

# Reconstitution of Clathrin-coated Pit Budding from Plasma Membranes

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**Abstract.** Receptor-mediated endocytosis begins with the binding of ligand to receptors in clathrin-coated pits followed by the budding of the pits away from the membrane. We have successfully reconstituted this sequence *in vitro*. Highly purified plasma membranes labeled with gold were obtained by incubating cells in the presence of anti-LDL receptor IgG-gold at 4°C, attaching the labeled cells to a poly-L-lysine-coated substratum at 4°C and then gently sonicating them to remove everything except the adherent membrane. Initially the gold label was clustered over flat, clathrin-coated pits. After these membranes were warmed to 37°C for 5–10 min in the presence of buffer that con-

tained cytosol extract, Ca<sup>2+</sup>, and ATP, the coated pits rounded up and budded from the membrane, leaving behind a membrane that was devoid of LDL gold. Simultaneous with the loss of the ligand, the clathrin triskelion and the AP-2 subunits of the coated pit were also lost. These results suggest that the budding of a coated pit to form a coated vesicle occurs in two steps: (a) the spontaneous rounding of the flat lattice into a highly invaginated coated pit at 37°C; (b) the ATP, 150 μM Ca<sup>2+</sup>, and cytosolic factor(s) dependent fusion of the adjoining membrane segments at the neck of the invaginated pit.

**W**ITHIN a cell, coated vesicles form from coated pits (Anderson et al., 1977; van Deurs et al., 1989). Besides transforming into endocytic vesicles, coated pits also control the clustering of receptors that ligands use to enter cells by receptor-mediated endocytosis (Anderson, 1991). Selective mutagenesis of these receptors has revealed that their cytoplasmic domains contain an amino acid sequence that is necessary for efficient internalization (Chen et al., 1990; Collawn et al., 1990; Ktistakis et al., 1990). However, the identification of the molecular element(s) within coated pits that recognize these cytoplasmic domains remains to be made.

Isolated coated vesicles have been useful for analyzing the structure, protein composition, and *in vitro* assembly of the clathrin lattice (Keen, 1990). The lattice is composed of two subunits: (a) the clathrin triskelion, which is constructed from three 180-kD and three 30–35 kD proteins and forms the polygonal mesh work and (b) the AP (Assembly Protein [Keen, 1990] or Adaptor Protein [Pearse, 1988])<sup>1</sup> subunit, which is created from two sets of three proteins (100–110, 50, and 16–18 kD) and appears to link the lattice to the plasma membrane (Vigers et al., 1986). There are two different forms of the AP subunit: AP-1 and AP-2. These two isoforms differ in protein composition and location within the cell.

Whereas AP-2 is thought to reside exclusively in coated pits at the cell surface (Robinson, 1987), AP-1 is preferentially associated with the Golgi region of the cell (Ahle et al., 1988).

Most of our knowledge about the function of coated pits is based on a morphological assessment of lattice behavior (Anderson et al., 1977; Heuser, 1980; van Deurs et al., 1989). The clathrin coat seems to behave like a molecular motor (Anderson, 1991) that shapes the membrane into a vesicle. Coincidentally, the polygonal lattice undergoes a rearrangement that results in an increase in the ratio of pentagons to hexagons (Heuser, 1980). This is clearly a different kind of motor, however, than the linear motors that move organelles along microtubules and microfilaments (Vale, 1987). After the deeply invaginated pit forms, it pinches off from the membrane. This process may involve the physical melding together of the membrane bilayers due to forces generated by the lattice, much like a clam closing its shell. Alternatively, there might be a critical protein(s) that mediate(s) membrane fusion and consequently allows the incipient vesicle to detach from the membrane.

## An *In Vitro* Model of Endocytosis

The conversion of coated pits into coated vesicles is a membrane budding reaction that most likely shares properties with vesicle forming machinery in other membranes. Since the introduction of the *in vitro* inter-Golgi transport system (Balch et al., 1984; Gruenberg and Howell, 1989), several semi-pure membrane preparations have been developed that

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1. *Abbreviations used in this paper:* AP, assembly protein or adaptor protein; LPPS, lipoprotein-deficient serum; NEM, *N*-ethylmaleimide.

mimic: endosome-endosome fusion (Davey et al., 1985; Gruenberg and Howell, 1986); ER to Golgi transport (Beckers et al., 1987); Golgi recycling (Goda and Pfeffer, 1988); and exocytosis (Tooze and Huttner, 1990). All of these transport events require the budding of vesicles from a donor membrane. There now is evidence that membrane budding may be controlled by more than one type of membrane coat complex (Orci et al., 1989); however, virtually nothing is known about the mechanism of action of these special coating materials.

One way to gain access to coated pits for functional analysis is to use broken cell preparations. Smythe et al. (1989) studied the internalization of transferrin by coated pits in permeabilized A431 cells. They found that ~12% of the pre-bound transferrin could be delivered to endosomes in these broken cells and that this delivery depended on ATP and a cytosolic factor. By contrast, Podbilewicz and Mellman (Podbilewicz and Mellman, 1990), using broken MDCK cells, were unable to demonstrate an ATP dependence on the internalization of transferrin, although the recycling of this receptor was dependent on an energy source. These discrepancies may in part be due to differences in the type of cell preparations used by each investigator and to the assay employed to measure endocytosis (Schmid and Carter, 1990).

We have focused on developing a pure membrane system that mimics coated pit budding. Moore et al. (1987) introduced a method for preparing highly purified plasma membranes that are rich in coated pits. These membranes have been extremely useful for elucidating the mechanism of coated pit assembly. When the endogenous clathrin is removed, the membranes are able to assemble new coated pits using either purified coat proteins isolated from coated vesicles (Mahaffey et al., 1990) or cytosol (Moore et al., 1987) as a source of clathrin. If both the endogenous clathrin and the AP complex are removed, then a high affinity binding site for purified AP-2 is unmasked on the inside surface of the plasma membrane (Mahaffey et al., 1990).

Under the right circumstances the coated pits in these same membrane preparations should also form coated vesicles. In preliminary trials to find the proper conditions (Moore et al., 1987), we found that the flat coated pits on these membranes invaginate at 37°C, without the addition of either ATP or cytosol. Concomitant with the shape change the clathrin disappeared from the membrane. Subsequent studies, however, showed that clathrin loss was not accompanied by a loss of the AP-2 subunit from the membrane, which suggested that in these membranes the rounding up of the pit caused a destabilization of the lattice leading to the disassembly of the coated pit without productive vesicle formation.

We now report on a modification of the original membrane preparation procedure that allows coated pits, which are pre-labeled with anti-LDL receptor IgG-gold, to actually invaginate and bud from the membrane. Whereas invagination occurs spontaneously in a defined buffer, the detachment of the incipient vesicle requires ATP, high  $Ca^{2+}$  and cytosolic factor(s). The reconstitution of coated pit budding from purified membranes represents a new methodological advance in defining the molecular requirements for the sequestration of receptors and the budding of vesicles during endomembrane traffic in the cell.

## Materials and Methods

### Materials

Coverslips (18 and 22 mm square, and 18 mm round) were obtained from Fisher Scientific (Pittsburgh, PA).  $^{125}I$ -labeled streptavidin (IM-236) at a specific activity of 20–40  $\mu Ci/\mu g$  was purchased from Amersham Corp. (Arlington Heights, IL). The following tissue culture reagents were obtained from Gibco Laboratories (Grand Island, NY): DMEM (320-1885), Eagle's minimal essential medium (330-1435), FCS (200-6140), penicillin-streptomycin (600-5145), Dulbecco's PBS (310-4190) and trypsin-EDTA (610-5300). Human lipoprotein-deficient serum (LPPS,  $d > 1.215$  g/ml) was prepared by ultracentrifugation of human plasma (Goldstein et al., 1983). The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO): poly-L-lysine (P-1524), Hepes (H-3375), 2-(*N*-morpholino) ethanesulfonic acid (MES (M-8250), Trizma base (Tris[hydroxymethyl]amino-methane [T-1503], Tris(hydroxymethyl)methylaminopropane sulfonic acid (TAPS; T-5130), PMSF (P-7626), BSA fraction V (A-7906), crystalline grade BSA (C-BSA; A-7638), *N*-ethylmaleimide (E-3876), ATP (A-5394), and GTP (G-5756). AMP-PNP (102547), ATP- $\gamma$ -S (102342), and GTP- $\gamma$ -S (220647) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Sepharose-S (17-0511-01) was purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Leupeptin (62070), DTT (43819), and paraformaldehyde (76240) were obtained from Fluka Chemical Corp. (Ronkonkoma, NY). Glutaraldehyde (16320) was from Electron Microscopy Sciences (Ft. Washington, PA). Normal horse serum (S2000), and biotinylated horse anti-mouse IgG (BA-2000), were purchased from Vector Laboratories, Inc. (Burlingame, CA). Rabbit anti-mouse IgG conjugated to FITC (61-6011) was from Zymed Laboratories, Inc. (South San Francisco, CA). The mouse mAbs used in these experiments include the following: X-22, which recognizes the clathrin heavy chain (Brodsky, 1988); AP6, which reacts with the  $\alpha$ -100-kD (a and c) polypeptides of the AP-2 (also designated HA II [Keen, 1990]) class of clathrin assembly proteins (Chin et al., 1989); and 4A4, which recognizes the COOH terminus of the LDL receptor (van Driel et al., 1987). A polyclonal anti-LDL receptor IgG was prepared as previously described (Anderson, 1986).

