Assembly of Clathrin-Coated Pits onto Purified Plasma Membranes

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During receptor-mediated endocytosis, coated pits invaginate to form coated vesicles, clathrin and associated proteins dissociate from the vesicle membrane, and these proteins form new coated pits at the cell surface. As a means of elucidating molecular mechanisms that govern the function of coated pits, the assembly phase of this cycle was reconstituted by incubating purified membranes that were treated to remove endogenous coated pits with cytoplasm extracted from cultured cells. The in vitro assembly of coated pits on these membranes satisfactorily mimics many features of coated pit formation in the intact cell. These studies indicate that: (i) the membranes contain a limited number of coated pit assembly sites that bind clathrin with high affinity; (ii) the half-time for assembly is 5 minutes both at 4°C and 37°C; (iii) during assembly, proteins with molecular sizes of 180, 110, and 36 kilodaltons are recruited to the plasma membrane; and (iv) assembly is not dependent on adenosine triphosphate, but this nucleotide triggers a temperature-dependent loss of coated pits that are assembled in the absence of adenosine triphosphate.

THE CLATHRIN-COATED PIT IS A SPECIALIZED REGION OF the cell surface that mediates the internalization of extracellular macromolecules (*1*). Coated pits derive their name from the presence of a distinctive polygonal lattice that decorates the inner surface of the membrane (*2*). This lattice is thought to be composed of multiple triskelion-shaped subunits that contain three clathrin heavy chains (180 kD) and three clathrin light chains (30 to 40 kD) plus a family of associated proteins with molecular sizes of 110 kD and 50 kD (*3*).

Coated pits are the primary portal of entry for receptors that deliver macromolecules into cells by receptor-mediated endocytosis (*4*). This process has two cardinal features. (i) The receptor migrates in the plane of the membrane and becomes associated with the coated pit; and (ii) mechanical forces exerted at the coated pit convert planar segments of membrane into highly curved coated vesicles by progressive invagination. Molecular studies have shown that the cytoplasmic portion of these receptors is responsible for directing them to coated pits (*5*). However, nothing is known about either the molecules in the coated pit that recognize receptors or the molecular determinants that cause membrane shape change during endocytosis.

Coated pits internalize at a rapid rate [1500 to 3000 per minute per cell (*6*)], and internalization does not appear to depend on the presence of a ligand-receptor complex (*7*). Once a coated vesicle forms, the clathrin and associated proteins dissociate from the vesicle membrane and return to the cell surface to form a new coated pit. This cycle continues for many hours in the absence of protein synthesis, but is inhibited in cells that are depleted of intracellular K^+ (*8*). These observations indicate that regulatory molecules exist—both within the cytoplasm and at the cell surface—and they control the coated pit–coated vesicle cycle.

To better understand the molecular properties of clathrin-coated membranes, investigators have focused their efforts on purified coated vesicles (*9*). These vesicles can be obtained in large quantity, and the coat proteins are easily purified by dissociating the lattice from the membrane with buffers of high *p*H or with high concentrations of tris (*3*). Under the proper conditions, these proteins assemble into spherical polygonal lattices, either in the presence or absence of membranes (*10*). However, purified clathrin appears unable to form planar lattices like those often found in coated pits (*11*). Therefore, the requirements for formation of coated pits have yet to be determined.

The relative inaccessibility of coated pits to experimental manipulations accounts for the paucity of information about the molecular mechanisms that govern the function of this specialized region of surface membrane. For this reason, much could be learned if all or part of the coated pit–coated vesicle cycle could be reconstituted in a cell free system. As a first step toward this goal, we have been able to assemble coated pits onto purified plasma membranes with the use of cytoplasm extracted from cultured cells and to make several new observations about coated pit formation.

Preparation of assembly competent plasma membrane. The in vitro coated pit assembly system depends upon the availability of purified plasma membrane oriented so that the cytoplasmic surface is exposed for experimental manipulation. To accomplish this, we cultured human fibroblasts, harvested them by trypsinization, and plated the separated cells onto cover slips that were coated with poly-L-lysine (*12, 13*). The cells were allowed to attach for 1.5 hours at 37°C and then chilled for 1 hour to maximize coated pit assembly (*14*). The cover slips were then sonicated, which destroyed the cells but left behind large sheets of the plasma membrane that remained attached to the poly-L-lysine.

Coated pits were visualized by indirect immunofluorescence staining with the immunoglobulin G (IgG) fraction of a monoclonal

