The Appendage Domain of the AP-2 Subunit Is Not Required for Assembly or Invagination of Clathrin-coated Pits

John S. Peeler, William C. Donzell, and Richard G. W. Anderson

Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235

Abstract. Coated pits contain a resident membrane molecule(s) that binds clathrin AP-2 with high affinity. AP-2 binding to this site is likely to be the first step in coated pit assembly because this subunit functions as a template for the polymerization of clathrin into flat polygonal lattices. Integral membrane proteins involved in receptor mediated endocytosis cluster in the newly assembled pits as they invaginate and bud from the membrane. The AP-2 subunit is a multi-domain, molecular complex that can be separated by proteolysis into a brick-shaped core and ear-like appendage domains. We have used this property to identify the domain involved in the various stages of coated pit assembly and budding. We found that the core of AP-2 is the domain that binds both to membranes and to triskelions during assembly. Triskelions are perfectly capable of forming lattices on the membrane bound cores. Clathrin lattices bound only to core domains were also able to invaginate normally. Limited proteolysis was also useful for further characterizing the AP-2 binding site. Elastase treatment of the inside membrane surface released a peptide fraction that is able to bind AP-2 in solution and prevent it from interacting with membranes. Affinity purification of binding activity yielded a collection of peptides that was dominated by a 45-kD species. This is the candidate peptide for containing the AP-2–binding site. Therefore, the appendage domain does not directly participate in any of the assembly or invagination events required for coated pit function.

CLATHRIN-coated pits at the surface of cells are composed of two subunits (18, 19): the triskelion and the clathrin AP complex (Assembly Protein; (12), Adapter Protein; (23, 25). The overlapping legs of the triskelions form the walls of each polygon in the clathrin lattice (12, 25). The location of the AP subunits between the lattice and the membrane (35) suggests that they attach the clathrin lattice to the membrane. The AP-2 subunit is a multi-protein complex that consists of two 100-kD proteins (termed α and β adaptin) (25), one 16-kD protein and one 50-kD protein (2, 24). Each type of subunit can be selectively removed from clathrin coated membranes: the triskelions by high pH and both the triskelions and the APs by high salt (12). These two treatments allow one to study how the two sets of subunits interact with each other and with resident components of the membrane (18, 19, 36).

The clathrin lattice most likely is the molecular device that shapes the coated pit into a coated vesicle because selective removal of triskelions prevents coated pit invagination (17). The conversion of hexagons into pentagons within the lattice occurs during the change in curvature of coated pits (9), but there is no evidence that polygon rearrangement is the force-generating mechanism for invagination.

One function of the AP-2 subunit may be to control the clustering of membrane receptors that enter cells by coated pits (5, 23, 25). It may also anchor the lattice to the membrane (18). The subunit has the capability of performing more than one task because it structurally contains multiple domains. EM has shown that the subunit is composed of a brick-shaped core with two ear-like appendages extending from one end (11). Elastase will remove the appendages without degrading the proteins in the core (38). The appendages released by elastase correspond to the 30-kD COOH terminus of both the α and β adaptin (11, 38).

Several years ago we introduced a method for preparing plasma membranes that is ideal for studying the assembly and function of coated pits in vitro (20). We have shown that the plasma membrane–associated clathrin AP, AP-2 (29, 31), binds to a high affinity site in coated pits (19). The Golgi-associated clathrin AP, AP-1, seems to bind much less tightly to the plasma membrane (19), which suggests that AP-2 binding is specific. The binding site shares some of the properties of an integral membrane protein; for example, it is resistant to high salt but sensitive to proteases (19).

This preparation of coated pits offers a unique opportunity to build on the knowledge gained from studying coated vesicle assembly in vitro and to identify the components required for coated pit assembly and function (12). We have used limited proteolysis and column chromatography to prepare purified cores and appendages from AP-2. We found that AP-2 binding to membranes, clathrin lattice formation, and invagination are all under the control of the core domain within the subunit. We also found that elastase releases AP-2 binding activity from the membrane and that this activity is

in a peptide fraction with a dominant peptide species of 45 kD. This result suggests that plasma membranes contain an integral membrane protein that functions as an AP-2 receptor during coated pit assembly.

Materials and Methods

Materials

Immulon I Removawell 96-well plates (011-010-6301) were purchased from Dynatech Laboratories, Inc. (Alexandria, VA). Human plasma fibronectin was obtained from the New York Blood Center (New York). Elastase (LSO6363) was from Worthington Biomedical Corp. (Freehold, NJ). Diisopropyl flurophosphate (DFP)[1] was from Sigma Chemical Company (DO879) (St. Louis, MO). Biotinylated horse anti-mouse IgG (BA-2000) was from Vector Laboratories, Inc. (Burlingame, CA). ^{125}I-labeled streptavidin (sp act 40 μCi/mg, IM-236) was purchased from Amersham Corp. (Arlington Heights, IL). Glutaraldehyde-activated silica gel was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). mAbs against the 180-kD clathrin heavy chain (X-22, IgG isotype) and against the carboxyl-terminal domain (Frances Brodsky, personal communication) of the 100 kD, α component of AP-2 (AP.6, IgG isotype) were prepared in mice as previously described (7) and kindly provided by Dr. Frances Brodsky. A mAb against the amino terminal domain (Robinson, M., personal communication) of the 100 kD, β component of AP-2 (B1-M_6, IgG2a isotype) was prepared in mice as previously described (29) and generously provided by Dr. Margaret Robinson. [^{35}S]methionine (Trans ^{35}S-label) was from ICN Biomedical, Inc. (Irvine, CA). All other reagents were analytical grade and obtained as previously described (18–20).

Methods

Buffers and Cell Culture Medium

Cell Attachment Medium. 20 mM Hepes-buffered (pH 7.4) MEM with Earles salts and without $NaHCO_3$ (330-1435; Gibco Laboratories, Grand Island, NY).

Cell Culture Medium. DME (320-1885; Gibco Laboratories) buffered with 20 mM Hepes (pH 7.4), and supplemented with 2 mM L-glutamine, 10% (vol/vol) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Protease Inhibitor Cocktail. 10 μM leupeptin, 1 mM 1,10-orthophenanthroline, 0.5 mM benzamidine, 2 μg/ml soybean trypsin inhibitor, 0.5 mM PMSF.

Sonication Buffer. 20 mM Mes (pH 6.2), 2.5 mM EGTA, 2.5 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, protease inhibitor cocktail.

Cytosol Buffer. 20mM Hepes (pH 7.2), 68 mM KCl, 4 mM Mg acetate, 1 mM DTT, protease inhibitor cocktail.