### Buffers and Media

Medium A was DMEM containing 10% FCS, 20 mM Hepes, 100 U/ml penicillin, and 100  $\mu g/ml$  streptomycin. Medium B was Eagles MEM (EMEM) containing 20 mM Hepes, pH 7.4. Buffer A was 50 mM Hepes-NaOH pH 7.4, 100 mM NaCl. Buffer B was 25 mM Hepes-KOH, pH 7.0, 25 mM KCl, 2.5 mM MgAcetate, 0.2 mM DTT. Buffer C was 2.3 mM  $NaH_2PO_4 \cdot H_2O$ , 7.7 mM  $Na_2HPO_4 \cdot 7H_2O$ , 150 mM NaCl, 2 mM  $MgCl_2$ , pH 7.4. Buffer D was 25 mM Hepes-KOH, pH 6.8, 1 mM DTT.

### Cell Culture

The cells used for these experiments were SV-40-transformed human fibroblasts (designated SV589 [Moore and Anderson, 1989]), normal human fibroblasts or internalization defective human fibroblasts. Whereas normal and internalization-defective fibroblasts were cultured as previously described (Goldstein et al., 1983), SV589 cells were grown in monolayers and set up for experiments according to a standard format. On day 0,  $2.0 \cdot 10^5$  cells were seeded into each petri dish (100 mm diameter) containing medium A. Fresh medium of the same composition was added on day 2. For experiments requiring the induction of LDL receptors, on day 1 of growth each monolayer received medium A that contained 10% LPPS instead of FCS. On day 3 of cell growth, the cells were harvested for use in the experiments.

### Preparation of Membranes

Transformed human fibroblasts (SV589 cells) were released from the culture dish by incubating the cells in the presence of DPBS ( $Ca^{2+}$ ,  $Mg^{2+}$  free) containing 0.02% (wt/vol) EDTA for 10 min at 37°C. The cell suspension was removed and an equal volume of ice-cold medium B (Eagle's MEM, 20 mM Hepes) was added before the cells were washed three times by repeated, alternate resuspension and centrifugation in medium B at 4°C. Cells were finally resuspended in ice-cold medium B to a concentration of  $1.75 \cdot 10^6$  cells/ml. 400  $\mu l$  of this suspension was added to the center of a poly-L-lysine-coated (1 mg/ml in distilled water), 18-mm round coverslip that was affixed to the bottom of an individual well of a 12-well plate (Costar Corp., Cambridge, MA) with double-stick tape. Care was taken to be sure

that the coverslip was in the center of the well. The plates containing the coverslips were chilled to 4°C before the addition of 400  $\mu$ l of cells. After the cells were added, the plates were incubated for 20 min on ice and then 600  $\mu$ l of ice-cold medium B was added to each well. The plates were centrifuged for 3 min at 3,800 rpm (1,800 g) in a tabletop centrifuge (model 4B, equipped with microplate rotor #244; International Equipment Co., Needham Heights, MA) that had been placed in a 4°C room for several hours before centrifugation. After centrifugation, the plates were placed on ice, the medium aspirated, and 2 ml of ice-cold medium B containing 2% BSA was added to each well. The plates were incubated on ice, at 4°C, for 30 min to 1 h. The wells of each plate were rinsed twice with 3 ml ice-cold buffer A (50 mM Hepes, pH 7.4, 100 mM NaCl) and then once with a 3-ml ice-cold buffer B (25 mM Hepes-KOH, pH 7.0, 25 mM KCl, 2.5 mM MgAcetate, 0.2 mM DTT). Each well was aspirated, and 5 ml ice cold buffer B was added. The plate was removed from ice, placed on a sonicator platform and each well was sonicated for one second at power setting of 2.5 using a W-380 ultrasonic processor (Heat Systems Inc., Farmingdale, NY) equipped with a 1/2-in. tapped horn. The sonicator horn was tuned according to the manufacturer's instructions before each experiment. The head of the sonicator was mounted on a drill press and the platform was adjusted before sonication so that when the sonicator probe was lowered into the well, the tip measured 12 mm above the platform. The drill press allowed us to position the horn at the same height in each well. After all of the coverslips were sonicated, they were rinsed twice in 2 ml of ice-cold buffer B and either fixed immediately or subjected to the indicated treatments. For incubations, the wells were aspirated and 400  $\mu$ l of ice-cold buffer B containing the indicated additions was added. The plate was then floated on a 37°C water bath for the indicated time. The plate was then placed on ice, rinsed three times with 2 ml ice-cold buffer B and fixed. For radioimmunoassay, the coverslips were fixed in 3% paraformaldehyde in buffer B (without DTT) for 15 min on ice, rinsed twice in buffer C, and held in buffer C at 4°C until assayed.

### Preparation of Cytosol Extract

All operations were performed at 4°C. One bovine brain maintained on ice in Dulbecco's PBS (Ca<sup>2+</sup>, Mg<sup>2+</sup> free) was rinsed several times in this buffer and then several times in cold buffer D (25 mM Hepes-KOH, pH 6.8, 1 mM DTT). 150 g of brain tissue was then added to 150 ml buffer D containing 1 mM EDTA, 1 mM PMSF, 10  $\mu$ g/ml leupeptin, and 10<sup>-6</sup> M pepstatin and homogenized in a Waring blender (New Hartford, CT) for three, 5-s bursts. The homogenate was centrifuged at 18,000 rpm in an SS-34 rotor (Sorvall Div., Newton, CT) (40,000 g), for 30 min in an RC-5 centrifuge (Sorvall Div.). The supernatant fluids were collected and centrifuged for 1 h in a Ti45 rotor (L8-70M ultracentrifuge; Beckman Instrument Co., Fullerton, CA) at 35,000 rpm (100,000 g). The supernatant fluids were collected and 100 ml of this was applied to a 20 ml Sepharose-S cation exchange column (2.5  $\times$  4 cm) that had been equilibrated with buffer D containing 1 mM EDTA. The sample was loaded by gravity flow at a flow rate of  $\sim$ 40 ml/h. The column was washed with 40 ml buffer D, and then eluted with 26 ml of buffer D containing 100 mM KCl. 2-ml fractions were collected during elution and the protein monitored by OD<sub>280</sub>. All of the fractions that contained protein were pooled (usually  $\sim$ 22 ml). The column was then washed with buffer D containing 1.0 M KCl. In the initial characterization of this column, separate peaks eluting in 100, 200, and 500 mM KCl as well as the flow through and wash fractions were assayed for their ability to stimulate clathrin loss from sonicated membranes and all of the activity was found in the pooled 100 mM eluate fraction. These pooled fractions were dialyzed overnight against a total of 8 liters of buffer C containing 1 mM DTT. After dialysis, the sample was centrifuged for 1 h at 35,000 rpm in a Ti50 rotor (Beckman Instruments) to remove any insoluble material, divided into aliquots, and frozen at -80°C. When this extract was used for experiments, it was thawed quickly at 37°C and then placed on ice. Dilution of the extract and the addition of any other components (ATP, CaCl<sub>2</sub>, et cetera) was done within an hour of use.