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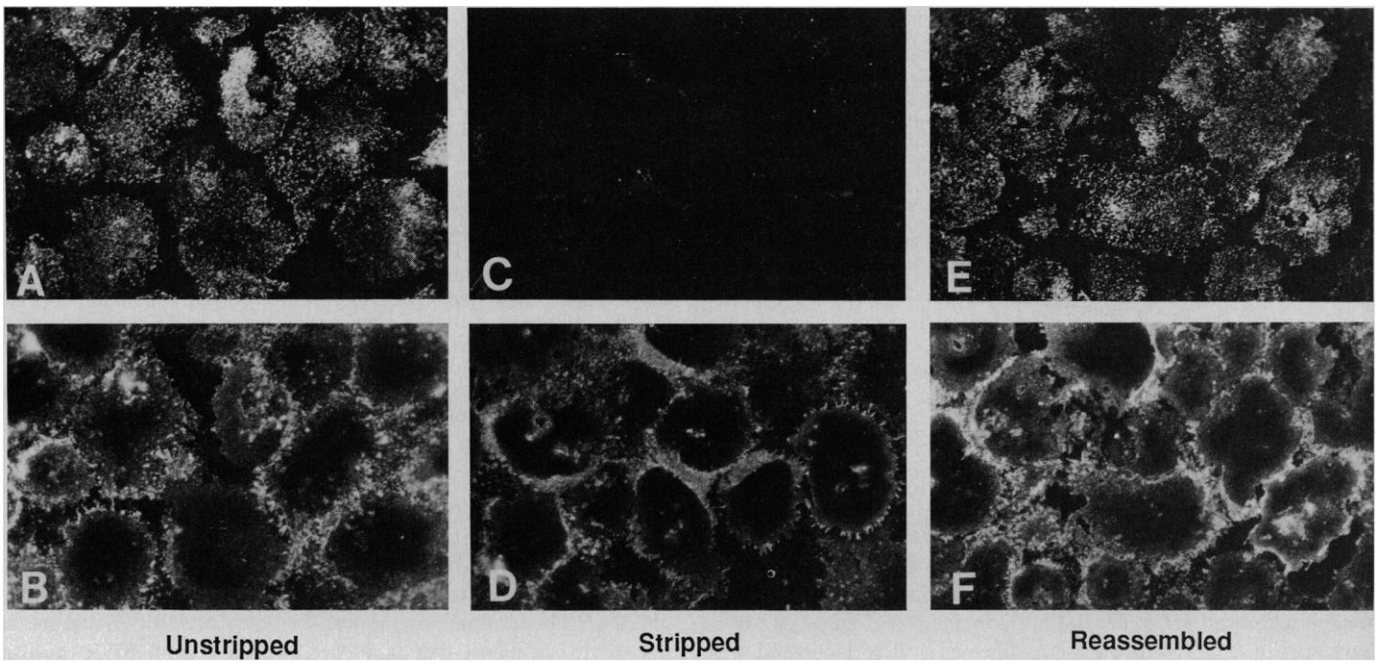

Fig. 1. Distribution of binding sites of antibody (IgG) to clathrin (top set, A, C, and E) or of wheat germ agglutinin (WGA) binding sites (bottom set, B, D, F) on isolated membranes attached to poly-L-lysine coated cover slips. Membranes from normal human fibroblasts were attached to cover slips (*12*). One cover slip in each set (**A** and **B**) was fixed immediately with 3 percent formaldehyde in buffer G (*13*). The other two cover slips in each set (**C** and **D** and **E** and **F**) were washed three times with ice-cold buffer C (*13*), transferred to chilled 60-mm dishes containing 3 ml of buffer C, and incubated on ice for 20 minutes. Each dish was then washed three times with buffer C and three times with buffer F (*13*) at 4°C. Cover slip C,D was fixed as above and cover slip E,F was transferred to another chilled 60-mm dish, overlaid with 80 μl of cytoplasm [7.8 mg/ml (*17*)] and incubated on ice for 30 minutes. After this incubation, the cover slip was washed four times with ice-cold buffer F (*13*) and fixed. Each dish was processed for localization by indirect immunofluorescence of clathrin and WGA binding sites (*25*). The same set of membranes was photographed (Zeiss Photomicroscope III) with the filter package first for fluorescein (A, C, and E) and then rhodamine (B, D, and F) (×500).

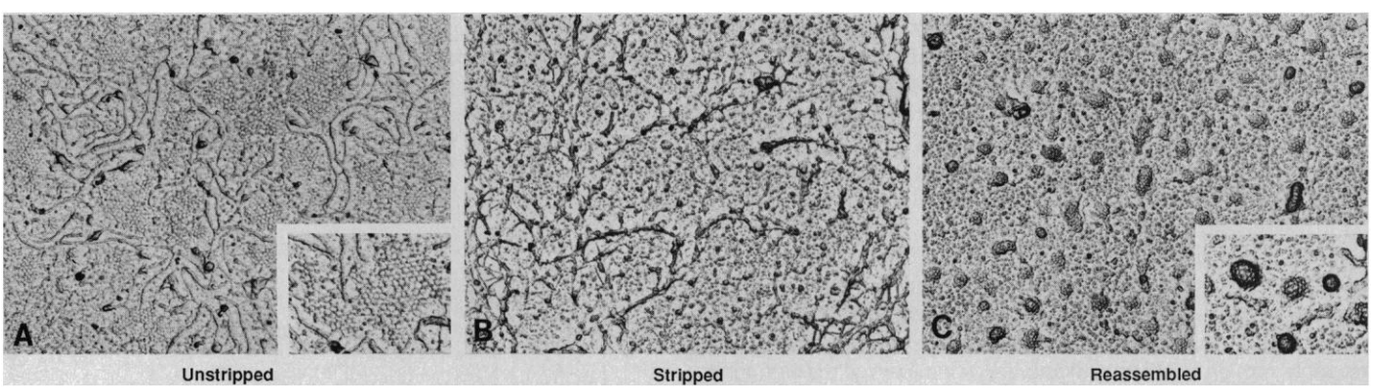

Fig. 2. Carbon-platinum replicas of isolated membranes attached to poly-L-lysine coated cover slips that had been unstripped (**A**), stripped (**B**), or stripped and incubated with cytoplasm (**C**). Membranes from normal human fibroblasts were attached to cover slips and subjected to the same treatments described in Fig. 1. At the end of the treatment, each set of membranes was fixed with 1 percent glutaraldehyde plus 1 percent formaldehyde in buffer I (*13*) and carbon-platinum replicas were prepared (*26*). These membranes were prepared in the same experiment as shown in Fig. 1 (×27,000; inset ×38,000).

antibody to clathrin (anti-clathrin) (*15*). For orientation purposes, the membranes were counterstained with fluorescent wheat germ agglutinin (WGA), which bound to the borders of each membrane segment. The surface of these membranes was virtually covered with coated pits (Fig. 1, A and B). The clathrin-coated pits were removed by incubation of the plasma membranes in a high *p*H buffer under conditions that are known to strip coat proteins from isolated coated vesicles (*9*). After this stripping procedure, coated pits were no longer visible (Fig. 1, C and D).