Tris-Stripping Buffer. cytosol buffer containing 0.6 M Tris (pH 7.2, 4°C), protease inhibitor cocktail.

Taps-Stripping Buffer. 20 mM Taps (pH 9.0), 1 mM DTT, protease inhibitor cocktail.

Cell Culture

Cultured fibroblasts were derived from a skin biopsy obtained from a normal human subject. Cells were grown in monolayer and set up for experiments according to a standard format (8). On day 0, 7 × 10^4 cells were seeded into each Petri dish (100 × 15 mm) containing 10 ml DME supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (vol/vol) CS. 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 μg/ml selenium, 5 μg/ml insulin, 5 μg/ml transferrin, and 10% (vol/vol) human lipoprotein-deficient serum. Cells were used for all binding experiments on day 7 of cell growth. SV-40 transformed human fibroblasts (SV-589 cells) were grown as a monolayer (100-mm-diam dishes) in cell culture medium as previously described (20). Cells were harvested in log phase of growth and used to prepare elastase fragments (see below).

Preparation of Coat Proteins

Clathrin and associated proteins were extracted from isolated bovine-coated vesicles with 0.6 M Tris and either used directly or used to purify clathrin triskelions and clathrin AP subunits by gel filtration (Superose 6) as previously described (18). The AP-1 and AP-2 subunits were separated by hydroxyapatite chromatography as described (24).

To prepare AP-2 cores and appendages, purified AP-2 (2–4 mg/ml) was dialyzed into cytosol buffer without protease inhibitors and 400 μl of this solution was treated with 1 μg/ml elastase at 4°C for 45 min. The reaction was stopped by adding DFP to a final concentration of 1.0 mM. The solution was loaded onto a 1 × 24 cm Sephacryl S-200 SF column and chromatographed with cytosol buffer containing the protease inhibitors PMSF and leupeptin. Fractions containing the appendages and the cores of the proteolysed AP-2 were identified by SDS-PAGE. Virtually complete removal of both the α and the β appendage domains was achieved by these treatments.

AP-2 Binding Assay

Immulon I removawells were coated with 1 mg/ml poly-L-lysine for 60 min at 37°C, followed by 1 μg/ml fibronectin for 60 min at 37°C. Normal human fibroblasts were harvested during log-phase growth, washed in serum-free cell attachment medium and 250,000 cells were added to each well. The cells were allowed to spread for 90 min at 37°C without serum and then 10% FBS was added to each well for an additional 15 min. The plates were placed on ice and all subsequent treatments were carried out at 4°C. Wells that did not contain cells were used as a control in all experiments. After 60 min, the wells were washed with sonication buffer, attached to the bottom of a buffer reservoir containing 700 ml of sonication buffer, and then sonicated at constant energy (15 joules) using a 3-mm-microtip step probe (Vibra Cell VC60; Sonics and Materials, Danbury, CT) positioned 2 mm above the top of each well. The wells were washed with sonication buffer and used for experiments as described below. In some experiments AP-2 binding was assessed by incubating membranes with unfractionated coat proteins whereas in other trials the purified AP-2 subunit was used (see figure legends). Wells were subsequently fixed with 3% paraformaldehyde and assayed for the presence of either clathrin or specific domains of the AP-2 subunit using an ^{125}I-streptavidin radioimmunoassay as previously described (19).

Preparation of Elastase Fragments

SV-40 transformed human fibroblasts (SV-589) were harvested with Trypsin-EDTA, washed with cell attachment medium and 400,000 cells were allowed to attach to the bottom of each polylysine/fibronectin-coated well of a 24-well culture dish. 10 to 20 24-well culture dishes were prepared for each experiment. The cells were incubated in a normal atmosphere at 37°C. After 90 min, 10% FBS was added to each well and the cells were incubated for an additional 15 min at 37°C before being placed on ice for 60 min. All subsequent treatments were carried out at 4°C. Each plate was washed twice in sonication buffer and attached to the bottom of a reservoir containing 700 ml of sonication buffer. The cells in each well were sonicated at constant energy (50 joules) using a 13-mm probe immersed in the buffer reservoir 2 mm above the top of each well. After sonication, the plates were washed twice in sonication buffer and then seven times in Tris-stripping buffer to remove clathrin and AP-2. The plates were washed seven times in cytosol buffer without protease inhibitors and incubated for 45 min on ice with 400 μl/well cytosol buffer containing 2 μg/ml elastase. Proteolysis was terminated by the addition of DFP to a final concentration of 2 mM. The proteolytic fragments were then harvested from each well and 20% glycerol was added. 10-ml aliquots were frozen in liquid nitrogen and stored at −80°C until use. In some experiments, the cells were incubated for 6 h in methionine-free DME culture medium containing 125 μCi/ml [^{35}S]methionine and 10% dialyzed FBS before plating and sonication. The radiolabeled fragments prepared from these membranes had a specific activity of 400,000 to 600,000 cpm/mg protein. Elastase fragments prepared from the outside membrane of fibroblasts were treated identically except that the cells were not sonicated.

Affinity Purification of Elastase Fragments

Purified APs were dialyzed at 4°C overnight against Ca^{2+}, Mg^{2+}-free PBS (pH 6.5) containing 1.0 M NaCl (coupling buffer). 4 ml of the AP solution (0.87 mg/ml) was mixed by gentle rocking with 0.5 grams glutaraldehyde-activated silica overnight at 4°C. The AP-coupled silica was placed in a 5-ml

1. *Abbreviations used in this paper:* BCA, bicinchoninic acid; DFP, diisopropyl fluorophosphate.

column and washed with coupling buffer until no protein eluted from the column. The remaining free aldehyde groups were quenched with coupling buffer containing 0.3 M ethanolamine. The column was then washed with cytosol buffer containing 1.0 mM DFP and 0.6 M Tris, pH 7.2, and then equilibrated in cytosol buffer without Tris before use. 95% of the protein was coupled to the column. Either unlabeled or ^{35}S-labeled elastase fragments prepared as described above were thawed and fresh DFP (1 mM) was added before pouring over the AP-2-silica column. The flow-through was recirculated over the column for two hours at 0.5 ml/min and then the column was washed extensively with cytosol buffer. The bound protein was then eluted with 5.0 ml of cytosol buffer containing 0.6 M Tris, pH 7.2. Fractions (0.5 ml) were collected and dialyzed into cytosol buffer containing 1.0 mM DFP before testing in competition binding assays. A portion of each ^{35}S-labeled sample was precipitated with ethanol and prepared for SDS-PAGE. The gels were impregnated with 22% PPO in DMSO and fluorographed using pre-flashed x-ray film.

