-227) were from Fisher Scientific Co. (Pittsburgh, PA). Glycine (161-0718) was from Bio-Rad Laboratories Inc. (South San Francisco, CA). 125I-labeled streptavidin (IM-236) at a specific activity of 20-40 ~Ci/~g, was purchased from American护肤品 Inc. (South San Francisco, CA). Poly-L-lysine (P-1524), Hepes (H-3375), NaC1 (S-9625), 2(N-morpholino)ethanesulfonic acid, 1 mM DTT, 10 ~M leupeptin, 1 mM orthophenanthroline, 0.5 mM benzamidine, 2 ~g/ml soybean trypsin inhibitor, pH 7.2 (KOH). Buffer F: 2.3 mM NaH2PO4.H2O, 77 mM NaHPO4, 7 H2O, 150 mM NaCl, 2 mM MgCl2, pH 7.4 (NaOH). Buffer G: 0.05 M NaHCl in Dulbecco's PBS. Buffer H: MEM, 20 mM HEPES, pH 7.4 (NaOH).

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 (9). On day 0, 7 × 10^5 cells were seeded into each petri dish (100 × 15 mm) containing 10 ml DME supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% (vol/vol) FCS. Fresh medium of the same composition was added on day 3. On day 5 of cell growth, each monolayer received 8 ml DME supplemented with penicillin, streptomycin, 5 ~g/ml transferrin, 5 ~g/ml selenium (ITS Premix), and 10% (vol/vol) human lipoprotein-deficient serum. On day 7 of cell growth, the media was removed and 3 ml of trypsin - EDTA was added to each dish and the cells were incubated for 5 min at room temperature. The cell suspension was removed and added to an equal volume of buffer H containing 10% (vol/vol) FCS. The suspended cells were centrifuged at 600 g for 5 min and washed three times by repeated, alternate resuspension and centrifugation in buffer H. This cell suspension was used in the various assembly assays described below.

Preparation of Coat Proteins

Coated vesicles were purified from bovine brain by the method of Nandi et al. (26) with the following modifications. Buffer I was used as the homogenization buffer. For the initial 100,000 g spin, we used a rotor (model 45 Ti; Beckman Instruments, Inc., Palo Alto, CA) operated at 100,000 g (49,000 rpm) in a rotor (model 100.3; Beckman Instruments, Inc.) operated at 36,000 rpm, and for the 8% sucrose cushion spin we used a rotor (model AH-627; Sorvall Instruments Div., Newtown, CT) at 25,000 rpm, 4°C. Immediately after purification, coated vesicles were pelleted for 1 h at 100,000 g (49,000 rpm) in a rotor (model 100.3; Beckman Instruments Inc.) to strip proteins from the vesicles, the coated vesicles were resuspended in buffer J using a homogenizer (Dounce, Vineland, NJ) and incubated at 4°C for 30 min. The stripped vesicles were pelleted for 1 h at 100,000 g in a rotor (model 100.3; Beckman Instruments Inc.) and the resulting supernatant fraction contained the coat proteins. This fraction was divided into 10-~l aliquots and stored at −80°C. Coat proteins were thawed just before assembly for each experiment. The coat proteins (4.75 mg/ml, in buffer J) were diluted to the concentration used for assembly (20 ~g/ml protein unless otherwise noted) in buffer E + 1% C-BSA and supplemented with a variable amount of Triton-X-100 such that the Triton-X-100 concentration was 7.8 mg/ml in all assembly experiments. Coat proteins were diluted 10-1000 min before exposing them to membranes for assembly.