### Indirect <sup>125</sup>I-Streptavidin Binding Assay

Coverslips were removed from the double-stick tape and transferred to individual wells of a new 12-well plate that contained buffer C. The buffer was removed from the well by aspiration and 2 ml of blocking solution (buffer C containing 100 mM glycine and 2% C-BSA) was added to each well. The plates were incubated for 30 min at 37°C and rinsed twice in 2 ml of buffer C. The wells were aspirated and 0.5 ml of the first antibody diluted in antibody buffer (buffer C containing 1% C-BSA and 2% horse serum)

was added. Both X-22 and AP.6 antibodies were used at a concentration of 1  $\mu$ g/ml. After a 1-h incubation at 37°C, the wells were washed five times in 2 ml buffer C and allowed to sit in the third rinse for 5 min. 0.5 ml of antibody buffer containing biotinylated horse anti-mouse IgG (1  $\mu$ g/ml) was added, incubated for 1 h at 37°C, and then the wells were washed as above. The wells were aspirated and 0.5 ml of <sup>125</sup>I-streptavidin (1  $\mu$ ci/ml in buffer C containing 1% C-BSA) was added. The wells were incubated for 20 min at room temperature, washed as above, and aspirated. 0.5 ml of 1 N NaOH was added to each well, incubated for 15 min, and then drawn off and placed in a 12  $\times$  75 mm tube. The well was rinsed with 0.5 ml of 1 N NaOH and this was pooled with the first eluate. The tubes were counted for 1 min in a gamma counter (Packard Instrument Co., Downers Grove, IL). Background radioactivity was routinely determined by measuring the radioactivity associated with identically treated coverslips that did not receive any cells. When an irrelevant mAb was substituted for the X-22 or the AP.6 antibody, only background radioactivity was observed.

### Immunofluorescence

Immunofluorescence localization of X-22 was carried out on membranes attached to coverslips as described above using the method of Moore et al. (1987).

### Electron Microscopy

**Labeling the LDL Receptor.** The LDL receptors on the plasma membrane of cells that were grown in the absence of lipoproteins were labeled by incubating cells in suspension (3.5  $\times$  10<sup>6</sup>/ml in medium B) with either LDL-gold (15 nm diameter, 15  $\mu$ g/ml) or anti-LDL receptor IgG-gold (15 nm diameter, 20  $\mu$ g/ml) for 45 min at 4°C. The labeled cells were then washed two times in medium B by centrifugation and repeated resuspension before being finally resuspended in 10 vol of ice-cold medium B to a concentration of 1.75  $\times$  10<sup>6</sup> cells/ml and kept on ice.

**Membrane Preparation.** Formvar supported, nickel electron microscope grids were attached to 18-mm round coverslips by applying several droplets of nail polish at the edge of each grid. After air drying, each coverslip was coated with poly-L-lysine as described above. Each coverslip, which contained several grids, was attached to the bottom of a well in a 12-well plate with double-stick tape and the plate was placed on ice. 400  $\mu$ l of the labeled cell suspension was then added to each well and incubated for 20 min on ice before the addition of an additional 600  $\mu$ l of ice-cold medium B. The plates were then centrifuged for 3 min, 1,800 g at 4°C as described above to attach the cells. The supernatant fluid was aspirated and 2 ml of ice-cold medium B containing 2% fraction V BSA (Sigma Chemical Co.) was added to each well and incubated on ice for 1 h. The coverslips were rinsed twice with 2 ml of buffer A (50 mM Hepes, 100 mM NaCl, pH 7.4) and once with 2 ml of buffer B (25 mM Hepes, 25 mM KCl, 2.5 mM MgAcetate, 0.2 mM DTT, pH 7.0). Each well was sonicated as described above. After the indicated treatment all coverslips were rinsed three times with ice-cold buffer B, and fixed with 4% glutaraldehyde in buffer B for 15 min on ice and 15 min at room temperature. After fixation each coverslip was washed three times with buffer B at room temperature. The grids were removed from the coverslip with a fine forceps and fixed in 1% osmium tetroxide in buffer B (without DTT) for 10 min at room temperature, washed three times for 5 min each in buffer B (without DTT) at room temperature, incubated with 1% aqueous tannic acid for 10 min, washed twice for 5 min each in distilled water, incubated with 1% uranyl acetate for 10 min at room temperature and washed twice for 1 min each with distilled water before air drying. Each grid was then coated with carbon to reduce contamination in the electron microscope and viewed with a JEOL 100CX electron microscope.

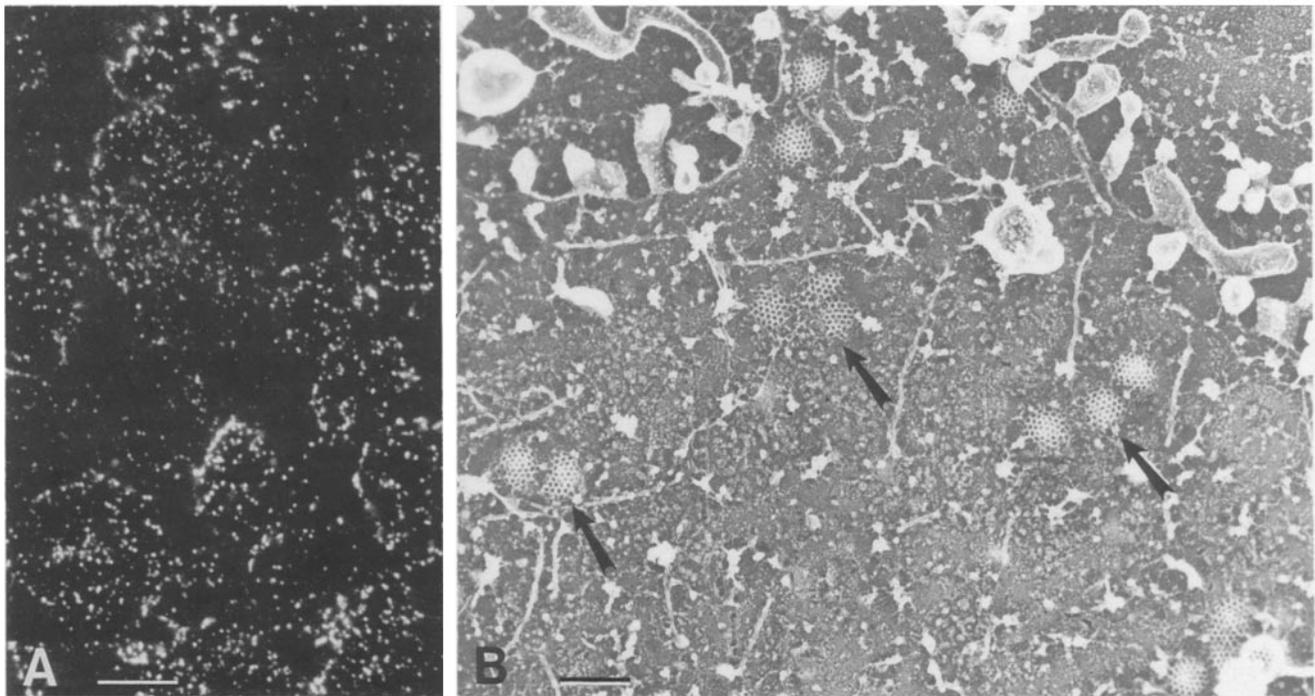
**Alternative Method for Preparing Labeled Membranes.** In some experiments cells were labeled with LDL-gold and membranes were prepared by the method of Sanan and Anderson (1991). The membranes were then washed and incubated as indicated.

**Rapid-Freeze Deep-Etch Preparations.** The rapid-freeze, deep-etch replicas of the inner membrane surface were prepared according to the method of Heuser (Heuser, 1980) as previously described (Moore et al., 1987).

## Results

### Purification of Plasma Membranes

Previously, coated pit membranes were prepared by first al-



**Figure 1.** Immunofluorescence (A) and rapid freeze, deep-etch replica (B) views of the inner surface of attached membranes. SV589 cells were released from the culture dish and attached to poly-L-lysine-coated coverslips by centrifugation at 4°C. After sonication to remove the cells, the attached membranes were fixed and processed either for indirect immunofluorescence microscopy (A) or electron microscopy (B) as described. (Arrows) Clathrin-coated pits. Bars: (A) 10  $\mu$ m; (B) 100 nm.

lowing human fibroblasts to adhere and spread on a poly-L-lysine-coated surface at 37°C and then gently sonicating the cells to remove everything except the adherent membrane (Moore et al., 1987). The goal of the current studies was to demonstrate endocytosis *in vitro*; therefore, the method was modified to allow labeling of the LDL receptor with gold probes before the membranes were isolated at 4°C. The strategy was to label the cells in suspension and then attach them to a poly-L-lysine-coated surface, all at 4°C, by centrifugation. Sonication and other manipulations were carried out as previously described (Moore et al., 1987). Since the cells never had an opportunity to interact with the substratum at 37°C, each isolated membrane is a sample of plasma membrane as it existed on the surface of the suspended cell at 4°C.

To verify the architecture of the membranes, we examined unlabeled preparations with both the light and the electron microscope. Indirect immunofluorescence staining with anti-clathrin IgG showed that these membranes had numerous coated pits scattered across the inner surface (Fig. 1 A). The rapid-freeze, deep-etch images confirmed that the lattices were intact and had a normal morphology (Fig. 1 B). The shape of the pits ranged from flat to slightly invaginated.