Other Methods

SDS-PAGE was carried out according to the method of Laemmli (16). Protein determinations were made using the micro bicinchoninic acid (BCA) assay (33). Rapid-freeze, deep-etch microscopy was carried out as previously described (18).

Results

The Core of AP-2 Links Triskelions to the Membrane

The domain within the AP-2 subunit that interacts with the high affinity binding site on the membrane should compete for the binding of intact AP-2 to isolated plasma membranes. We used elastase and column chromatography to purify AP-2 appendages and cores. We then mixed different concentrations of each domain with a constant amount of intact AP-2 subunits and incubated the membranes in the presence of the mixture (Fig. 1). The cores abolished the binding of AP-2 to the membrane in a concentration dependent manner (Fig. 1, ●) but the appendages had no effect on binding (Fig. 1, ○). In other trials, we used a core specific mAb (29) to

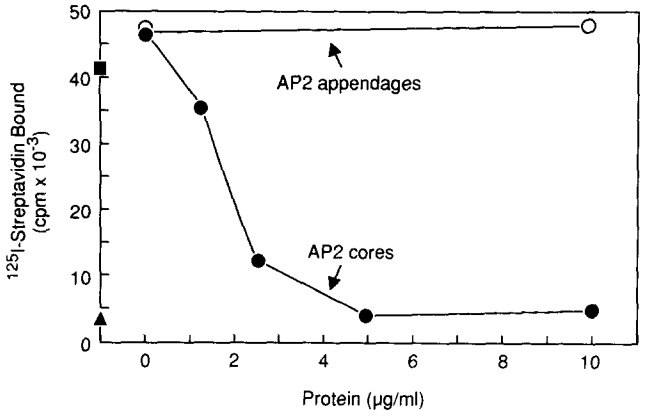


Figure 1. The core of AP-2 binds to the membrane binding site. AP-2 was treated with elastase and the appendages were separated from the cores by gel filtration. The indicated amount of either the appendages (○) or the cores (●) was added to each well containing sonicated membranes. While keeping the volume constant, a constant amount of AP-2 was added to each well and the mixture was incubated at 4°C for 30 min. The amount of bound AP-2 was measured using monoclonal AP.6 as described in Materials and Methods. The amount of AP-2 on the membrane before (■) and after (▲) the membranes were treated with Tris is indicated on the ordinate. All values are the average of duplicate measurements.

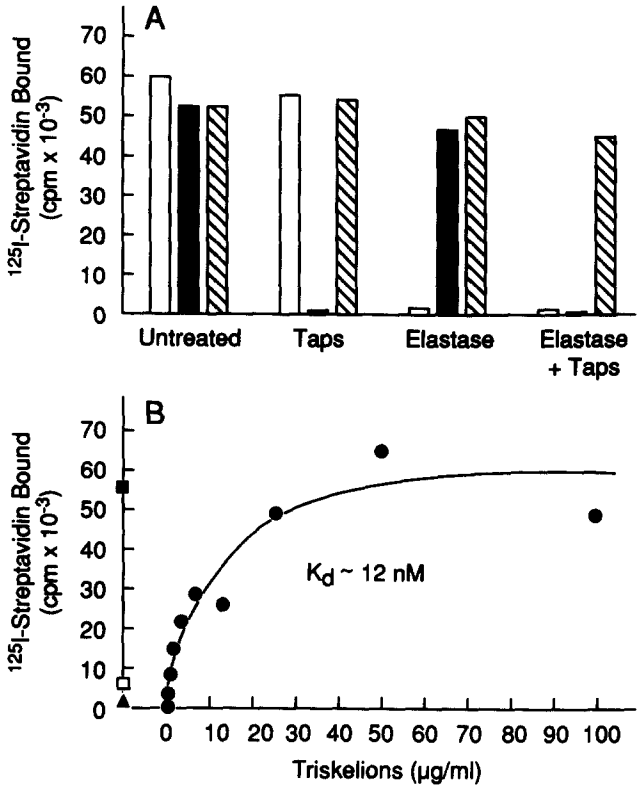
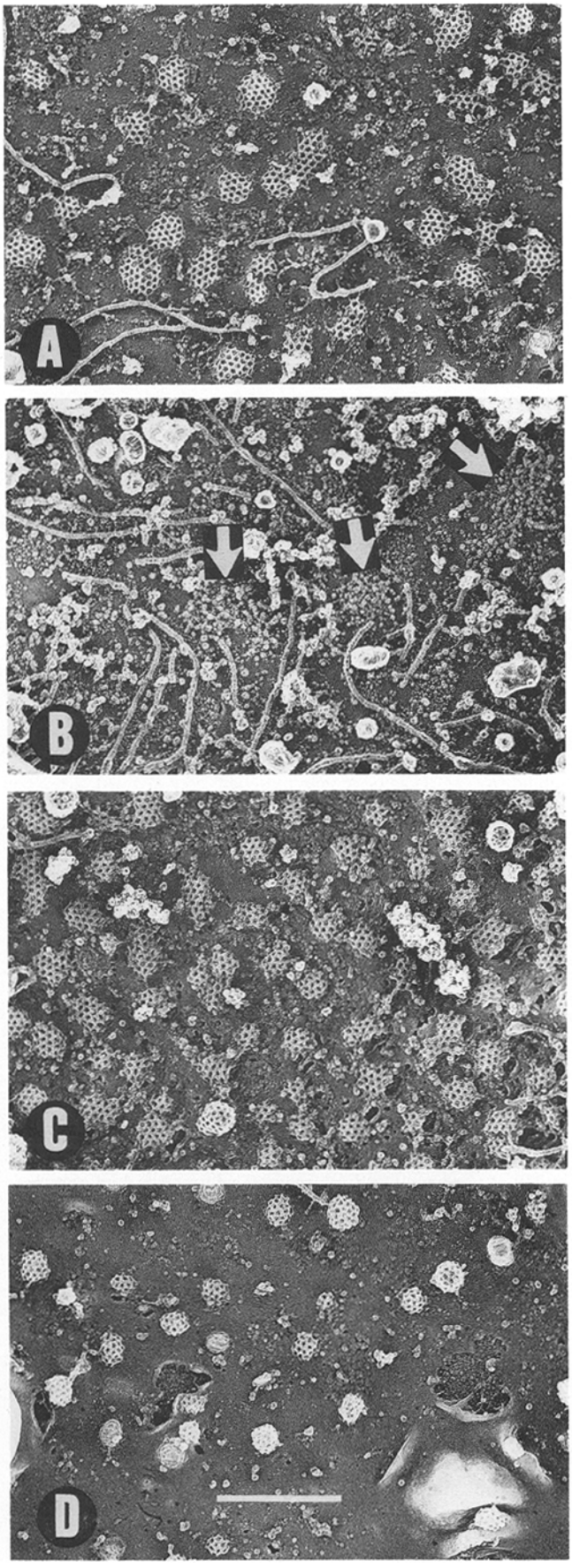