Buffers

The following buffers were used where indicated. The pH for each buffer was adjusted with either NaOH or KOH, as shown in parenthesis. Buffer A: 50 mM Hepes, 100 mM NaCl, pH 7.4 (NaOH). Buffer B: 20 mM Mes, 1 mM EDTA, 2.5 mM MgCl2, 100 mM KCl, 1 mM DTT, 10 ~g/ml leupeptin, 1 mM orthophenanthroline, 0.5 mM benzamidine, 2 ~g/ml soybean trypsin inhibitor, pH 6.2 (NaOH). Buffer C: 20 mM Hepes, 3 mM EDTA, 5 mM MgCl2, 100 mM KCl, pH 6.8 (NaOH). Buffer D: 20 mM Tris(hydroxymethyl)methylaminopropyl sulfonic acid, 1 mM DTT, 10 ~g/ml leupeptin, 1 mM orthophenanthroline, 0.5 mM benzamidine, 2 ~g/ml soybean trypsin inhibitor, pH 9.0 (NaOH). Buffer E: 36.4 mM HEPES, 68.2 mM KCl, 4.1 mM Mg acetate, 1 mM DTT, 10 ~M leupeptin, 1 mM orthophenanthroline, 0.5 mM benzamidine, 2 ~g/ml soybean trypsin inhibitor, pH 7.2 (KOH). Buffer F: 2.3 mM NaH2PO4.H2O, 77 mM NaHPO4, 7 H2O, 150 mM NaCl, 2 mM MgCl2, pH 7.4 (NaOH). Buffer G: 0.05 M NaHCl in Dulbecco's PBS. Buffer H: MEM, 20 mM HEPES, pH 7.4 (NaOH). Buffer I: 0.1 M Mes, 1 mM EDTA, 0.5 mM MgCl2, 3 mM NaN3, 1 mM orthophenanthroline, 0.5 mM benzamidine, 2 ~g/ml soybean trypsin inhibitor, 10 ~M leupeptin, 5 mM sodium fluoride, pH 6.5 (NaOH).

Indirect 125I-Streptavidin Binding Assay

Each well of a 96-well plate (Immuno I Removewell) was washed once with glass-distilled H2O and incubated with 100 ~g of poly-L-lysine (1 mg/ml in distilled H2O) for 1 h at 37°C. Wells were washed five times with distilled water and incubated with 100 ~g of human plasma fibronectin (1 mg/ml in buffer H) for 30 min at 37°C. Wells were washed three times with buffer H and 200 ~g of a cell suspension containing 1.25 × 10^5 cells/ml in buffer H was added to each well. The cells were incubated for 1.5 h at 37°C and then 350 ~g of warm (37°C) buffer H containing 10% (vol/vol) FCS was added to each well. After a 15-min incubation at 37°C, the wells were placed...
Figure 1. Gel electrophoresis of coated vesicles, stripped vesicles, and coat proteins. Coat proteins were prepared from bovine brain and treated with 0.5 M Tris-HCl, pH 7.0, as described. Coat proteins correspond to the 0.5-M Tris-HCl extract. Electrophoresis was performed as described. Each lane was loaded with 20 μg of proteins and the gels were stained with Coomassie brilliant blue.

on ice for 1 h. Each well was then washed three times with buffer A at 4°C. Sets of three wells were washed twice with 4°C buffer B (200 μl first wash; 400 μl second wash) and submerged in a 20 x 13-cm tray containing 400 ml of ice-cold buffer B. Each well was sonicated for 2 s at a power setting of 3.5 using a 1/8-inch tapered microtip probe centered 2 mm above the top of the well (model W380; Heat Systems-Ultrasonics, Inc., Farmingdale, NY). The cells were then washed once with ice-cold buffer B. After sonication, the wells were either fixed by adding 100 μl/well of ice-cold 3% formaldehyde in buffer C for 15 min, or processed to remove coated pits at 4°C by the method of Heuser (11) using a Cryopress freezing device (Med Vac, Inc., St. Louis, MO) and liquid nitrogen as the coolant. Samples were flooded with 2 ml/dish of buffer H containing 10% FCS and incubated at 37°C. The dishes were placed on ice and incubated an additional 15 min at 37°C. The remaining coverslips were processed to remove coated pits by washing each coverslip four times (3 ml/wash) with buffer D, incubating in buffer D for 5 min followed by five washes with buffer E, all at 4°C. Coverslips were either fixed with ice-cold 3% formaldehyde in buffer C or incubated for the indicated time with coat proteins (100 μl/coverslip at the indicated concentration) at 4°C. Assembly was terminated by removing the coat proteins and washing each coverslip four times with buffer F (3 ml/wash, 4°C) and fixing with ice-cold 3% formaldehyde in buffer C. After fixation, coverslips were washed twice with buffer F and once with buffer G at room temperature. Each coverslip was incubated for 30 min at 37°C in Dulbecco's PBS containing 1% BSA. Each coverslip was incubated with 60 μl of monoclonal anti-clathrin IgG (1 μg/ml of X-22 [4], in Dulbecco's PBS containing 0.1% BSA) for 30 min at 37°C, washed four times in buffer F (5 min/wash, room temperature) and then incubated with 60 μl of anti–mouse IgG coupled to fluorescein isothiocyanate (50 μg/ml) in Dulbecco's PBS containing 0.1% BSA for 30 min at 37°C. The wash procedure was repeated and the coverslips were incubated for 10 min at 37°C with 60 μl of wheat germ agglutinin (1 μg/ml in Dulbecco's PBS) coupled to rhodamine tetramethylisothiocyanate and washed as above. All antibodies and WGA were centrifuged for 3 min at 11,000 g before use.