### Coated Pit Budding *In Vitro*

The most convincing way to determine if these isolated coated pits are capable of budding *in vitro* is to visualize the incorporation of a receptor bound ligand into a budding coated pit. Membranes were prepared at 4°C from cells that had been labeled in suspension with anti-LDL receptor IgG-gold. As seen in Fig. 2 A, numerous coated pits were present

that were labeled with IgG-gold and most of the lattices were not invaginated. The same labeling pattern was observed when LDL-gold was used as the probe; moreover, binding of this probe was blocked by excess LDL (Sanan and Anderson, 1991).

When these same membranes were warmed to 37°C in buffer alone, the flat lattices transformed into deeply invaginated pits (Fig. 2 B) and the gold remained tightly associated with the coated pit. The addition of ATP and Ca<sup>2+</sup> to the buffer gave the same results (Fig. 2 C). Sometimes a membranous stalk was visible (arrows, Fig. 2 B) connecting the pit to the plasma membrane, which suggests that under these conditions the pit is arrested at the pinching-off step. Exactly the same results were obtained when either Ca<sup>2+</sup> (Fig. 2 D) or ATP (Fig. 2 E) was omitted from buffer that contained 1 mg/ml of cytosol. Only when the membranes were incubated with the complete cocktail consisting of 1 mM ATP, 150  $\mu$ M Ca<sup>2+</sup> and 1 mg/ml of cytosol did both the anti-LDL receptor IgG-gold and the clathrin lattices disappear from the membrane (Fig. 2 F). These conditions routinely caused >80% of the IgG-gold clusters to disappear from the membrane.

Numerous studies have shown that in intact cells both clustered receptors and a functional clathrin lattice are required for receptor-mediated endocytosis (Anderson, 1991). Therefore, we subjected the membrane preparations to these same tests to show that loss of gold label and coated pits occurred by coated pit budding. First, membranes were labeled with LDL-gold conjugates and warmed to 37°C in pH 5.5 buffer, conditions that cause dissociation of LDL from its receptor and paralyze the clathrin lattice (Anderson, 1991). The lattices remained on the membrane along with the LDL-

gold (data not shown). Second, membranes labeled with LDL-gold at 4°C were prepared by the method of Sanan and Anderson (Sanan and Anderson, 1991) and washed with either neutral buffer (Fig. 3 C) or with pH 9.0 buffer (Fig. 3 A) to remove selectively the clathrin lattice (Mahaffey et al., 1990). When both sets of membranes were warmed to 37°C in a complete cytosol cocktail, the clathrin-stripped membranes retained the gold in clustered arrays (Fig. 3 C) but the unstripped membranes were devoid of gold (Fig. 3 A). Finally, gold-labeled membranes were prepared in an identical fashion from human fibroblasts that express the internalization-defective form of the LDL receptor (Fig. 3 B). When these membranes were washed in neutral buffer and warmed to 37°C, the gold remained on the membrane in a random distribution (circles, Fig. 3 B) but the clathrin coated pits disappeared. Just as internalization defective receptors fail to internalize LDL in intact cells (Goldstein et al., 1985), these receptors also failed to incorporate LDL-gold into budding-coated pits.

A more rapid method for monitoring coated pit budding used mAbs directed against either the heavy chain of clathrin or the  $\alpha$ -100-kD component of AP-2 to monitor the presence of coated pits. Fig. 2 (insets A-F) shows that by immunofluorescence the anti-clathrin IgG faithfully detects the presence or absence of clathrin on the membranes under the same conditions used for the EM experiments. Only when the membranes were incubated in a complete cytosol cocktail did the clathrin disappear from the membrane (compare inset F with insets A-E in Fig. 2).

We next used a radioimmuno assay to measure the presence of clathrin or AP-2 on the membrane (Mahaffey et al., 1990). When membranes from cells that had been labeled with LDL-gold were assayed to determine if the cytosol cocktail caused a loss of clathrin coats, there was an exact concordance between the disappearance of clathrin or AP-2 and the disappearance of LDL-gold (data not shown).

### Characterization of the Budding Reaction

The loss of both clathrin and AP-2 was dependent on the concentration of cytosol in the buffer (Fig. 4). With increasing amounts of cytosol protein there was a progressive increase in the loss of clathrin (Fig. 4, *clathrin*) and AP-2 (Fig. 4, *AP-2*) until the concentration reached  $\sim 1$  mg/ml. Above this concentration, no further loss was seen. At saturation, cytosol in the presence of 150  $\mu$ M  $Ca^{2+}$  typically caused a 50–70% decrease in the amount of both subunits on the membrane.

In our initial trials, a crude, unfractionated preparation of cytosol was used. After passing this material over a cation exchange column, the eluate had a  $\sim 17$ -fold increase in specific activity, suggesting that a specific factor(s) in the cytosol that binds to the cationic column is responsible for the activity. This material was used in all subsequent trials. If cytosol was replaced with either 1 mg/ml of albumin or 1 mg/ml of cytosol that had been heated to 100°C for 3 min, clathrin remained on the membrane. Finally, to rule out the possibility that protease activity in the cytosol was causing the clathrin loss, we added a variety of different protease inhibitors (0.5 mM PMSF, 1  $\mu$ M pepstatin, or 10  $\mu$ g/ml leupeptin) to the cytosol; however, none of these inhibitors affected the activity of the cytosol.

The disappearance of either LDL-gold or anti-LDL recep-

tor IgG-gold from the membrane should be accompanied by a loss of LDL receptors. Since the COOH terminus of the receptor tail is accessible in our membrane preparations, we used a tail-specific mAb to detect the receptor by radioimmuno assay. In eight different trials using two different mAbs there was on average a 27% (range 15.6 to 36.1%) loss of receptor under conditions that cause the loss of the anti-LDL receptor IgG-gold.

Receptor-mediated endocytosis is rapid (Goldstein et al., 1985). The half-time for uptake of LDL, for example, is just 3 min. Using clathrin loss as our measure, we found that coated pits disappeared from the purified membranes with similar, rapid kinetics (Fig. 5). The half-time for maximal loss was 2.8 min. In the same experiment, membranes that were kept at 4°C did not lose clathrin ( $\Delta$ , Fig. 5) nor did membranes that were incubated in the absence of cytosol ( $\nabla$ , Fig. 5).

Calcium was required for cytosol-dependent clathrin loss from the membrane. Fig. 6 shows the effect of calcium concentration. Between 0 and 100  $\mu$ M  $Ca^{2+}$ , there was little effect of the cytosol. 100  $\mu$ M and above, however, caused a substantial loss with maximal disappearance plateauing at  $\sim 500$   $\mu$ M  $Ca^{2+}$ . Half-maximal loss of clathrin was estimated to occur between 75 and 150  $\mu$ M  $Ca^{2+}$ .

Clathrin loss in these membrane preparations was strictly dependent on the presence of nucleotides. Whereas we routinely found that 1 mM ATP, without a regenerating system, caused a 50–70% loss (Fig. 7), ADP was nearly as effective (data not shown) and GTP stimulated a 25% loss (Fig. 7). There was not any loss of clathrin if ATP was replaced with the nonhydrolyzable analogue AMP-PNP (Fig. 7); however, ATP- $\gamma$ -S was as effective or better than ATP (Fig. 7).