Figure 2. Elastase removes the appendages from endogenous AP-2 on isolated membranes but does not release either the cores or clathrin (*A*) and does not affect the ability of purified triskelions to rebind (*B*). (*A*) Isolated membranes were prepared and either not treated (*Untreated*), washed with high pH buffer (*Taps*), treated with 1 μg/ml of elastase for 30 min at 4°C (*Elastase*), or treated with elastase followed by washing with high pH buffer (*Elastase + Taps*). Membranes were fixed and then processed to measure the presence of either the AP-2 appendages with monoclonal AP.6 (□) The clathrin heavy chain with monoclonal X-22 (■), or the AP-2 cores with monoclonal BIM_6 (▣) as described. (*B*) In the same experiment, membranes that had been treated with elastase and stripped of clathrin were incubated with increasing amounts of purified triskelion (●) for 30 min at 4°C. The membranes were then fixed and assayed for the presence of clathrin heavy chain using mAb X-22. The amount of clathrin on the membrane before (■) and after (▲) the high pH treatment is indicated on the ordinate. Half maximal binding was calculated to be 12 nM. Clathrin did not bind to membranes that had been stripped of elastase-treated AP-2 before incubation with 100 μg/ml of purified triskelions for 30 min at 4°C (□, ordinate). All values are the average of duplicate measurements.

confirm that the isolated core binds to the membrane with about the same affinity as the whole AP-2 (data not shown).

We used the same approach to determine if the core also contains the triskelion binding site. We prepared membranes and treated them with a concentration of elastase that removes both the α and the β appendages from purified coat proteins (32) but does not effect the AP-2 binding site (19). Domain specific antibodies showed that these membranes had normal levels of the AP-2 cores (Fig. 2 *A*; elastase, □) and triskelion subunits (Fig. 2 A; elastase, ■) but lacked the appendages (Fig. 2 *A*; elastase, □). We then washed elastase-treated membranes with high pH to remove both the appendages (Fig. 2 *A*; Elastase + Taps, □) and the clathrin



(Fig. 2 *A*; Elastase + Taps, ■), but not the core (Fig. 2 *A*; Elastase + Taps, □). These membranes supported normal triskelion binding (Fig. 2 *B*). Binding was saturable with half maximal occupancy occurring at a triskelion concentration of 12×10^{-9} M. Binding did not occur if the elastase-treated AP-2 core was removed from the membrane before the addition of triskelions (Fig. 2 *B*, □ on the ordinate).

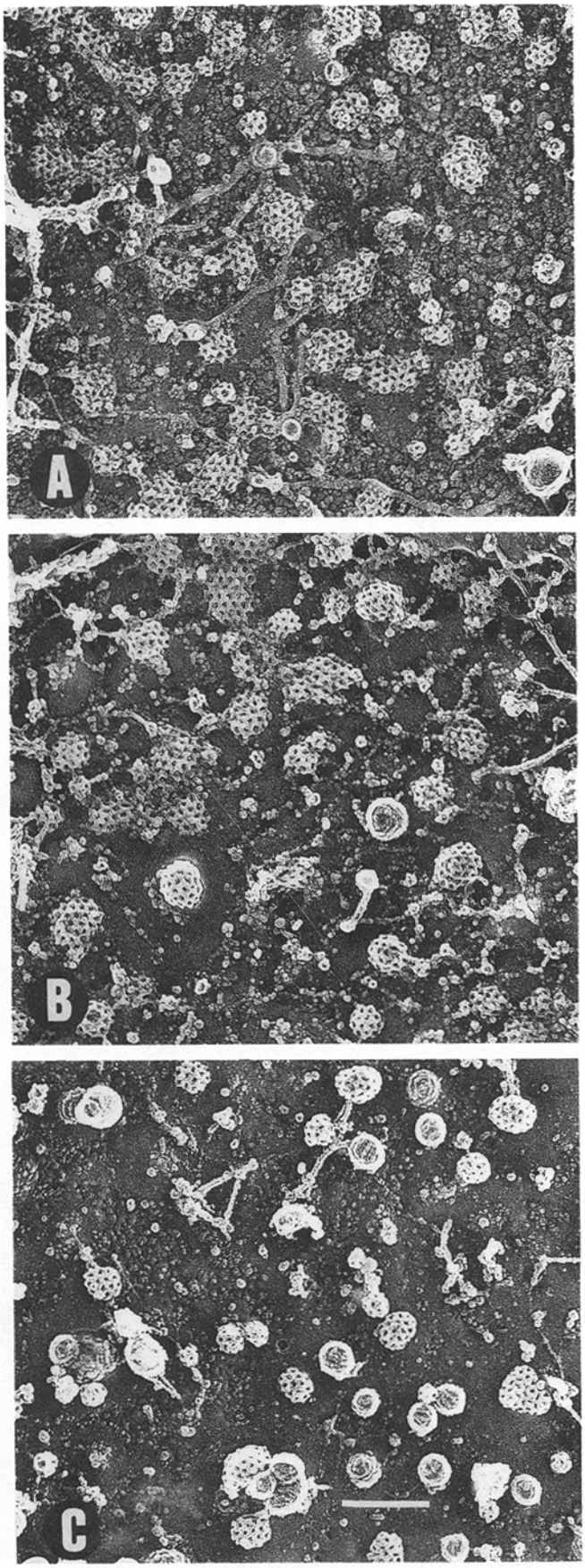
The previous experiment showed that high affinity binding of triskelions is not dependent on the AP-2 appendages (Fig. 2). We next used rapid-freeze, deep-etch microscopy to determine if normal lattices form after triskelions bind to elastase-treated, pH-stripped membranes (Fig. 2, Elastase + Taps). We prepared four different sets of membranes: untreated (Fig. 3 *A*); stripped of clathrin with high pH (Fig. 3 *B*); stripped and incubated in the presence of triskelions (Fig. 3 *C*); or elastase treated, stripped, and incubated in the presence of triskelions (Fig. 3 *D*). Intact AP-2 supported lattice assembly (compare Fig. 3, *B*, *C*) but the lattices were generally smaller than native lattices (compare Fig. 3, *A* with *C*). Elastase-treated AP-2 also supported lattice assembly (Fig. 3 *D*), although we consistently found that triskelions tended to form much more rounded lattices on these membranes.