Procedure for assembly of coated pits. To assemble coated pits in vitro, we took advantage of the observations of Louvard and coworkers (*16*) who showed that cultured fibroblasts contain a pool of clathrin that is free in the cytoplasm. Human fibroblast membranes attached to cover slips that were coated with poly-L-lysine were prepared, stripped of coated pits with buffer of high *p*H, and incubated for 30 minutes at 4°C with cytoplasm prepared from SV40 transformed human fibroblasts (*17*). As determined by indirect immunofluorescence, this procedure restored the coated pits on the plasma membrane (Fig. 1, E and F). The newly formed coated pits were found only on the membranes and not on the adjacent cover slip, indicating that the clathrin was bound specifically to the stripped membranes. Furthermore, coated pits did not form when isolated human erythrocyte membranes, which do not have coated pits, were incubated with cytoplasm.

To visualize coated pit assembly with the electron microscope, we prepared carbon-platinum replicas of membranes (*18*). In a replica

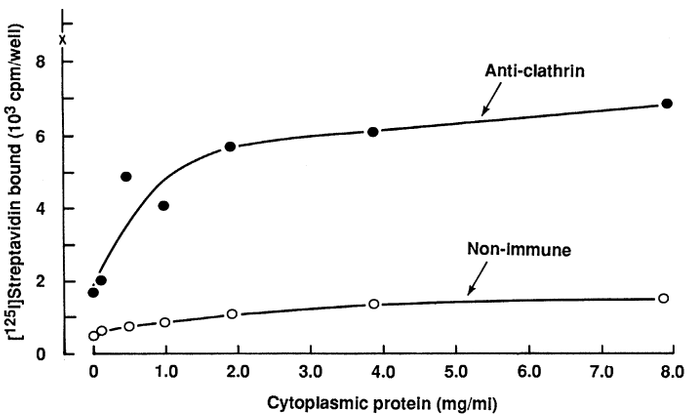

Fig. 3. Dependence of coated pit formation in vitro on the concentration of cytoplasmic protein. Membranes from normal human fibroblasts (2.5×10^5) were attached to the bottom of individual wells of a 96-well plate (*27*). Each well was washed twice with 250 μl of buffer C (*13*) and then incubated with 250 μl of buffer C for 20 minutes, all at 4°C. At the end of the incubation period, each well was washed three times with 250 μl of buffer C and then washed three times with 250 μl of buffer F (*13*). Each well then received 50 μl of ice-cold cytoplasm at the indicated concentration of protein and incubated at 4°C for 1 hour. The various concentrations of cytoplasmic protein were prepared by diluting cytoplasm (*17*) with buffer F (*13*). At the end of the incubation period, each well was washed four times with 250 μl of buffer F at 4°C, fixed with 3 percent formaldehyde, and assayed for the binding of either anti-clathrin IgG (●) or non-immune IgG (○) by an indirect ^{125}I-labeled streptavidin binding assay (*27*). Each point is the average of three wells. The (X) on the ordinate corresponds to the amount of ^{125}I-labeled streptavidin that bound to unstripped membranes. The amount of ^{125}I-labeled streptavidin bound to wells that did not contain membranes but were treated identically averaged 248 cpm per well. Nonimmune IgG was an irrelevant monoclonal IgG (*28*).

of a freshly isolated plasma membrane (Fig. 2A), numerous planar polygonal lattices, which correspond to coated regions, were scattered over the membrane. Only an occasional deeply invaginated coated pit was seen. A companion set of membranes that had been treated with high *p*H buffer did not contain either planar or curved lattices (Fig. 2B). Stripped membranes that were incubated with cytoplasm, however, contained numerous coated pits, each of which had the typical lattice morphology (Fig. 2C). Most of the newly assembled coated pits were smaller in diameter than the original coated pits (compare Fig. 2, C and A); moreover, the lattices were slightly curved (Fig. 2C, inset).

The amount of clathrin bound to isolated membranes was quantified by preparing membranes in individual wells of a 96-well plate and measuring clathrin with an indirect ^{125}I-labeled streptavidin binding assay (*15*). When the membranes were stripped with buffer at high *p*H, 30 percent of the clathrin remained associated with the membranes (Table 1). After the membranes were incubated with cytoplasm, the level of bound clathrin rose to 78 percent of the amount that was originally on the membrane. Subtracting the amount of clathrin remaining after stripping (30 percent), coated pits assembled in vitro contained 48 percent as much clathrin as the coated pits on isolated membranes before stripping.

Characterization of coated pit assembly. The cytoplasmic extract, together with the isolated membranes, appear to contain the essential elements for coated pit assembly. The formation of coated pits may be regulated by the availability of assembly sites at the inner surface of the membrane. To see if there were a limited number of assembly sites on the plasma membrane, we measured the amount of clathrin that bound to purified membranes in response to incubation with different concentrations of cytoplasm. As seen in Fig. 3, maximal binding of clathrin occurred at a cytoplasmic protein concentration of 0.5 to 1.0 mg/ml and did not increase further when more cytoplasmic protein was added. This suggests that there are high affinity interactions between coated pit proteins and the membrane during assembly.