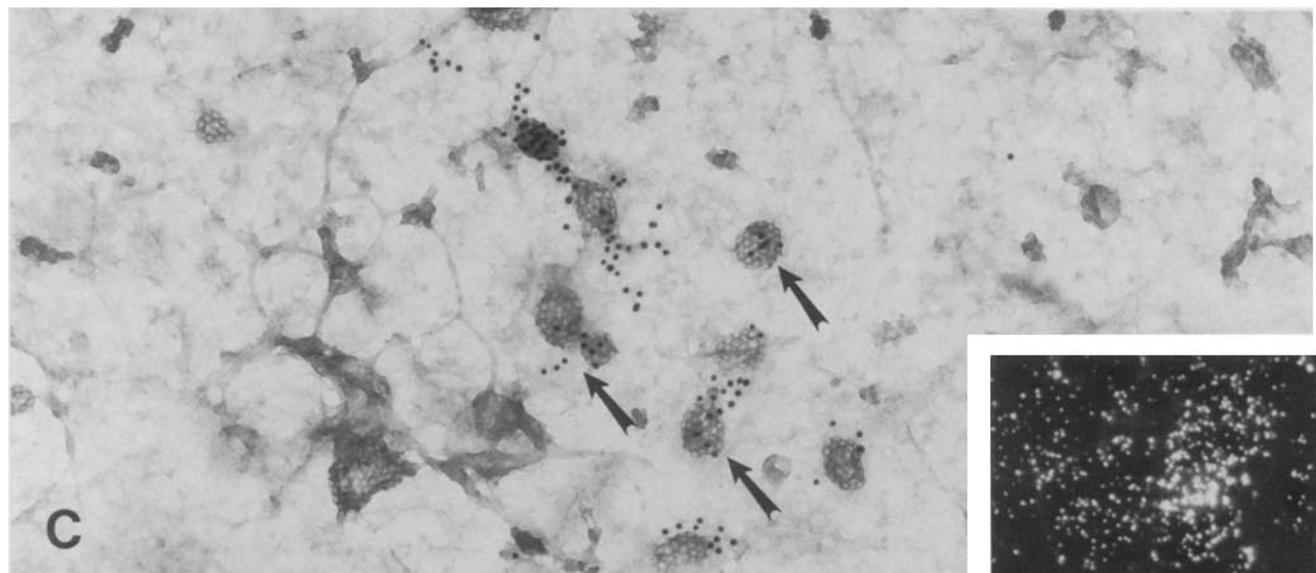
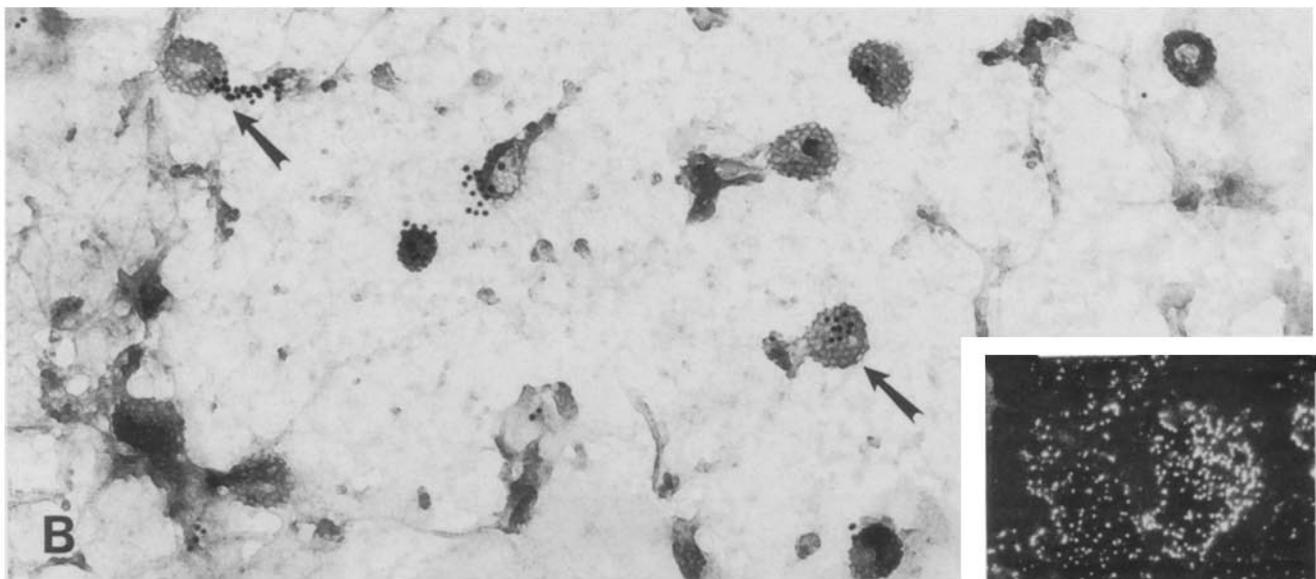
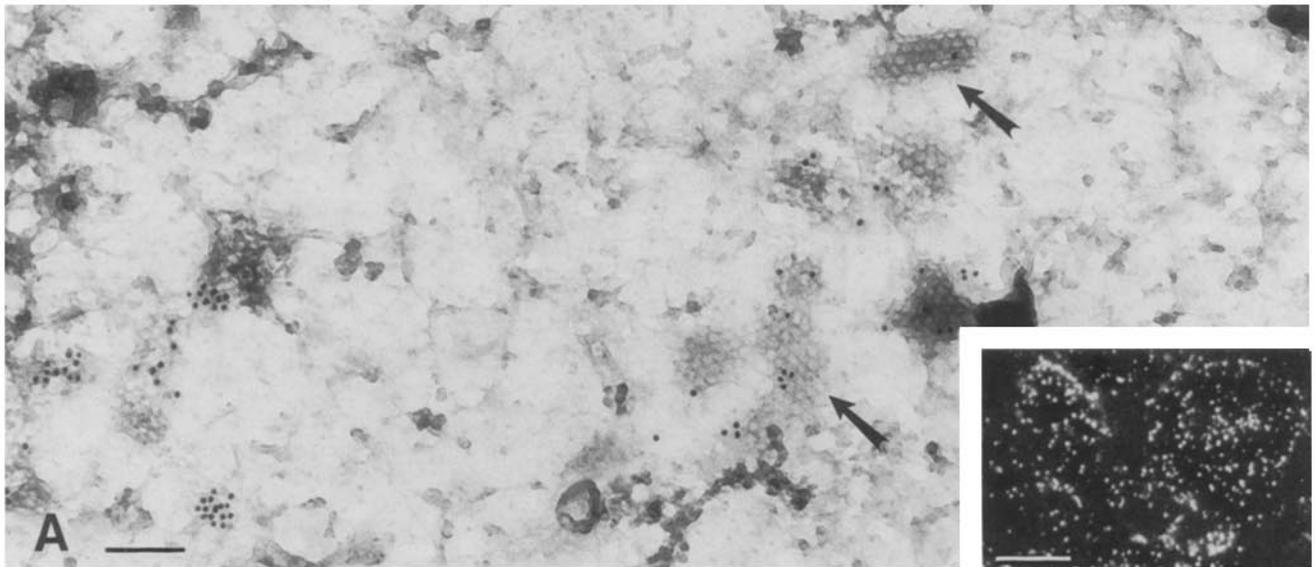
Since GTP-binding proteins have been implicated in certain phases of endomembrane traffic (Gruenberg and Howell, 1989), we tested the effects of GTP- $\gamma$ -S on clathrin loss using conditions that have been found by other investigators to be inhibitory. As seen in Fig. 7, the addition of 200  $\mu$ M GTP- $\gamma$ -S to the standard ATP containing cocktail had no effect on the loss of clathrin.

One of the most effective inhibitors of LDL internalization in human fibroblasts is *N*-ethylmaleimide (NEM) (Brown and Goldstein, 1976). After exposure to  $>0.1$  mM NEM, virtually all receptor-bound LDL remains at the cell surface in cells that are incubated at 37°C. To determine whether it is the membrane or the cytosolic factor(s) that is NEM sensitive, each was incubated with 3 mM NEM and then tested for their ability to support clathrin loss. As seen in Fig. 8, NEM-treated cytosol was as effective as untreated cytosol in causing a decrease in membrane-bound clathrin. NEM-treated membranes, on the other hand, retained clathrin after being incubated with untreated cytosol. Therefore, the membranes are the NEM-sensitive component of the budding machinery and activity can not be restored to inactivated membranes by incubation with cytosol.