Keen and co-workers (12, 13) previously showed that the appendage domain is essential for AP-stimulated assembly of cages. Cage formation is analogous to the rounding of a coated pit into a vesicle. Therefore, we used the purified membranes to see if elastase removal of the appendage domain affected coated pit invagination (Fig. 4). Initially all of the pits were flat (Fig. 4 *A*). Digestion at 4°C with elastase had little effect on the organization of the polygons (Fig. 4 *B*), although we noted that the lattices tended to have more curvature. All of the elastase-treated lattices became deeply invaginated (Fig. 4 *C*) after warming the membranes to 37°C.

Membrane Binding Site Is Released by Elastase

All that we know about the AP-2 binding site is that it is destroyed by elastase and that it is in coated pits (19). Several studies have suggested that clathrin coat proteins can bind to lipid vesicles (6, 28, 34), which raises the possibility that the AP-2 binding site might be a lipid. Therefore, we performed further tests to determine the macromolecular nature of the binding site. We started by determining whether or not elastase released a molecule that retained the ability to bind AP-2. We treated the cytoplasmic surface (inside) and the environmental surface (outside) of human fibroblast membranes with 0.5 M Tris to remove any AP-2 and clathrin be-

Figure 3. Reassembly of clathrin lattices on untreated membranes (*A–C*) or membranes that had been treated with elastase (*D*). Membranes were isolated on coverslips as described. One set was either fixed immediately (*A*), stripped of clathrin with a high pH buffer (*B*) or stripped and incubated in the presence of 20 μg/ml of triskelions for 1 h at 4°C (*C*). Another set (*D*) was first treated with elastase as described in Fig. 2 to remove the AP-2 appendage domain, stripped of clathrin and then incubated in the presence of 20 μg/ml purified triskelions for 1 h at 4°C. All samples were then fixed and processed for rapid-freeze, deep-etch microscopy as described. Arrows (*B*) indicate the location of coated pits that have been stripped of clathrin. Bar, 0.5 μm.



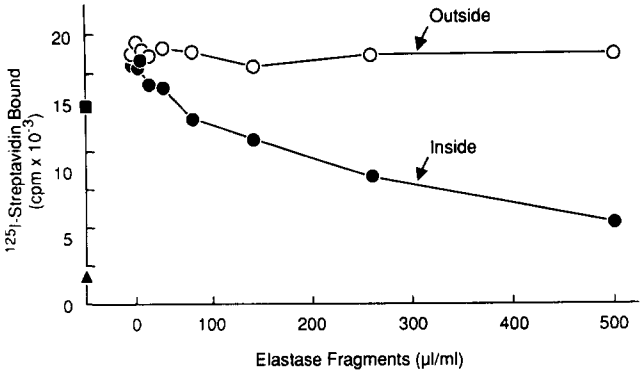


Figure 5. The effect of elastase fragments prepared from either the inside (●) or the outside (○) membrane surface on AP-2 binding. Elastase fragments were prepared as described. Keeping the volume of the incubation mixture constant (100 µl), varying amounts of either outside (○) or inside (●) fragments were added to purified AP-2 (10 µg/ml) and incubated for 10 min at 4°C. Membranes were then stripped of AP-2 and clathrin and incubated with this mixture for 30 min at 4°C. At the end of the incubation, the samples were fixed and processed for the detection of membrane bound AP-2 using monoclonal AP.6 (1 µg/ml) as described. The amount of AP-2 on the membrane before (■) and after (▲) the membranes were treated with Tris is indicated on the ordinate. All values are the average of duplicate measurements.

fore incubating them in the presence of 2 µg/ml of elastase. We added protease inhibitors to each fraction, mixed different amounts of each fraction with a constant amount of coat proteins (containing both AP-2 and clathrin) and incubated the mixture for 10 min at 4°C. Finally, we removed AP-2 and clathrin from isolated membranes and incubated these stripped membranes in the presence of the mixture for 30 min at 4°C. Fig. 5 shows that the addition of increasing amounts of inside fragments to the mixture caused a progressive inhibition of AP-2 binding to the membranes (Fig. 5, ●). By contrast, the same concentrations of outside fragments did not affect binding (Fig. 5, ○).

These results suggest that elastase releases a polypeptide capable of interacting with AP-2 in solution. To see if AP-2 would bind directly to the fragment, we prepared inside and outside elastase fragments and dried different amounts to the bottom of individual wells of a 96-well plate. We used gel electrophoresis to determine that the two preparations contained numerous peptides (data not shown). We then incubated each well in the presence of 10 µg/ml of coat proteins (Fig. 6). AP-2 did not bind to wells containing even the highest concentration of outside fragments (Fig. 6, ○). By contrast, inside fragments did support binding (Fig. 6, ●) and the amount of AP-2 that bound was proportional to the concentration of fragments added to the well. AP-2 bound to wells that received as little as 100 ng of inside elastase fragments.

Figure 4. Effect of elastase on clathrin coated pit invagination. Membranes were prepared as described and either not treated (*A*), incubated in the presence of 1 µg/ml of elastase for 30 min at 4°C (*B*), or incubated in the presence of elastase and then warmed to 37°C for 3 min (*C*). The samples were then fixed and processed for rapid-freeze, deep-etch microscopy as described. Bar, 0.25 µm.

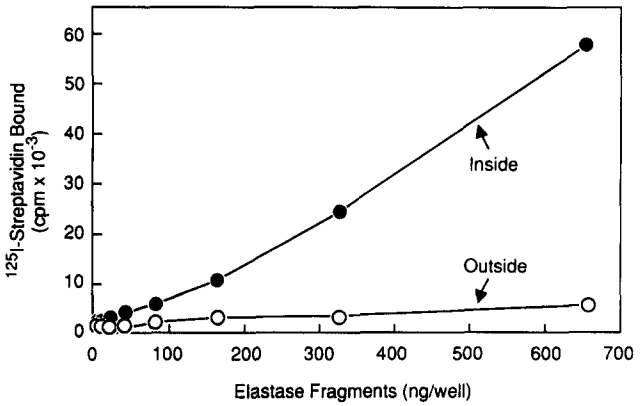


Figure 6. Binding of AP-2 to wells that contain varying amounts of either inside fragments (●) or outside fragments (○). Elastase fragments were prepared as described and the indicated amount was dried onto the surface of individual wells of a 96-well plate. Each well was then incubated with 10 μg/ml of purified AP-2 for 30 min at 4°C, fixed, and processed to detect AP-2 binding using monoclonal AP.6 (1 μg/ml) as described. All values are the average of duplicate measurements.