To account for the dynamics of coated pit turnover, assembly should be rapid. We again used the ^{125}I-labeled streptavidin assembly assay to measure directly the kinetics of coated pit formation both at 4° and 37°C (Fig. 4). Regardless of temperature, maximal assembly occurred after a 10-minute incubation with cytoplasm, although the amount of clathrin on the membrane at this time was nearly two times higher at 4°C than at 37°C. With longer incubation, the level of clathrin membranes treated at 4°C declined slightly; however, at 37°C there was a marked decline in the amount of membrane-bound clathrin. The temperature-dependent disappearance of coated pits was also observed when we used the indirect immunofluorescence assay described in Fig. 1.

We next determined what proteins are recruited to the plasma membrane during coated pit assembly. Purified plasma membranes were prepared and the coated pits were removed by treating the membranes with either *p*H 9.0 buffer or 0.5*M* tris buffer at neutral *p*H (*9*). Each set of membranes was then incubated for 15 minutes at 4°C with cytoplasm that had been prepared from SV40 human fibroblasts grown in the presence of [^{35}S]methionine for 24 hours to label cellular proteins. Companion sets of membranes were either processed for immunofluorescence localization of coated pits (Fig. 5, A and B) or solubilized in detergent and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography (Fig. 5, A′ and B′). Whereas membranes treated at high *p*H had normal numbers of coated pits (Fig. 5A), coated pits did not assemble onto membranes treated with 0.5*M* tris (Fig. 5B). In the autoradiogram of the polyacrylamide gel, several labeled proteins were common to both membranes (Fig. 5, A′ and B′). However, only membranes treated at *p*H 9.0 contained labeled proteins with molecular sizes of 180, 110, and 36 kD (arrows, Fig. 5A′). Therefore, coordinate with

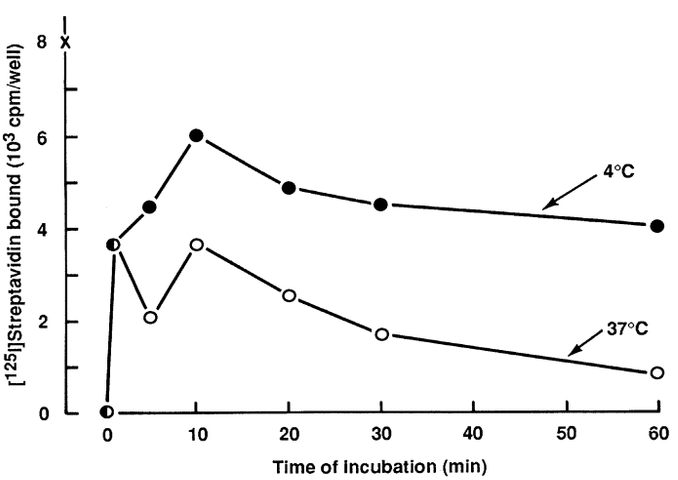

Fig. 4. Time course of coated pit assembly at 4°C (●) and at 37°C (○) on isolated membranes attached to poly-L-lysine. Membranes from 2.5×10^5 normal human fibroblasts were attached to the bottom of individual wells of a 96-well plate (*27*). Each well was treated to remove coated pits as in Fig. 3. Each well then received 50 μl of cytoplasm (6.3 mg/ml) and was incubated for the indicated time at either 4°C (●) or 37°C (○). At the end of the incubation, each well was assayed for anti-clathrin IgG binding as described in Fig. 3. The average amount of ^{125}I-labeled streptavidin that bound to stripped membranes (3924 cpm per well) was subtracted from each value. X on the ordinate corresponds to the amount of ^{125}I-labeled streptavidin that bound to unstripped membrane. Each value is the average of three wells. The amount of ^{125}I-labeled streptavidin bound to wells that did not contain membranes but were treated identically averaged 567 cpm per well.