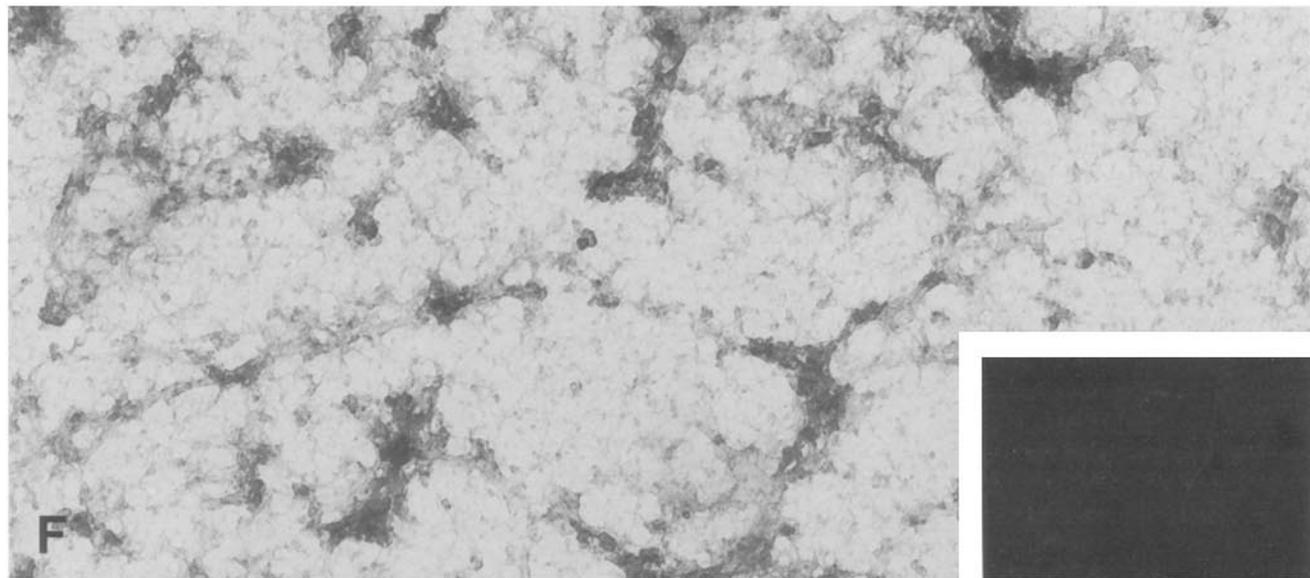
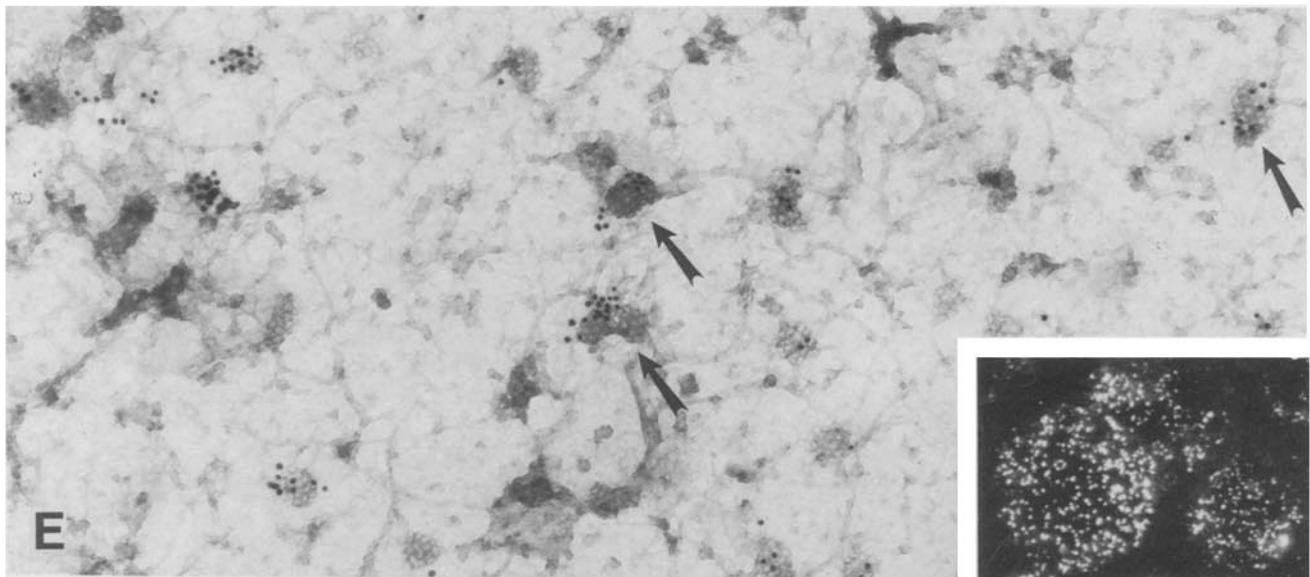
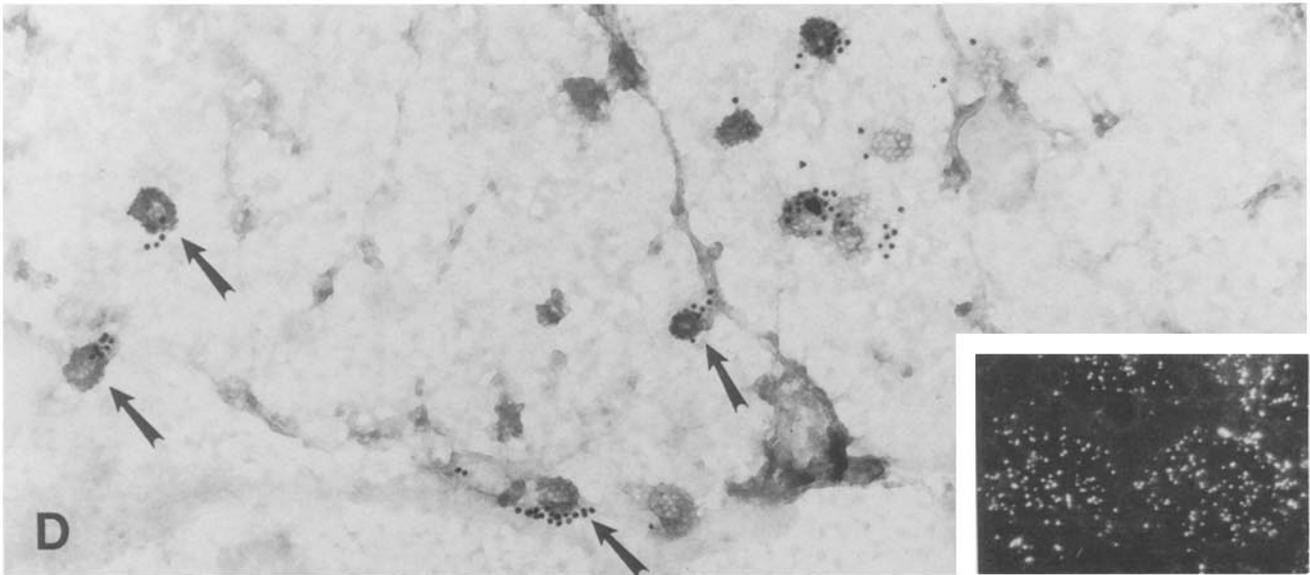
## Discussion

### Purified Plasma Membranes Support Coated Pit Budding

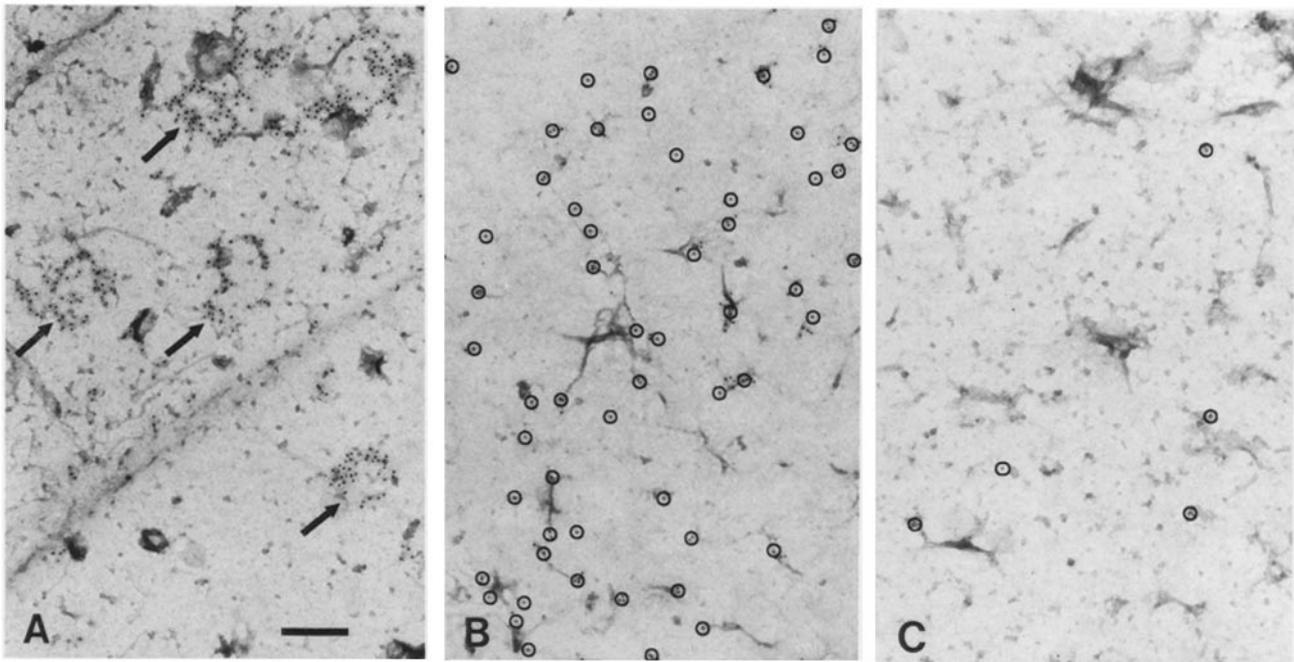
We have used three different assays to show that in the presence of a cytosol cocktail, coated pits will bud from purified plasma membranes. First, gold-labeled ligands bound to