Elastase fragments also bound to immobilized AP-2. We prepared an affinity column to isolate the elastase fragments by attaching bovine brain APs (a mixture of AP-2 and the Golgi specific AP, AP-1) to glutaraldehyde-activated silica gel. We passed the elastase fragments from the cytoplasmic membrane surface over the column and washed extensively until there was no more protein in the flow through (Fig. 7, *B*, ●). The addition of 0.6 M Tris-HCl immediately released additional protein from the column (Fig. 7, *C*, ●). We collected each fraction and tested it for AP-2 binding activity

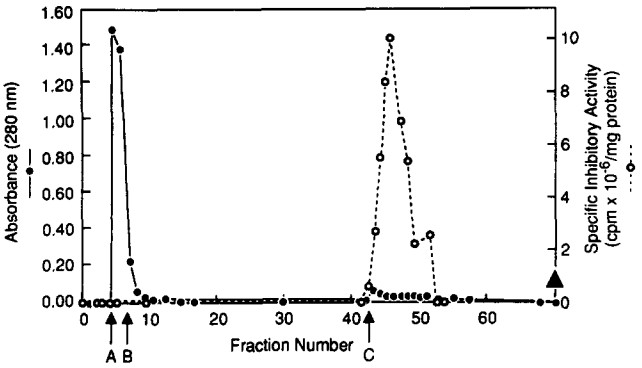


Figure 7. Affinity purification of an elastase fragment that contains the AP-2 binding site. Elastase fragments from the inside surface of the plasma membrane were prepared as described. 5 ml of this preparation was applied to an AP affinity column by repeated circulation. The flow-through was collected (*A*) and then the column was washed (*B*) until the absorbance at 280 nm (●) declined to zero. 0.6 M Tris was added (*C*) to release any proteins that had bound to the AP-2 and 0.5-ml fractions were collected. Each fraction was then tested for its ability to inhibit AP-2 binding to the inside surface of the plasma membrane (○). Specific inhibitory activity in the starting material is shown on the right ordinate (▲). Specific inhibitory activity equals the amount of binding in the absence of the fraction minus the amount of binding in the presence of the fraction per milligram of protein. All values are the average of duplicate measurements.

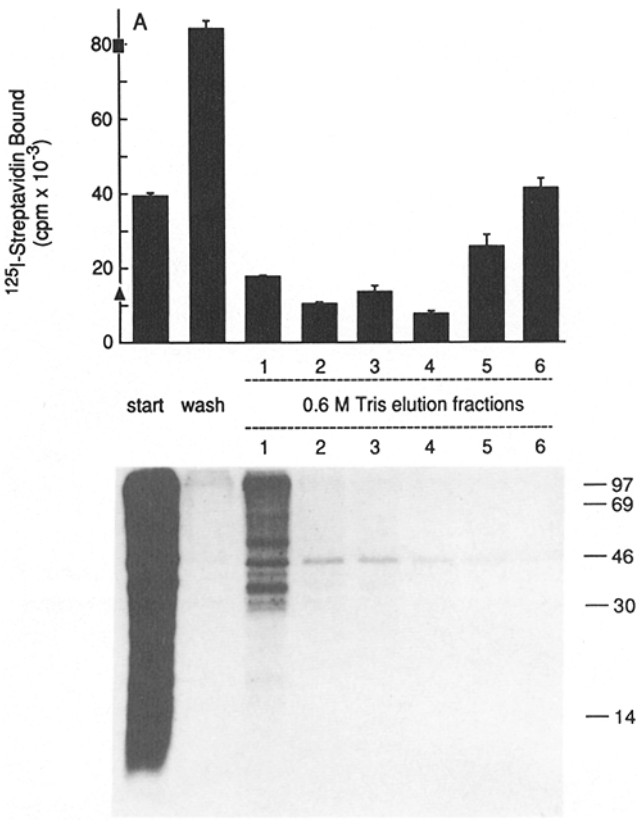


Figure 8. Identification of the AP-2 binding fragment. Elastase fragments were prepared from cells that had been grown in the presence of [^{35}S]methionine. The radiolabeled fragments were applied to an AP affinity column as described in Fig. 7. The column was washed, 0.6 M Tris-HCl was added, and the eluate was collected in six fractions. The starting material (*start*), the wash fraction (*wash*), and each eluate fraction (*1–6*) was tested for its ability to inhibit AP-2 binding to purified membranes (*A*) and analyzed by SDS-PAGE (*B*). The amount of AP-2 on the membrane before (■) and after (▲) the membranes were treated with Tris is indicated on the ordinate (*A*). All values are the average of duplicate measurements.

using the competition assay (Fig. 7, ○). The starting material had a modest amount of specific inhibitory activity (Fig. 7, ▲), but there was not any activity in the initial washes from the column. By contrast, the Tris eluate fractions (Fig. 7 *C*) contained significant inhibitory activity. The fraction with the highest specific activity had 30-fold more binding activity than the starting material.

We wanted to determine the molecular weight range of the peptides that bound to AP-2. Therefore, we radiolabeled elastase fragments from fibroblasts by growing the cells for 6 h in the presence of [^{35}S]methionine. We then applied fragments to the AP column. We eluted specifically bound fragment(s) with 0.6 M Tris and collected 0.5-ml fractions. The competition assay was used to assay each fraction for AP-2 binding (Fig. 8 *A*). The fractions were also analyzed by gel electrophoresis (Fig. 8 *B*). The first fraction off the column had multiple protein bands (Fig. 8 *B*, lane *1*). In succeeding fractions (lanes *2* to *4*), many bands disappeared until a band of ~45 kD was the dominant species on the gel (Fig. 8 *B*, lanes *3–5*). We found a correspondence between the presence of this band and the ability of the fraction to in-

hibit AP-2 binding to membranes (Fig. 8 *A*). Fraction 1 (Fig. 8 *A*, column *1*) had more inhibitory activity compared to the starting material (Fig. 8 *A*, *start*) and fractions *2–4* had comparable amounts of activity (Fig. 8 *A*, columns *2–4*) even though there were fewer bands on the gel (Fig. 8 *B*, lane *2–4*). As the intensity of the band declined in fraction *5* and *6*, there was also a decline in the ability of the fraction to inhibit AP-2 binding (compare Fig. 8 *B*, lanes *5* and *6* with Fig. 8 *A*, columns *5* and *6*).