Rapid-Freeze, Deep-Etch Assembly Assay

Membranes were assembled and assembled as described earlier (Indirect Immunofluorescence Assay) except that the fixative was either 1% paraformaldehyde plus 1% glutaraldehyde or 4% glutaraldehyde in buffer C at 4°C. Each coverslip was cut into 3-4-mm squares and rapidly frozen by the method of Heuser (11) using a Cryopress freezing device (Med Vac, Inc., St. Louis, MO) and liquid nitrogen as the coolant. Samples were etched and coated with platinum and carbon using a freeze fracture unit (model 301; Balzers, Hudson, NH) with a rotating stage set at 136 rpm. Samples were coated with ~9 Å of platinum using an electron gun at an angle of 15°. The replica was then recombined with ~120 Å of carbon using an electron gun at an angle of either 90° or 50°. Replicas were floated free of the coverslips in 30% hydrofluoric acid and digested with Clorox for 1 min before being mounted on formvar-coated grids.

Other Procedures

Protein determinations were determined by the method of Lowry et al. (21) using BSA as a standard.

Results

Coat Proteins Assemble into Coated Pits

Membranes that support coated pit assembly were prepared by the method of Moore et al. (23) with the modification that the substratum was coated with both poly-L-lysine and human fibronectin. Membranes prepared on this substratum were covered with numerous coated pits that were revealed after indirect immunofluorescence staining using a monoclonal antibody to the clathrin heavy chain (Fig. 2, A and B). The coated pit staining pattern was markedly reduced, however, after the membranes were briefly treated with a high pH buffer (Fig. 2, C and D), conditions that are known to remove coat proteins from isolated coated vesicles (37). The membranes were still present on the substratum after this treatment because they stained with rhodamine-labeled wheat germ agglutinin (Fig. 2 D).
Coat proteins isolated by extracting purified coated vesicles with 0.5 M Tris–HCl will spontaneously reassemble into empty cages when the Tris concentration is reduced to 20 mM (44). Therefore, to assemble coated pits, the high pH–treated membranes were incubated with Tris-extracted coat proteins that had been diluted with buffer E to lower the Tris concentration immediately before addition to the membranes. After a 30-min incubation at 4°C with 20 μg/ml of coat proteins, the membranes regained a normal anti-clathrin IgG immunofluorescence staining pattern (Fig. 2, E and F). The frequency of punctate dots as well as the size of the dots were indistinguishable from freshly prepared, untreated membranes (compare Fig. 2 A with 2 E).

To document that the immunofluorescence staining pattern reflected the formation of coated pits, membranes from the same experiment were visualized by rapid-freeze, deep-etch, electron microscopy. As seen in Fig. 3, untreated membranes had numerous clathrin lattices distributed on the inner surface of the membrane (Fig. 3 A). The majority of these lattices had a planar configuration. The membranes that had been treated with a high pH buffer had very few organized clathrin lattices (Fig. 3 B). After the incubation of pH-treated membranes with coat proteins (Fig. 3 C), normal appearing lattices were present; their frequency, size, and degree of curvature was similar to that observed on untreated membranes (compare Fig. 3 A with 3 C).

The immunofluorescence and electron microscopic experiments established that coat proteins, which are ordinarily competent to assemble into empty cages, will also associate with the membrane to form coated pits. We did not find any empty cages associated with the membrane or the surrounding substratum, which gave us confidence that the majority of the membrane-associated clathrin was in the form of coated pits. On the basis of these observations, we used an indirect 125I-streptavidin binding assay to quantify coated pit assembly on these membranes.

The dependence of coated pit assembly on the concentration of coat proteins is shown in Fig. 4. As found previously (23), the high pH treatment removed 70–80% of the clathrin from the membranes (Fig. 4). However, when the membranes were incubated with increasing amounts of coat protein, there was an increase in the amount of clathrin that became associated with membrane, reaching a maximum at 20 μg/ml. At this concentration, the amount of clathrin on the membrane was approximately equal to the amount that was on the membrane before the pH treatment. Increasing the concentration of coat protein to 20 μg/ml did not cause an increase in clathrin binding to the membrane.