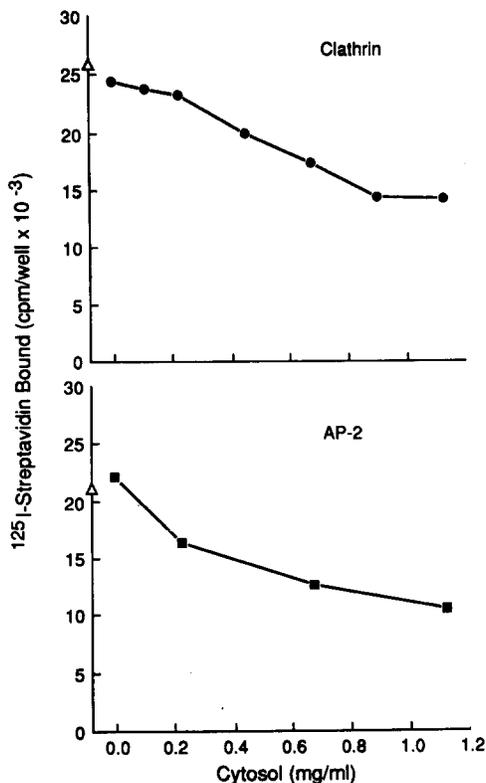
**Figure 2.** Electron microscopic localization of anti-LDL receptor IgG-gold and clathrin coated pits (A-E) or indirect immunofluorescence localization of clathrin (insets) on membranes that received either: (A) no treatment; (B) 37°C in buffer B; (C) 37°C in buffer B containing 150  $\mu\text{M}$   $\text{Ca}^{2+}$  and 1 mM ATP; (D) 37°C in buffer B containing 1 mM ATP and 1 mg/ml of cytosol; (E) 37°C in buffer B containing 150  $\mu\text{M}$   $\text{Ca}^{2+}$  and 1 mg/ml of cytosol; (F) 37°C in buffer B containing 1 mM ATP, 150  $\mu\text{M}$   $\text{Ca}^{2+}$ , and 1 mg/ml of cytosol. SV589 cells were incubated in suspension in the presence of anti-LDL receptor IgG-gold for 1 h at 4°C. The cells were then washed at 4°C and attached



by centrifugation to poly-L-lysine-coated coverslips containing several formvar-coated electron microscope grids that were held in place with nail polish. The cells were disrupted by sonication. One set of membranes was fixed immediately (*A*), whereas the other sets received the indicated treatment before being processed for electron microscopy as described. For immunofluorescence, the membranes were treated identically except that they were not pre-labeled with the anti-LDL receptor IgG-gold. (*Arrows*) Clathrin-coated pits. Bars: 100 nm; (*inset*) 10  $\mu$ m.



**Figure 3.** The effect of a clathrin lattice (*A* and *C*) and receptor clustering (*B*) on loss of LDL-gold from isolated membranes. Membranes were prepared by the method of Sanan and Anderson (1991) from either normal human fibroblasts (*A* and *C*) or internalization-defective human fibroblasts (*B*) that had been incubated in the presence of 17  $\mu\text{g/ml}$  of LDL-gold for 1 h at 4°C. Membranes were washed at 4°C either with neutral buffer (*B* and *C*) or pH 9.0 buffer (*A*) before warming to 37°C in cytosol cocktail for 10 min. The membranes were then fixed and processed as described. The circles (*B* and *C*) indicate the location of one or more LDL-gold particles on the membrane, whereas the arrows (*A*) point to clusters of LDL-gold. Bar, 500 nm.

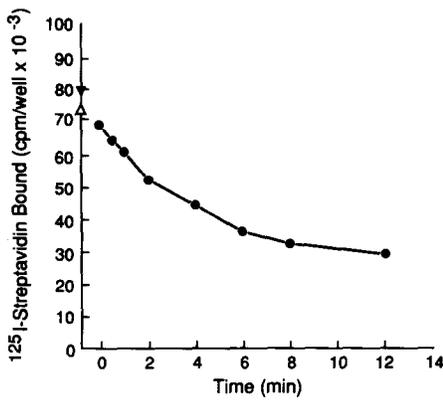


**Figure 4.** Cytosol-dependent loss of clathrin triskelions (*clathrin*) and AP-2 complex (*AP-2*) from isolated plasma membranes. Membranes attached to coverslips at 4°C were prepared as described.

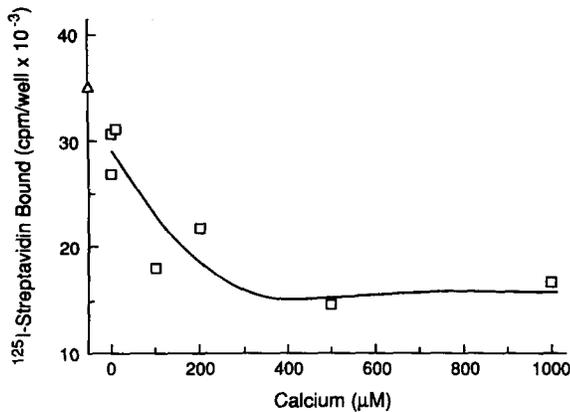
receptors in coated pits disappear from the membrane under permissive conditions. The plasma membrane and the formvar support are barriers to the movement of the gold because if the pits are inactive (4°C; absence of either cytosol,  $\text{Ca}^{2+}$ , ATP or clathrin lattice) the gold remains trapped between the two layers (see Fig. 2, *A-E* and Fig. 3). Furthermore, gold-labeled receptors that are unable to cluster in coated pits are not lost from the membrane (Fig. 3 *B*). Therefore, there is not any other way for the gold to leave the membrane except by coated pit budding. Second, the radioimmune assay showed that concomitant with LDL-gold loss, both clathrin and the AP-2 complex are also lost from the membrane. Finally, these same conditions cause a substantial portion of the LDL receptors to disappear from the membrane.

Based on the amount of anti-LDL receptor IgG-gold clusters that disappeared (>80%), neither the amount of receptor loss (~27%) nor the amount of clathrin loss (~60%) was as high as anticipated. The most likely explanation for this discrepancy is that the radioimmune assay un-

Coverslips were incubated at 37°C in buffer B containing 100  $\mu\text{M}$   $\text{Ca}^{2+}$ , 1 mM ATP and the indicated concentration of cytosol for 10 min. At the end of the incubation, the membranes were fixed and processed for radioimmune detection of either triskelions (●) or AP-2 (■) as described. The amount of each subunit initially present on the members is indicated on the ordinate (Δ). Background radioactivity of 1,959 and 1,723 cpm for X-22 and AP-6 antibodies, respectively, was subtracted from the values shown.



**Figure 5.** Kinetics of cytosol-dependent clathrin loss at 37°C. Membranes were prepared as described in Fig. 1. Buffer B containing 1.2 mg/ml of cytosol, 150  $\mu$ M  $\text{Ca}^{2+}$ , and 1 mM ATP was added to each dish before warming to 37°C for 12 min. At the indicated time, the reaction was stopped by the addition of 2 ml of buffer B containing 6 mM EDTA. The coverslips were washed, fixed and processed for radioimmune detection of clathrin (●) as described. The amount of clathrin present on membranes that were not treated ( $\Delta$ ) and the amount remaining after incubation in buffer B that did not contain any cytosol ( $\nabla$ ) are shown on the ordinate. A background value of 2,327 cpm was subtracted from all values.

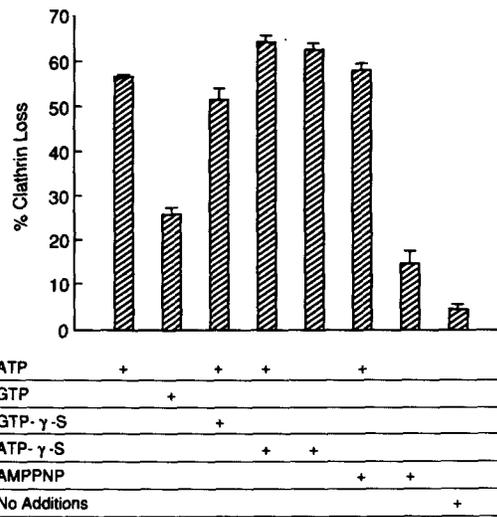


**Figure 6.** Effect of  $\text{Ca}^{2+}$  on clathrin loss at 37°C. Membranes were prepared as described in Fig. 1. Membranes were incubated for 10 min at 37°C in buffer B that contained 2 mg/ml of cytosol, 1 mM ATP, and the indicated concentration of  $\text{Ca}^{2+}$ . At the end of the incubation, the membranes were fixed and processed for radioimmune detection of clathrin ( $\square$ ) as described. The amount of clathrin initially present on the members is indicated on the ordinate ( $\Delta$ ). A background value of 1,635 cpm was subtracted from all values.