Discussion

Different Functions for the Core and the Appendage Domains

Ordinarily investigators study the interaction between AP-2 and clathrin using clathrin in solution, clathrin immobilized on a support or clathrin assembled into cages (12). Keen and co-workers (13, 37, 38) have used these techniques to show that the core of AP-2 will not promote clathrin cage assembly but is able to bind to intact clathrin trimers. There appear to be two binding sites for the core of AP-2 on the triskelion legs (21, 27): one on the terminal domain and one near the hub region. In contrast to these studies, Schroder and Ungewickell (32) found that in a tartrate buffer system only intact APs would bind to clathrin. They concluded from their studies that tartrate had its affect by preventing the APs from aggregating.

The primary interaction between these two subunits ordinarily occurs within the cell during coated pit formation. We designed the in vitro assay used in the current study to mimic the conditions that favor these interactions (18, 20). When we treated membranes with elastase concentrations that are known to remove both the α and the β appendages from purified coated vesicle APs (32), clathrin coated pit assembly appeared to be unimpaired. We can not rule out the possibility that these conditions did not completely remove both appendages from the membrane bound APs. Nevertheless the affinity of interaction between clathrin and the bound, elastase treated AP-2 was unaltered (compare Fig. 2 *B* with Fig. 3 in reference 19). Furthermore, the bound triskelions formed into lattices that were normal in appearance.

Rapid-freeze, deep-etch images have previously shown that triskelions make contact with solid surfaces through the terminal portion of each leg (14) and that this is the region that interacts with APs in solution (11). Murphy et al. (21) recently showed that this region of the leg binds to the AP core domain. We propose that coated pit lattice assembly requires the high affinity binding of the triskelion terminal region to the core of AP-2. Possibly β-adaptin contains the binding site for clathrin (1).

The core is also the domain that mediates AP-2 binding to the inner membrane surface. Elastase treatment of intact coated pits removed the appendages without releasing the AP-2 core from the membrane, which indicates that the appendage does not have a high affinity for the binding site (Fig. 2 *A*). What is more important is that only the purified cores inhibited AP-2 binding to membranes (Fig. 1). The AP-2 domain that binds to the membrane could reside in the non-proteolysed, amino terminal portion of either α or β adaptin.

We conclude that all of the major interactions that occur between the membrane and clathrin during assembly of coated pits are mediated by the AP-2 core domain. This leaves the appendage domain available to carry out other key reactions necessary for coated pit function. One activity may be to cluster receptors that enter cells by receptor mediated endocytosis (3) while another may be to control the shape of the lattice.

Molecular evidence has suggested a role for the appendage domain in the adaptive ability of the AP-2 complex to recognize the cytoplasmic tails of multiple receptors that enter cells by coated pits (15, 26, 30). On the other hand, Beltzer and Spies (5) have found that the amino terminal domain of β-adaptin, which is present in the AP-2 core, binds a fusion protein that contains the cytoplasmic tail of the asialoglycoprotein receptor. Therefore, the exact role of the appendages in receptor clustering is not known.

Elastase treatment did not prevent coated pits from rounding up at 37°C. In fact, there seemed to be a tendency for elastase by itself to cause coated pits to change shape. We observed this in the shape change experiments (Fig. 4 *B*) and in the reassembly trials (Fig. 3 *D*). This raises the possibility that the appendage domain plays an active role in keeping the lattice in a planar configuration during assembly. This may be important for the proper clustering of receptors before budding and for the ordered addition of clathrin trimers into the forming polygonal lattice.

The AP-2 Receptor

The protein fragments released by elastase from the inside surface of the plasma membrane contain the high affinity, AP-2 binding site. The predominant band in the affinity-purified fraction has an apparent molecular weight of 45,000. This peptide is a candidate for containing the AP-2 binding site. Most likely the peptide or peptides that have the binding activity are derived from integral membrane proteins that are essential for coated pit assembly. Therefore, AP-2 seems not to be binding with high affinity to a specific class of plasma membrane lipids.

The isolation of peptide fragments, released specifically from the inside surface of membranes, that have AP-2 binding activity raises the possibility that plasma membranes contain an AP-2 receptor. The major function of this receptor would be to bind the core of the AP-2 and initiate coated pit assembly. The appendage domain, on the other hand, would be available to interact with membrane proteins that contain a reverse-turn, three-dimensional conformation in their cytoplasmic tail (4) and cause them to cluster before coated pit budding (3). This model agrees with rapid-freeze, deep-etch microscopy studies that show coated pit assembly precedes membrane receptor clustering (10). A hierarchical arrangement of domains within the AP-2 subunit gives each cell type the flexibility of controlling the uptake of a specific ligand without affecting the whole endocytic process (22).

We would like to thank Renée Stone for her dedicated technical assistance during the course of this study and Edith Womack for her assistance in providing the cultured human fibroblasts. This study would not have been possible without the generous supply of mAbs X-22 and AP-6 provided by Dr. Frances Brodsky and B1-M_6 provided by Dr. Margaret Robinson.

This work was supported by grants from the American Cancer Society, PF-3168 to J. S. Peeler and the National Institutes of Health, HL 20948.

Received for publication 27 July 1992 and in revised form 7 October 1992.

References

1. Ahle, S., and E. Ungewickell. 1989. Identification of a clathrin binding subunit in the HA2 adaptor protein complex. *J. Biol. Chem.* 264:20089–20093.
2. Ahle, S., A. Mann, U. Eichelsbacher, and E. Ungewickell. 1988. Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *EMBO (Eur. Mol. Biol. Org.) J.* 7:919–929.
3. Anderson, R. G. W. 1991. Molecular motors that shape endocytic membrane. *In* Intracellular Trafficking of Proteins. C. J. Steer and J. Hanover, editors. Cambridge University Press, London. 13–46.
4. Bansal, A., and L. M. Gierasch. 1991. The NPXY internalization signal of the LDL receptor adopts a reverse-turn conformation. *Cell.* 67:1195–1201.

4a. Beck, K. A., M. Chang, F. M. Brodsky, and J. H. Keen. 1992. Clathrin assembly protein AP-2 induces aggregation of membrane vesicles: a possible role for AP-2 in endosome formation. *J. Cell Biol.* 119:787–796.