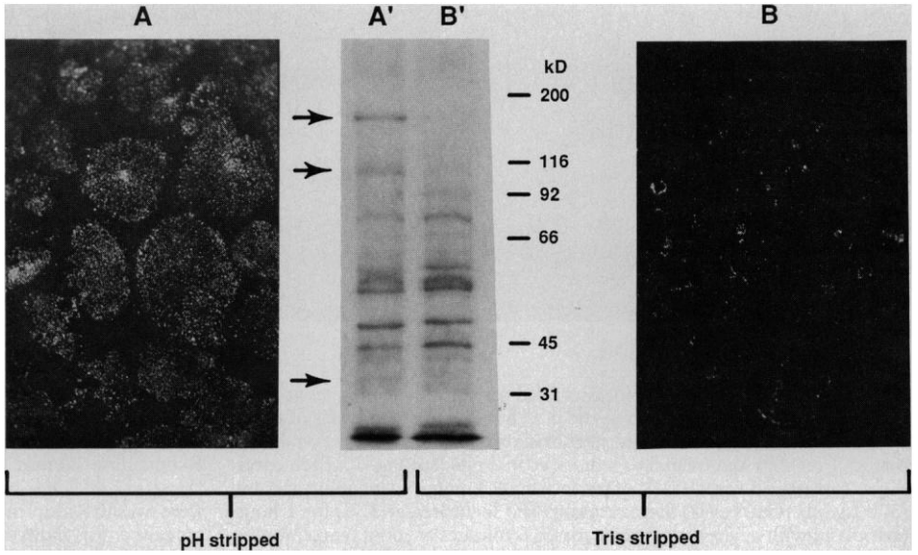

Fig. 5. Identification of cytoplasmic proteins that associate with isolated membranes during coated pit formation in vitro. Membranes from normal human fibroblasts were attached to two sets (set **A** and **A'**, and set **B** and **B'**) of poly-L-lysine coated cover slips (*12*). Both sets of membranes were subjected to the coated pit stripping procedure described in Fig. 1; however, one set (A and A') was stripped with buffer C (*13*), whereas the other set (B and B') was stripped with buffer E (*13*). Each set was then incubated at 4°C for 15 minutes with 70 μl of cytoplasm (*17*) prepared from SV40-transformed human fibroblasts that had been grown for 24 hours in the presence of methionine free DMEM (K C Biological LMC 237) containing 10 percent fetal calf serum, 20 m*M* Hepes, penicillin at 100 U/ml, streptomycin at 100 μg/ml, 10 μ*M* methionine, and [^{35}S]methionine (100 μCi/ml; 1120 Ci/mmol, New England Nuclear NEG-009C). The specific activity of the cytoplasm was 10^6 cpm/μl. At the end of the incubation, each cover slip was washed six times with ice-cold buffer F (*13*). One cover slip from each set (A and B) was processed for indirect immunofluorescence localization of clathrin heavy chains (*25*). The other cover slip from each set (A' and B') was incubated for 1 minute with 50 μl of boiling sample buffer (0.0625*M* tris-HCl, *p*H 6.8, 10 percent glycerol, 5 percent 2-mercaptoethanol, 0.001 percent bromophenol blue and 2.3 percent sodium dodecyl sulfate). Sample buffer was removed, and 50 μl of fresh boiling sample buffer was added to the cover slips. The two portions of sample buffer were pooled, boiled for 3 minutes, centrifuged at 11,600*g* for 5 minutes (microfuge) and subjected to electrophoresis into a 7.5 percent SDS polyacrylamide gel slab according to the method of Laemmli (*31*). After electrophoresis, the gel was stained with Coomassie blue, destained, incubated for 30 minutes at room temperature with Amplify (Amersham DAMP.100) and dried. The dried gel was overlaid with XAR-5 x-ray film, and stored at −80°C for 4 days. Molecular size markers correspond to standard proteins (Bio-Rad 161-0303,0304) processed in the same gel. Arrows mark proteins of 180 kD, 110 kD, and 36 kD (×500).

the assembly of coated pits, the membrane acquired cytoplasmic proteins that correspond in molecular size to the heavy chain of clathrin (180 kD), one of the light chains of clathrin (36 kD), and one or more of the 110-kD family of clathrin-associated proteins.

The effect of adenosine triphosphate (ATP) on coated pit assembly was determined by incubation of stripped membranes with cytoplasm that contained apyrase, a potato enzyme that hydrolyses ATP (*19*). Normal numbers of coated pits assembled after the stripped membranes were incubated with ATP-depleted cytoplasm for 30 minutes at 37°C; moreover, the number of coated pits did not decline after incubation at 37°C for 60 minutes (Fig. 6A).

These results suggested that the temperature-dependent disappearance of newly assembled coated pits observed in Fig. 4 required ATP. Therefore, two sets of membranes that contained coated pits assembled from apyrase-treated cytoplasm were washed to remove cytoplasm and incubated for 10 minutes at 37°C in buffer that contained either ATP or apyrase. A comparison of Fig. 6B with Fig. 6C shows that after incubation with ATP, coated pits had disappeared from the membrane.

New insights. Membranes that have been treated to remove endogenous coated pits support the formation of new coated pits when they are incubated in the presence of cytoplasm from a cultured cell. As judged by immunofluorescence, just as many coated pits formed as were initially present on the membrane. Moreover, the lattice morphology appeared to be normal, although the lattices were smaller and somewhat more curved than the endogenous lattices. By quantitative assay, the amount of clathrin that bound to the membranes during reassembly was only half of that present before stripping. This is in agreement with the smaller size of the reassembled lattices.

Having achieved coated pit assembly in vitro, and with the confidence that this process mimics many features of coated pit formation in situ, we proceeded to use this system to obtain information about the assembly process. Our results indicate that (i) there are a limited number of high affinity assembly sites on the membrane; (ii) assembly is rapid, both at 4° and 37°C; (iii) in addition to clathrin heavy chain and light chain, the 110-kD class of clathrin-associated proteins is recruited to the plasma membrane during assembly; and (iv) ATP is not directly required for assembly but appears to trigger a temperature-dependent loss of coated pits.

In tissue-cultured cells, 2 percent of the cell surface has coated pits (*1*), and 50 to 60 percent of the total clathrin (*16*) resides in an unassembled pool of 9*S* clathrin (triskelions) (*20*). Our results suggest that assembly is regulated in part by the number of assembly sites present on the plasma membrane. Assembly in vitro was a saturable process and half-maximal assembly occurred at a cytoplas-

Table 1. Relative amount of clathrin on isolated membranes. Membranes from 2.5 × 10^5 normal human fibroblasts were attached to the bottom of individual wells of a 96-well plate (*27*). One set of wells (unstripped) was fixed with 3 percent formaldehyde. The other wells were washed twice with 250 μl of buffer C (*13*) and then incubated with 250 μl of buffer C for 20 minutes, all at 4°C. At the end of the incubation, each well was washed three times with 250 μl of buffer C at 4°C. One set of wells (stripped) was washed with buffer F and fixed with 3 percent formaldehyde. The other set (reassembled) was washed three times with ice-cold buffer F (*13*) and then incubated for 1 hour at 4°C with 50 μl of cytoplasm (7.7 mg/ml) in each well. At the end of the incubation, the wells were washed four times with 250 μl of buffer F and fixed with 3 percent formaldehyde. All wells were then assayed for the binding of either anti-clathrin IgG or non-immune IgG [a monoclonal IgG_{2b} that does not bind to human fibroblasts (*28*)] with an indirect ^{125}I-labeled streptavidin binding assay (*27*). Each value is the mean (cpm ± SEM) of each of three wells. The average amount of ^{125}I-labeled streptavidin bound to wells that did not contain membranes but were treated identically was 248 cpm per well.