In vivo, clathrin coated pit assembly occurs with a half-time of 5 min (19). To determine if the formation of coated pits from coat proteins was this rapid, we measured the time course of assembly at 4°C (Fig. 5). With time at 4°C, there was a progressive increase in the amount of clathrin associated with the membrane, reaching equilibrium after 30 min. In this experiment, at equilibrium somewhat more clathrin was bound to the membranes than was present before the membranes were subjected to the high pH treatment (Fig. 5).

Ionic Requirements for Assembly

The standard assembly buffer contained 4.1 mM magnesium acetate. We measured the effects of divalent cations on assembly by either making no additions or by adding CaCl2, EDTA, or EGTA to the assembly buffer. As seen in Table I, without any additions, the amount of clathrin that bound to the membrane was nearly equal (98%) to the amount originally on the membranes. However, the addition of either
Figure 4. Dependence of coated pit formation on the concentration of coat protein. Membranes were attached to the bottom of individual wells of a 96-well plate and treated to remove endogenous clathrin as described. Wells that contained membranes (∗) and wells that did not contain membranes (●) were incubated with the indicated amount of coat protein for 30 min at 4°C and assayed for the presence of clathrin as described. The amount of clathrin on untreated membranes (●) and the amount of clathrin remaining on membranes after high pH treatment (○) are indicated on the ordinate. All values are the average of triplicate wells.

Ca++, EDTA, or EGTA slightly inhibited the amount of clathrin that bound to the membrane (84, 86, and 71%, respectively).

Ionic strength and pH are known to influence both coated vesicle and clathrin cage assembly in vitro (15, 24, 25, 37, 39, 44). Therefore we measured the effects of these two conditions on coated pit assembly.

The standard assembly reaction contained 83.2 mM K+. Fig. 6 shows the results of an experiment where assembly was measured at different K+ concentrations ranging from 15 to 180 mM. Very little assembly was seen at the highest K+ concentration. As the concentration was lowered, however, there was a progressive increase in the amount of clathrin that bound to the membrane. The amount that bound dramatically increased when the K+ concentration was lowered <80 mM. We obtained similar results when we used Na+ as the cation (data not shown).

pH also affected the amount of clathrin that bound to membranes (Fig. 7). When we varied the pH of the assembly reaction from pH 8.2 to pH 6.1, there was a progressive increase in the amount of membrane bound clathrin. The amount of clathrin associated with the membrane at pH 7.2 (the pH of the standard assembly reaction) was equal to the amount initially on the membrane; however, below pH 6.8 there was a dramatic increase in the amount of membrane associated clathrin.

Figure 3. Carbon-platinum replicas of isolated membranes attached to poly-L-lysine/fibronectin-coated coverslips that had been fixed before treatment (A), treated to remove coated pits (B), or treated and then incubated with coat proteins (20 μg/ml) for 30 min at 4°C (C). These membranes were prepared in the same experiments as shown in Fig. 2. At the end of the treatment, the membranes were fixed with 1% formaldehyde plus 1% glutaraldehyde and processed by the rapid-freeze, deep-etch procedure. Bars, 0.2 μm.
The rise in membrane-bound clathrin at low pH and low ionic strength could be due to a nonspecific association of clathrin with the membranes. Therefore, we prepared rapid-freeze, deep-etched replicas of membranes that had been incubated with coat proteins at normal pH and high ionic strength (Fig. 8A), normal ionic strength and low pH (Fig. 8B), and normal pH and low ionic strength (Fig. 8C). Membranes incubated with the low pH buffer had few normal-sized lattices; instead, the membrane was decorated with numerous, small clathrin cages (Fig. 8B). On the other hand, membranes incubated with low K⁺ buffer had normal-appearing coated pits with only an occasional clathrin cage evident (Fig. 8C). Although the coated pits tended to be larger, they did not appear to be large enough to account for the substantial increase in bound clathrin detected by the radioimmunoassay (Fig. 6).