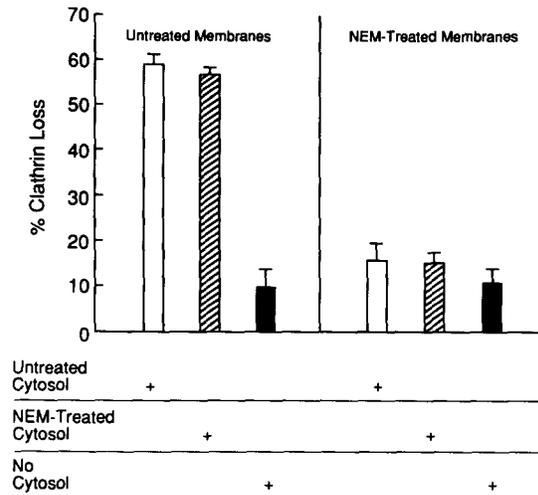
derestimates the amount of loss because some of the budded vesicles and associated material nonspecifically stick to the bottom and sides of each well in the assay plate. In support of this explanation, we routinely saw in the electron microscope released vesicles that had stuck to the substratum around the membranes. Taking this into account, the measured loss of receptors from the membrane is very close to the expected loss (30%) if 60% of the receptors were initially in coated pits (Anderson et al., 1977).

### Vesicle Formation and Endomembrane Transport

Coated pit budding is one example of a class of reactions that



**Figure 7.** Effect of nucleotides and nucleotide analogues on clathrin loss at 37°C. Membranes were prepared as described in Fig. 1. Each set of membranes was incubated for 10 min at 37°C in buffer B that contained 1 mg/ml of cytosol, 150  $\mu$ M  $\text{Ca}^{2+}$ , and 1 mM of the indicated nucleotide or nucleotide analogue. At the end of the incubation, the membranes were fixed and processed for radioimmune detection of clathrin as described. The untreated membranes that were not warmed to 37°C bound 78,986 cpm of  $^{125}\text{I}$ -streptavidin, which was used as the 100% value. Background was 2,854 cpm.



**Figure 8.** Effects of *N*-ethylmaleimide (*NEM*) on clathrin loss at 37°C. Membranes were prepared as described in Fig. 1, with the exception that DTT was deleted from sonication buffer C. In *A*, the membranes were incubated at 4°C with buffer C for 15 min, whereas in *B* the membranes were incubated at 4°C with buffer C containing 3 mM *NEM* for 15 min. At the end of each pretreatment, the membranes were washed and the *NEM* quenched with 6 mM DTT. Both sets of membranes were warmed to 37°C for 10 min in the presence of either no cytosol, 2 mg/ml untreated cytosol, or 2 mg/ml *NEM* treated cytosol (+ marks) all in buffer B that contained 150  $\mu$ M  $\text{Ca}^{2+}$  and 1 mM ATP. At the end of the incubations, the membranes were fixed and processed for radioimmune detection of clathrin as described. The untreated membranes that were not warmed to 37°C bound 68,934 cpm of  $^{125}\text{I}$ -streptavidin, which was used as the 100% value. Background was 2,093 cpm.

are essential for endomembrane movement. Whereas vesicle formation in the *trans*-Golgi region and the plasma membrane use the clathrin-coated pit, Orci and co-workers (Orci et al., 1989) have identified a non-clathrin-coated membrane intermediate in inter-Golgi traffic. In addition, the inner membrane surface of caveolae (plasmalemmal vesicles), which are membrane specializations thought to have an endocytic function (Anderson, 1991; van Deurs et al., 1989), are also decorated with a morphologically unique coat (Anderson, 1991; Peters et al., 1985). The composition of the clathrin coat is known to consist of at least six proteins, many of which have been identified, purified and cloned (Keen, 1990). Likewise, Golgi apparatus-derived coated vesicle coats have an equally complex protein composition (Malhotra et al., 1989). The coat material surrounding each membrane specialization presumably plays an important role in the formation of the vesicle (Anderson, 1991). Therefore, a molecular understanding of coated pit-coated vesicle transformation will give important clues about all membrane budding reactions.

Whereas AMP-PNP did not support coated pit budding, ATP- $\gamma$ -S was as effective as ATP. This is in marked contrast to transport between the ER and Golgi (Beckers et al., 1990) as well as within Golgi stacks (Rothman and Orci, 1990), which is very sensitive to this analogue. In these systems, however, the ATP- $\gamma$ -S-sensitive step is after budding from the donor membrane (Beckers et al., 1990). If budding from Golgi and ER membranes requires nucleotides, then ATP- $\gamma$ -S can substitute in these reactions too.

Considerable work will be required to determine how nucleotides function during coated pit budding. Several possibilities come to mind. (a) ATP may be used directly as an energy source. Other nucleotides can substitute because the enzymatic machinery has an extremely high affinity for ATP and as a consequence can get by with less desirable substrates under in vitro conditions (Anderson, 1991). (b) Since ATP- $\gamma$ -S can substitute for ATP, maybe ATP is used in a phosphorylation reaction. Biochemical studies have shown that some of the protein components of the AP-2 and triskelion subunits are substrates for protein kinase(s); moreover, isolated coated vesicles have both protein kinase activity and phosphatase activity (Keen, 1990). (c) ATP may act catalytically during coated pit budding.

Coated pit budding is not inhibited by GTP- $\gamma$ -S. By contrast, endomembrane transport has been found to be sensitive to this GTP analogue. This implies that inter-organelle membrane traffic is regulated by GTP binding protein(s) (Gruenberg and Howell, 1989). As in the case of the ATP- $\gamma$ -S sensitive step, however, GTP- $\gamma$ -S inhibits a reaction that is distal to vesicle formation.

NEM inhibits endosome-endosome fusion (Braell, 1987; Diaz et al., 1989), inter-Golgi traffic (Balch et al., 1984), Golgi recycling (Goda and Pfeffer, 1988), and ER-to-Golgi transport (Beckers et al., 1987) in vitro. This commonality has led to the isolation and cloning of NEM-sensitive factor (Rothman and Orci, 1990), a cytosolic factor that is required for membrane-membrane fusion between vesicles in these membrane transport pathways. Since NEM treatment of cytosol did not affect coated pit budding in vitro, apparently this factor is not required. Nevertheless, there is an NEM-sensitive step that resides in the plasma membrane. The inactivation of the membranes by NEM could not be reversed by the

addition of cytosol; therefore, most likely the sensitive molecule(s) is a structural component of the coated pit. Treatment of membranes with NEM also inactivated transferrin receptor recycling in vitro (Podbilewicz and Mellman, 1990).

Since the outer surface of the plasma membrane ordinarily is bathed in high concentrations of calcium (>1 mM), this is the candidate site of action for  $\text{Ca}^{2+}$  during coated pit budding. Calcium may function to promote fusion of the opposing lipid bilayers during the final closure of the coated pit. In liposome model systems, high concentrations of  $\text{Ca}^{2+}$  have been found to promote fusion of liposomes composed of a mixture of neutral and anionic lipids (Gennis, 1989). In combination with the force generated by the change in membrane shape that occurs during the rearrangement of the lattice, the  $\text{Ca}^{2+}$  may be sufficient to cause fusion. Most likely this step is also dependent on special properties of the lipids in the coated pit membrane (Anderson, 1991).

Although ER to Golgi traffic is dependent on  $\text{Ca}^{2+}$ , optimal transport occurs at 10  $\mu\text{M}$   $\text{Ca}^{2+}$  (Beckers et al., 1990). The  $\text{Ca}^{2+}$  appears to be required late in the transport process. Because the luminal concentration of  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -binding proteins in the ER is quite high (Sambrook, 1990), there is a possibility that  $\text{Ca}^{2+}$  has a critical function in the budding of vesicles from these membranes as well.

Our results show that the clathrin lattice is a device that actively changes the shape of the membrane. Without the lattice (Fig. 3 A) the membranes do not invaginate even though the receptors remain clustered. Since coated pits ordinarily round up spontaneously at 37°C, then some intrinsic property of the lattice causes invagination. Heuser (1980) has suggested that polygon rearrangement within the lattice accompanies shape change, but whether or not this is an active or a passive activity remains to be elucidated.

### Future Applications

This plasma membrane preparation has the potential for revealing the secrets of endocytosis via coated pits. This is virtually the purest preparation of membranes available for studying a fundamental event in endomembrane traffic. Not only can these membranes recapitulate coated pit budding, but also when the endogenous coat proteins are removed they support coated pit assembly. Therefore, in the future the coated pit can be assembled from components that have been modified in informative ways to probe the function of each structural protein.

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