Treatment	^{125}I-Streptavidin bound		Percent of control
	Anti-clathrin IgG	Non-immune IgG	
Unstripped	8823 ± 1264	711 ± 52	100
Stripped	2686 ± 186	283 ± 25	30
Reassembled	6875 ± 641	1631 ± 66	78

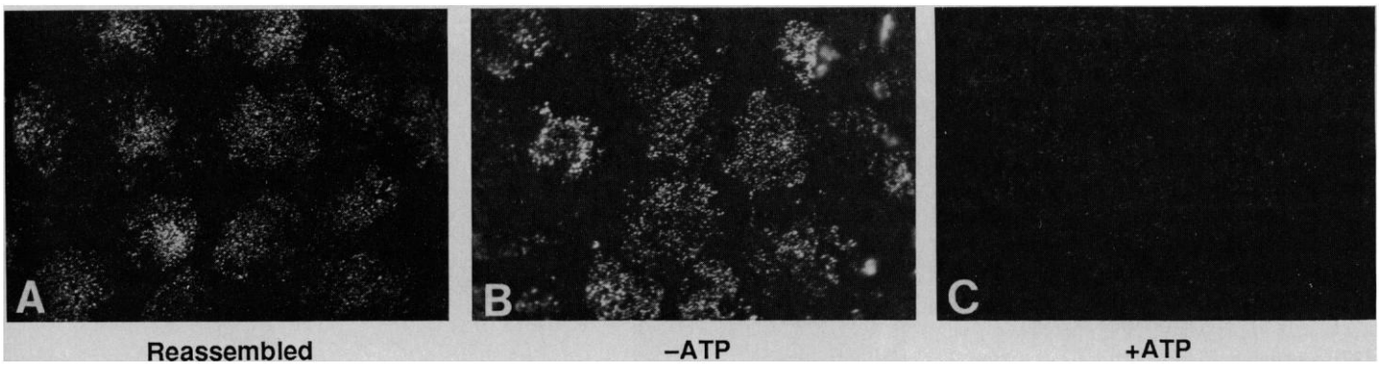

Fig. 6. Distribution of coated pits assembled on isolated membranes at 37°C (**A**) or assembled and incubated in the presence (**C**) or absence (**B**) of ATP. Membranes from normal human fibroblasts were attached to poly-L-lysine coated cover slips and treated to remove coated pits (see Fig. 1). Each cover slip was overlayed with 100 μl of cytoplasm (4.8 mg/ml) containing apyrase (500 μg/ml) (Grade VII, Sigma A6160) and incubated at 37°C for 1 hour (cytoplasm with apyrase was preincubated 5 minutes at room temperature). At the end of the incubation, each cover slip was washed four times with buffer F (*13*) at 4°C. Cover slip A was fixed with 3 percent formaldehyde. Cover slip B was incubated with 100 μl of buffer F containing apyrase (500 μg/ml) for 10 minutes, and cover slip C was incubated with 100 μl of buffer F containing 3.2 m*M* ATP, 16 m*M* phosphocreatine, and creatine phosphokinase (12.8 IU/ml) at 37°C. At the end of the incubation, both cover slips were washed four times with buffer F and fixed. All cover slips were processed for indirect immunofluorescence localization of clathrin (*25*) (×500).

mic protein concentration of about 0.3 mg/ml. On the basis of the studies of Goud *et al.* (*16*), we estimate the clathrin concentration to be 0.2 to 0.4 μg/ml. Therefore, the plasma membrane contains a limited number of assembly sites, and clathrin appears to have a high affinity for these sites. Our method should facilitate the biochemical identification of the assembly sites.

Coated pit assembly was just as rapid at 4°C as it was at 37°C. Moreover, the half-time of assembly (about 5 minutes) was similar to what has been estimated from studies on the time course of assembly in the intact cell (*18*). These results support a role for high-affinity interactions between coated pit proteins and the cell surface during assembly.

Whereas isolated coated vesicles usually have five proteins associated with the lattice (*9*), we only detected three proteins in coated pits assembled from cytoplasmic extract labeled with [^{35}S]methionine; the 50-kD clathrin-associated protein and one of the clathrin light chains were missing. These proteins may not have appeared on the autoradiograms because they did not incorporate a sufficient amount of [^{35}S]methionine (*15*). However, coated pits may not contain these proteins.

Attempts to determine the intracellular ATP requirements for internalization of molecules by coated pits has yielded conflicting results. Larkin *et al.* (*21*) depleted ATP in human fibroblasts by 98 percent without affecting the rate of low density lipoprotein (LDL) internalization. Likewise, ATP-depleted hepatocytes are able to internalize asialoglycoproteins (*22*). Hertel *et al.* (*23*), however, have found that the internalization of epidermal growth factor is inhibited in ATP-depleted astrocytoma cells. Our results indicate that at least the assembly phase of the coated pit–mediated endocytic cycle can occur in the absence of ATP because pits formed when membranes were incubated with ATP-depleted cytoplasm. In addition, assembly occurred at 4°C.