**Substructure of the Clathrin Lattice**

Computer enhanced images of isolated coated vesicles embedded in vitreous ice show that the assembly protein com-

![Figure 5](image)

*Figure 5.* Time course of coated pit assembly. Membranes were attached to the bottom of individual wells of a 96-well plate and treated to remove endogenous clathrin as described. Coat proteins (20 μg/ml) were added to wells containing membranes (●) or to wells that did not contain membranes (○) and incubated for the indicated times at 4°C. At the end of each incubation, the wells were assayed for the presence of clathrin as described. The amount of clathrin on untreated membranes (●) and the amount of clathrin remaining bound to membranes after high pH treatment (○) are indicated on the ordinate. All values are the average of triplicate wells.

![Figure 6](image)

*Figure 6.* Effect of K⁺ on coated pit assembly. Membranes were attached to the bottom of individual wells of a 96-well plate and treated to remove endogenous clathrin as described. Wells with attached membranes (●) or with no membranes (○) were incubated with 20 μg/ml of coat proteins in buffer E containing the indicated concentration of K⁺ for 30 min at 4°C. Wells were assayed for clathrin as described. The amount of clathrin on untreated membranes (●) and the amount of clathrin remaining bound to membranes after high pH treatment (○) are indicated on the ordinate. All values are the average of triplicate wells.

![Figure 7](image)

*Figure 7.* Effect of pH on coated pit assembly. Membranes were attached to the bottom of individual wells of a 96-well plate and treated to remove endogenous clathrin as described. Wells with attached membranes (●) or with no membranes (○) were incubated with 20 μg/ml of coat proteins in buffer E at the indicated pH for 30 min at 4°C. Mes buffer was substituted for Hepes buffer below pH 6.8. Wells were assayed for clathrin as described. The amount of clathrin on untreated membranes (●) and the amount of clathrin remaining bound to membranes after high pH treatment (○) are indicated on the ordinate. All values are the average of triplicate wells.

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**Table I. The Effect of Divalent Cations on Assembly**

| Conditions                  | 125I-Streptavidin-bound (cpm/well × 10⁴) | Percent of control |
|----------------------------|------------------------------------------|-------------------|
| Untreated                  | 19,010                                   | 100               |
| High pH-treated            | 7,123                                    | 37                |
| Coat proteins              | 18,611                                    | 98                |
| Coat proteins + 5.0 mM Ca²⁺| 15,988                                    | 84                |
| Coat proteins + 5.0 mM EDTA| 16,299                                    | 86                |
| Coat proteins + 5.0 mM EGTA| 13,427                                    | 71                |

Membranes were attached to the bottom of individual wells of a 96-well plate. Membranes were either not treated, treated to remove endogenous coated pits, or treated to remove coated pits and then incubated with 20 μg/ml of coat protein in buffer E with the indicated additions for 30 min at 4°C. Wells were assayed for clathrin as described. Each value is the average of triplicate wells after subtraction of nonspecific binding to wells that did not contain membranes.
the presence of numerous particles. The clusters of particles were the size (Fig. 10, A and B) and shape (C and D) of coated pits. Moreover, the clusters were easily recognized when there were not any partial lattices present (Fig. 10, B and D). Invaginated areas afforded an edge-on-view of the particles covering the membrane (Fig. 10 D) where they appeared like stalks or shelves that projected from the membrane.

Discussion

Previously we reported that isolated membranes attached to a substratum will support coated pit formation when incubated with cytoplasm from donor cells (23). The current results indicate that isolated coat proteins will induce coated pit formation on a similar preparation of membranes. This assembly reaction occurs best at physiologic pH and ionic concentration and is maximal at a protein concentration of 20 µg/ml. Therefore, cytoplasmic factors are not required for coated pits to form under these conditions nor does there appear to be a need for the addition of cofactors such as ATP. The coated pits that assemble are indistinguishable from the native coated pits.

Just as was found when cytoplasm was used as a source of coat proteins, these membranes can only assemble a limited number of coated pits. There are several reasons to believe that this is due to the presence of specific assembly sites on the membrane. First, with the purified coat protein preparation we can estimate that the clathrin has a high affinity for the membrane. Second, coated pit formation was markedly impaired on membranes that had been washed with 0.5 M NaCl after the high pH treatment (data not shown), which implies that essential peripheral membrane proteins are required to build the lattice and that lattices do not form indiscriminately on the membrane. Third, we observed that each lattice was built upon a specialized region of membrane that was composed of numerous particles aggregated together into patches the size and shape of a coated pit.