5. Beltzer, J. P., and M. Spiess. 1991. In vitro binding of the asialoglycoprotein receptor to the beta adaptin of plasma membrane coated vesicles. *EMBO (Eur. Mol. Biol. Org. J.)* 10:3735–3742.
6. Blumenthal, R., M. Henkart, and C. J. Steer. 1983. Clathrin-induced pH-dependent fusion of phosphatidylcholine vesicles. *J. Biol. Chem.* 258: 3409–3415.
7. Brodsky, F. M. 1985. Clathrin structure characterized with monoclonal antibodies. I. Analysis of multiple antigenic sites. *J. Cell Biol.* 101:2047–2054.
8. Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of LDL in cultured cells. *Methods Enzymol.* 98:241–260.
9. Heuser, J. E. 1980. Three-dimensional visualization of coated vesicle formation in fibroblasts. *J. Cell Biol.* 84:560–583.
10. Heuser, J. E., and R. G. W. Anderson. 1988. Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J. Cell Biol.* 108:389–400.
11. Heuser, J. E., and J. H. Keen. 1988. Deep-etch visualization of proteins involved in clathrin assembly. *J. Cell Biol.* 107:877–886.
12. Keen, J. H. 1990. Clathrin and associated assembly and disassembly proteins. *Annu. Rev. Biochem.* 59:515–538.
13. Keen, J. H., and K. A. Beck. 1989. Identification of the clathrin-binding domain of assembly protein AP-2. *Biochem. Biophys. Res. Commun.* 158:17–23.
14. Kirchhausen, T., S. C. Harrison, and J. E. Heuser. 1986. Configuration of clathrin trimers: evidence from electron microscopy. *J. Ultrastruct. Res.* 94:199–208.
15. Kirchhausen, T., K. L. Nathanson, W. Matsui, A. Vaisberg, E. P. Chow, C. Burne, J. H. Keen, and A. E. Davis. 1989. Structural and functional division into two domains of the large (100- to 115-kDa) chains of the clathrin-associated protein complex AP-2. *Proc. Natl. Acad. Sci. USA.* 86:2612–2616.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 27:680–685.
17. Lin, H. C., M. S. Moore, D. A. Sanan, and R. G. W. Anderson. 1991. Reconstitution of coated pit budding from plasma membranes. *J. Cell Biol.* 114:881–891.
18. Mahaffey, D. T., M. S. Moore, F. M. Brodsky, and R. G. W. Anderson. 1989. Coat proteins isolated from clathrin coated vesicles can assemble into coated pits. *J. Cell Biol.* 108:1615–1624.
19. Mahaffey, D. T., J. S. Peeler, F. M. Brodsky, and R. G. W. Anderson. 1990. Clathrin-coated pits contain an integral membrane protein that binds the AP-2 subunit with high affinity. *J. Biol. Chem.* 265:16514–16520.
20. Moore, M. S., D. T. Mahaffey, F. M. Brodsky, and R. G. W. Anderson. 1987. Assembly of clathrin-coated pits onto purified plasma membranes. *Science (Wash. DC).* 236:558–563.
21. Murphy, J. E., and J. H. Keen. 1992. Recognition sites for clathrin-associated proteins AP-2 and AP-3 on clathrin triskelia. *J. Biol. Chem.* 267:10850–10855.
22. Pathak, R. K., M. Yokode, R. E. Hammer, S. L. Hofmann, M. S. Brown, J. L. Goldstein, and R. G. W. Anderson. 1990. Tissue-specific sorting of the human LDL receptor in polarized epithelia of transgenic mice. *J. Cell Biol.* 111:347–359.
23. Pearse, B. 1988. Receptors compete for adaptors found in plasma membrane coated pits. *EMBO (Eur. Mol. Biol. Org.) J.* 7:3331–3336.
24. Pearse, B. M. F., and M. S. Robinson. 1984. Purification and properties of 100-kd proteins from coated vesicles and their reconstitution with clathrin. *EMBO (Eur. Mol. Biol. Org.) J.* 3:1951–1957.
25. Pearse, B. M. F., and M. S. Robinson. 1990. Clathrin, adaptors, and sorting. *Annu. Rev. Cell Biol.* 6:151–171.
26. Ponnambalam, S., M. S. Robinson, A. P. Jackson, L. Peiperl, and P. Parham. 1990. Conservation and diversity in families of coated vesicle adaptins. *J. Biol. Chem.* 265:4814–4820.
27. Prasad, K., and J. H. Keen. 1991. Interaction of assembly protein AP-2 and its isolated subunits with clathrin. *Biochemistry.* 30:5590–5597.
28. Privat, J.-P., M. Egret-Charlier, H. Labbe, and M. Ptak. 1989. Interaction of clathrin coat proteins with unilamellar and multilamellar vesicles of phosphatidylcholine. *Biochim. Biophys. Acta.* 979:257–267.
29. Robinson, M. S. 1987. 100-kD coated vesicle proteins: Molecular heterogeneity and intracellular distribution studied with monoclonal antibodies. *J. Cell Biol.* 104:887–895.
30. Robinson, M. S. 1989. Cloning of cDNAs encoding two related 100-kD coated vesicle proteins (α-adaptins). *J. Cell Biol.* 108:833–842.
31. Robinson, M. S., and B. M. F. Pearse. 1986. Immunofluorescent localization of 100K coated vesicle proteins. *J. Cell Biol.* 102:48–54.
32. Schröder, S., and E. Ungewickell. 1991. Subunit interaction and function of clathrin-coated vesicle adaptors from the Golgi and the plasma membrane. *J. Biol. Chem.* 266:7910–7918.
33. Smith, P. K., R. J. Krohn, G. T. Hermanson, A. K. Malin, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76–85.
34. Steer, C. J., R. D. Klausner, and R. Blumenthal. 1982. Interaction of liver clathrin coat protein with lipid model membranes. *J. Biol. Chem.* 257: 8533–8540.
35. Vigers, G. P. A., R. A. Crowther, and B. M. F. Pearse. 1986. Location of the 100kD-50kD accessory proteins in clathrin coats. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2079–2085.
36. Virshup, D. M., and V. Bennett. 1988. Clathrin-coated vesicle assembly polypeptides: physical properties and reconstitution studies with brain membranes. *J. Cell Biol.* 106:39–50.
37. Zaremba, S., and J. H. Keen. 1983. Assembly polypeptides from coated vesicles mediate reassembly of unique clathrin coats. *J. Cell Biol.* 97: 1339–1347.
38. Zaremba, S., and J. H. Keen. 1985. Limited proteolytic digestion of coated vesicle assembly polypeptides. *J. Cell Biochem.* 28:47–58.