The temperature-dependent disappearance of coated pits assembled in the absence of ATP, on the other hand, was stimulated by ATP. We do not know why the coated pits disappeared. The loss of pits may involve the invagination of the lattice and the pinching off of membrane. Alternatively, the coated pit lattice may disassemble directly at the membrane. Lattice disassembly could be mediated by the ATP-dependent enzyme that uncoats coated vesicles, which was discovered by Rothman and co-workers (*24*); however, this seems unlikely because the disappearance of coated pits was not dependent on the presence of cytoplasm or any other potential source of the enzyme.

The results obtained thus far from studying coated pit assembly in vitro suggest that it should be possible to characterize assembly sites and identify the proteins in the coated pit lattice. In addition, further studies of the temperature-dependent disappearance of reassembled coated pits may lead to new insights about how coated pits mediate endocytosis.

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12. Cultured human fibroblasts, derived from a skin biopsy of a normal subject, were grown in monolayer and set up for experiments in 60-mm dishes according to a standard format (*29*). On day 7 of cell growth, the media was removed, and 1 ml of trypsin-EDTA (Gibco, 610-5300) was added to each dish and the cells were incubated for 5 minutes at 37°C. The cell suspension was removed and added to an equal volume of DMEM (Dulbecco's modified Eagle medium, Gibco 320-1885) containing 20 m*M* Hepes, *p*H 7.3, and 10 percent fetal calf serum. The suspended cells were centrifuged at 600*g* for 5 minutes, and washed three times by repeated, alternate resuspension and centrifugation in Hepes-DMEM. The cells were finally resuspended in Hepes-DMEM to a concentration of 2.5×10^6 cells per milliliter, and 200 μl of this suspension was pipetted onto the top of a poly-L-lysine (>300 kD) coated cover slip incubated for 1 hour at 37°C in poly-L-lysine (1 mg/ml in water) that had been fixed to the center of a 60-mm petri dish with double stick tape. The cells were incubated 1.5 hours at 37°C, and 2 ml of warmed (37°C) Hepes-DMEM that contained 10 percent fetal calf serum was added to each dish and further incubated 15 minutes at 37°C. This media was removed, and 2 ml of ice-cold Hepes-DMEM containing 10 percent fetal calf serum was added to each dish and incubated for 1 hour at 4°C. Each dish was rinsed twice with ice-cold buffer A (*13*) and then once with ice-cold buffer B (*13*). Each dish was then filled with 25 ml of ice-cold buffer B, and the cells were immediately sonicated by placing a 1/2-inch tapped horn 1 cm above the center of the cover slip and sonicating for 2 seconds at a power setting of 5.5 (Heat Systems Ultrasonics, model W185G). The buffer was removed, 2 ml of ice-cold buffer B was added, and the dish was held at 4°C until the indicated treatment.
13. Buffer A consisted of 50 m*M* Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) *p*H 7.4, 100 m*M* NaCl; buffer B consisted of 20 m*M* MES [2(*N*-morpholino) ethanesulfonic acid], *p*H 6.0, 2.5 m*M* $MgCl_2$, 2.5 m*M* EGTA, 1 m*M* benzamidine, 1 m*M* 1,10-phenanthroline, 10 μ*M* leupeptin; buffer C consisted of 20 m*M* TAPS [tris(hydroxymethyl)methylaminopropane sulfonic acid], *p*H 9.0, 1

mM benzamidine, 1 mM 1,10-phenanthroline, 10 μM leupeptin; buffer D consisted of 1 M tris, pH 7.0, 1 mM benzamidine, 1 mM 1,10-phenanthroline, 10 μM leupeptin; buffer E consisted of equal volumes of buffer B and buffer D; buffer F consisted of 36.4 mM Hepes-KOH, pH 7.2, 68.2 mM KCl, 4.1 mM magnesium acetate, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM 1,10-phenanthroline, 10 μM leupeptin; buffer G consisted of 20 mM Hepes, pH 6.8, 100 mM KCl, 5 mM $MgCl_2$, 3 mM EGTA; buffer H consisted of 2.3 mM $NaH_2PO_4 \cdot H_2O$, 7.7 mM $Na_2HPO_4 \cdot 7\ H_2O$, 150 mM NaCl, 2 mM $MgCl_2$, pH 7.4; buffer I consisted of 8.1 mM $Na_2HPO_4 \cdot 7\ H_2O$, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4; buffer J consisted of 10 mM Hepes-KOH, pH 7.2, 15 mM KCl, 1.5 mM magnesium acetate, 1.1 mM dithiothreitol, 1.1 mM benzamidine, 1.1 mM 1,10-phenanthroline, 11 μM leupeptin; buffer K, consisted of 300 mM Hepes-KOH, pH 7.2, 600 mM KCl, 30 mM magnesium acetate; and buffer L consisted of 50 mM NH_4Cl in Dulbecco's phosphate-buffered saline (Gibco, 310-4190).