The formal proof that the patches of particles are assembly sites must await the immunological localization of resident proteins. We feel, however, that the residual lattices observed associated with a subset of these patches in high pH-treated membrane preparations are a good endogenous marker because they were only found associated with membrane that had these morphological features. In addition, patches like these were not observed on membranes that had fully formed lattices. The recent findings of Heuser and Keen (13) suggest that an assembly site should have a particulate substructure if it contains assembly proteins. These proteins exist as a dimeric, bilaterally symmetrical particle that can be visualized.

Figure 8. Rapid freeze, deep-etch view of membranes incubated with coat proteins at normal pH and ionic strength (A), low pH (B) or low K⁺ (C) buffer conditions. Membranes were prepared as in Fig. 3 and treated to remove coated pits. Each membrane then was incubated with 20 µg/ml of coat proteins for 30 min at 4°C in either buffer E (A), buffer E adjusted to pH 6.0 (B), or buffer E with 15 mM K⁺ (C). Fixation and replica preparation were as described. Bars, 0.3 µm.
in deep-etched images on the surface of coated vesicles stripped of clathrin. The high pH treatment that we used should have left the assembly protein complex on the membrane (reference 37, data not shown).

The molecules within these sites that are responsible for initiating the assembly reaction remain to be identified. Since the high pH treatment does not remove all of the clathrin (only 70–80%), there is the possibility that residual clathrin at each assembly site nucleates assembly. The identification of the initiator molecule(s) will be possible once conditions are found for assembling coated pits using purified protein components.

In addition to initiating lattice assembly, our results indicate that the assembly sites also control the size of the lattice. Unlike coated vesicle assembly where the formation of the coat is limited by the closure of the polygonal lattice, the margins of the planar coated pit lattice have the potential for supporting the polymerization of an unlimited number of triskelion subunits. We were unable to increase the size of these lattices, as judged by RIA, even when the coat protein concentration was increased twofold above that required for maximal assembly. Therefore, the existing lattice appears not to act like a template and induce polygon formation.

Acidification of the cytoplasm paralyzes the conversion of coated pits to coated vesicles (12, 35). K⁺ depletion of the cytoplasm appears to inhibit coated pit formation (19, 20). These observations stimulated us to measure the effect of pH and K⁺ concentration on coated pit assembly in vitro. Lowering the concentration of K⁺ did not inhibit coated pit assembly. The large amounts of clathrin on these membranes detected by the RIA most likely is not in a lattice and corresponds to bound triskelions that cannot be visualized by the rapid-freeze, deep-etch technique. Therefore, factors other than low ionic strength must account for the inhibition of coated pit assembly within cells (20). By contrast, acidic pH clearly inhibited coated pit formation without affecting cage assembly. We cannot conclude, however, that pit formation is specifically inhibited at low pH because the rapid induction of cage assembly most likely removed the free triskelions that would be needed to assemble the coated pits. Since acidification of cytoplasm inhibits the conversion of coated pits to coated vesicles (12, 35), there is not any way to determine if low pH directly prevents pit assembly in situ. Nevertheless, these in vitro experiments demonstrate that conditions that favor cage assembly will inhibit coated pit formation and that clathrin cages adhere to membranes (19).
The results of these studies focus attention on the assembly site as the fundamental controlling unit of the clathrin coated pit endocytic cycle. In the absence of these sites, coated pits will not form in vitro; we never saw planar lattices that were not on the membrane. This implies that for a cell to replenish surface coated pits at rates up to 3,000/min (22), the formation of assembly sites must precede the polymerization of the lattice. Although some of the molecules that make up the assembly site may belong to the assembly protein complex (1, 16, 32, 41), there must also be integral membrane proteins.

Figure 10. Four rapid-freeze, deep-etch, stereo views of coated pit assembly sites. Membranes were prepared, treated with buffer D, and processed as described in Fig. 3. Stereo images are ±10°. (A) Arrows, assembly sites with remnants of a clathrin lattice. (B) Arrows, assembly sites composed of clustered particles that protrude above the plane of the membrane. Bars, 0.2 μm.
that control the binding of the assembly proteins at these sites (41). These membrane proteins may correspond to the receptors that use the coated pit pathway for carrying molecules into cells (10), or they may be a unique, coated pit “receptor” that controls the organization of the assembly site. The ability to assemble coated pits from purified subunits should allow the dissection of the various steps in the assembly process.

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