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17. Cytoplasm was extracted from cells by a modification of the method of Balch *et al.* (*30*). SV40-transformed human fibroblasts were seeded into 20 tissue culture flasks (75 cm^2) at a density of 2×10^6 cells per flask. They were cultured in DMEM supplemented with 10 percent fetal calf serum, 20 mM Hepes, penicillin at 100 U/ml and streptomycin at 100 μg/ml; media was changed on days 2, 4, and 6. On day 7, the media was replaced with 5 ml of trypsin-EDTA and incubated for 5 minutes at 37°C. The cells were collected in this solution and an equal volume of buffer I (*13*) containing 10 percent fetal calf serum was added; the cells were then centrifuged at 600*g* for 5 minutes, and washed twice by resuspension and centrifuging with 40 ml of buffer I. The final pellet of cells was resuspended in 20 ml of ice-cold buffer J (*13*) and incubated on ice for 5 minutes. The cells were centrifuged at 600*g* for 3 minutes at 4°C, and the supernatant was removed, leaving a volume of fluid equal to 1.5 times the volume of cell pellet. The cells were resuspended in the remaining buffer and transferred to a 7-ml Dounce homogenizer (Fischer 06-4354) and homogenized (20 strokes). The volume of the homogenate was measured and 0.1 volume of buffer K (*13*) was added before centrifuging at 11,600*g* for 5 minutes at 4°C (Beckman II microfuge). The supernatant was removed and centrifuged at 107,000*g* for 1 hour at 4°C in a Beckman TL-100 ultracentrifuge (TLA-100.2 rotor). The supernatant, which corresponded to the cytoplasm, was divided into multiple portions and stored at −80°C. Prior to each experiment, a portion of extract was thawed briefly at 37°C and then placed on ice. From 20 flasks of cells, 5×10^8 to 8×10^8 cells were harvested, which yielded 3 to 5 ml of cytoplasm with a protein concentration of 4 to 8 mg/ml.
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25. Isolated membranes attached to cover slips were subjected to the indicated treatment and fixed with 3 percent formaldehyde in buffer G (*13*) for 15 minutes at 4°C. The membranes were washed once with buffer L (*13*), rinsed twice with buffer H, and incubated with anti-clathrin [1 μg per milliliter of monoclonal heavy chain immunoglobulin G (IgG); X-22, (*13*)] for 30 minutes at 37°C. The membranes were washed four times (5 minutes each) in buffer H (*13*) and incubated for 30 minutes at 37°C with rabbit antibody to mouse IgG (50 μg/ml) coupled to fluorescein isothiocyanate (Zymed, 61-6511). The washing procedure was repeated, and the membranes were then incubated for 15 minutes at 37°C with WGA (1 μg/ml) coupled to rhodamine tetramethylisothiocyanate (Vector, RL-1022) and washed as above. Buffer H containing 0.1 percent bovine serum albumin was used for all antibody dilutions.
26. Isolated membranes attached to cover slips were treated as indicated and fixed with 1 percent glutaraldehyde (v/v) plus 1 percent formaldehyde (w/v) in buffer G for 30 minutes at 4°C. The membranes were postfixed with 0.5 percent OsO_4 in buffer G for 30 minutes at room temperature, dehydrated through ethanol into amyl acetate, and dried at the critical point (Balzars CPD 020) with bone dry CO_2. Carbon-platinum replicas (7.5 nm thick) of the membranes were made in a Balzars 301 equipped with a rotating cold stage (120 rev/min). The electron gun was maintained at an angle of 13°. After the replicas were reinforced with carbon (15 nm thick), they were floated free of the cover slip in a distilled water bath and digested with Clorox for 2 minutes before being mounted on Formvar-coated grids.
27. Each well of a 96-well plate (Immulon I Removawell 96-well plates, Dynatech) was rinsed with water and incubated with 100 μl of poly-L-lysine (1 mg/ml, >300 kD) for 1 hour at 37°C. Each well was rinsed five times with water and 200 μl of cell suspension (1.25×10^6 cells per milliliter in Hepes-DMEM) was added. The cells were incubated 1.5 hours at 37°C and then 400 μl of warmed Hepes-DMEM containing 10 percent fetal calf serum was added to each well. After a 15-minute incubation at 37°C, the wells were placed on ice for 1 hour. Each well was then washed three times with ice-cold buffer A followed by a washing with cold buffer B (200 μl per washing). The wells were moved to the sonicator, 200 μl of fresh buffer B (4°C) was added, and a 1/8-inch tapered microtip tapped probe (Heat Systems) was placed 5 mm above the bottom of each well. The cells were sonicated for 2 seconds at a power setting of 2.5, the buffer removed, and the wells washed with cold buffer B (200 μl per well). The membranes in each well were subjected to the indicated treatment, rinsed four times with 250 μl of the ice-cold buffer F and fixed (15 minutes at 4°C) with 3 percent formaldehyde in buffer G. Each well was washed six times with 200 μl of buffer H (*13*) and held overnight in buffer H at 4°C. Each well was then washed twice with 250 μl of buffer H and incubated for 1 hour at 37°C with 400 μl of buffer H containing 2 percent crystalline grade bovine serum albumin (Sigma A7638) and 100 mM glycine. Each well was washed twice with buffer H containing 0.1 percent crystalline bovine serum albumin (BSA) and incubated for 1 hour at 37°C with 50 μl of anti-clathrin (heavy chain IgG) diluted to 1 μg/ml in buffer H containing 1 percent of crystalline BSA and 2.0 percent horse serum (Vector, 5-2000). Each well was washed seven times with buffer H containing 0.1 percent crystalline BSA (third washing was for 5 minutes). Each well then received 50 μl of biotinylated horse antibody to mouse IgG (Vector BA-2000) diluted to 1 μg/ml in buffer H containing 1 percent crystalline BSA and 2.0 percent horse serum and incubated for 1 hour at 37°C. Wells were washed again as above and incubated for 15 minutes at room temperature with ^{125}I-labeled streptavidin (50 μl per well; 20 to 40 μCi/μg, Amersham IM.112) diluted to a final concentration of 1 μCi/ml in buffer H containing 1 percent crystalline BSA. The ^{125}I-labeled streptavidin was incubated with a small amount of Dowex 1-X8 (Baker 1904-1) for 10 minutes at room temperature before use. Each well was then washed as before, placed in a glass tube (12 by 75 mm) and counted in a gamma counter. All assay buffers were filtered through a 0.2-μm filter and all antibodies were centrifuged for 5 minutes at 11,600*g* before use.
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