

Cytosol- and Clathrin-dependent Stimulation of Endocytosis In Vitro by Purified Adaptors

Elizabeth Smythe, Laura L. Carter, and Sandra L. Schmid

Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037

Abstract. Using stage-specific assays for receptor-mediated endocytosis of transferrin (Tfn) into perforated A431 cells we show that purified adaptors stimulate coated pit assembly and ligand sequestration into deeply invaginated coated pits. Late events in endocytosis involving membrane fission and coated vesicle budding which lead to the internalization of Tfn are unaffected. AP2, plasma membrane adaptors, are active at physiological concentrations, whereas AP1, Golgi adaptors, are inactive. Adaptor-dependent stim-

ulation of Tfn sequestration requires cytosolic clathrin, but is unaffected by clathrin purified from coated vesicles suggesting that soluble and assembled clathrin pools are functionally distinct. In addition to adaptors and cytosolic clathrin other, as yet unidentified, cytosolic factors are also required for efficient coated pit invagination. These results provide new insight into the mechanisms and regulation of coated pit assembly and invagination.

THE cellular uptake of many essential nutrients, growth factors, and hormones is mediated by clathrin-coated pits and coated vesicles (recently reviewed by Smythe and Warren, 1991). Ligands bind with high affinity to specific transmembrane receptors which become concentrated, as a consequence of signals contained within the receptor cytoplasmic tail (reviewed by Trowbridge, 1991), in specialized regions of the plasma membrane termed coated pits. The coated pits invaginate and a membrane fission event results in coated vesicle formation. After an uncoating reaction in the cytosol, the released coat constituents recycle for additional rounds of assembly, invagination and budding. The uncoated transport vesicles deliver their contents to the endosomal compartment.

Coated pits and vesicles have been well characterized both morphologically and biochemically. The structure and biochemistry of the major coat components, clathrin and adaptors (originally referred to as assembly proteins), has been extensively studied (reviewed by Brodsky, 1988; Morris et al., 1989). Clathrin triskelions, composed of three 180-kD heavy chains and three tightly associated ~30-kD light chains, form the polygonal lattice of the coat. Adaptors (APs)¹ are heterotetrameric molecules consisting of two distinct ~100-kD subunits, referred to as "adaptins" and two smaller subunits of unknown function. APs were first described in the functional context of mediating clathrin assembly into coat structures in the absence of membranes

(Zaremba and Keen, 1983). There are two classes of APs in all cells which differ in their subunit composition and in their subcellular localization (reviewed by Morris et al., 1989; Pearse and Robinson, 1990; Keen, 1990). AP1 is specifically associated with coated pits in the Golgi region of the cell and consists of γ and β' adaptins and smaller subunits of ~47 and ~19 kD. AP2 is specifically associated with coated pits at the plasma membrane and consists of α and β adaptins and smaller subunits of ~50 and ~17 kD. APs underlie the clathrin lattice and appear to interact directly with the membrane (reviewed by Pearse and Crowther, 1987). The nature of clathrin-AP interactions and their reassembly into coat structures in vitro has been extensively studied. In addition, recent studies suggest that APs might interact directly with the cytoplasmic tails of receptors concentrated in coated pits (Pearse, 1988; Glickman et al., 1989; Beltzer and Spiess, 1991). These results have led to the model that APs mediate clathrin assembly onto the membrane and perhaps function in concentrating receptors into coated pits (reviewed by Pearse and Robinson, 1990; Keen, 1990).

Despite considerable biochemical understanding of the structural components of coated pits, very little information is available concerning the regulation of coat protein assembly from the soluble pool onto the membrane to form coated pits. Similarly the mechanisms of coated pit invagination and coated vesicle budding remain obscure (for review see Schmid, 1992). A clearer understanding of these mechanisms would benefit from the use of cell-free assays for coat proteins in the functional context of receptor-mediated endocytosis. Towards this goal, we have developed stage-specific assays using perforated A431 cells which measure distinct events leading to the internalization of transferrin

Elizabeth Smythe's present address is Department of Biochemistry, University of Dundee, Dundee, DDI 4HN, Scotland.

1. *Abbreviations used in this paper:* AP, adaptor; Tfn, transferrin.

into sealed coated vesicles and/or to its sequestration into deeply invaginated coated pits (Smythe et al., 1989; Schmid and Smythe, 1991). Within this system a variety of partial reactions have been shown to occur, including de novo coated pit assembly, coated pit growth and invagination, and a membrane fission event leading to coated vesicle budding. Each of these partial reactions contributing to the productive sequestration or internalization of ligand requires elevated temperatures. In addition, using both morphological and biochemical approaches (Smythe et al., 1989; Schmid and Smythe, 1991), we have demonstrated that both coated pit assembly and coated vesicle budding require ATP hydrolysis and cytosol.

Here, we demonstrate that clathrin and adaptors are among the cytosolic factors required for receptor-mediated endocytosis in perforated A431 cells. Using these stage-specific assays, we have examined the role of purified coat proteins in the functional context of coated vesicle formation. We demonstrate that adaptors purified from coated vesicles stimulate coated pit assembly and enhance both the rate and extent of ligand sequestration into deeply invaginated coated pits. Results from preincubation protocols suggest that adaptors bind to membranes in an ATP, cytosol, and temperature independent manner to initiate coated pit assembly. These results are in agreement with earlier studies on adaptor binding to isolated plasma membranes (Mahaffey et al., 1989, 1990). However, subsequent events leading to productive coated pit formation as assessed by ligand sequestration require elevated temperatures and are ATP and cytosol dependent. Cytosol pretreated with anticlathrin antibodies is inactive and a cytosolic fraction enriched in clathrin is ~40-fold more active than unfractionated cytosol in mediating AP-stimulated ligand sequestration. Surprisingly, clathrin purified from coated vesicles is inactive. These results indicate a previously undetected functional distinction between assembled and unassembled pools of clathrin. In addition to adaptors and cytosolic clathrin other, as yet unidentified, cytosolic factors are required for productive coated pit invagination and for coated vesicle budding.

Materials and Methods

Cell Lines and Antibodies

The human adenocarcinoma cell line, A431, was obtained from Graham Warren (Imperial Cancer Research Fund, London) and was grown in DME (Gibco Laboratories, Grand Island, New York) supplemented with 10% FCS and 100 U/ml each of penicillin and streptomycin. Cells were passaged every 2–3 d by trypsinization. Cells required for an in vitro experiment were seeded overnight as previously described (Schmid and Smythe, 1991; Smythe et al., 1992).

Sheep antitransferrin antibody was a gift of the Scottish Antibody Production Unit. Anticlathrin monoclonal X22 was a generous gift from Frances Brodsky (University of California, San Francisco, CA). Antiadap-tin mAbs 100/1 and 100/2 were generously provided by Ernst Ungewickell (Max-Planck Institute for Biochemistry, Martinsried, Germany).

Preparation of Coat Proteins from Bovine Brain

Coat proteins were isolated from bovine brains which had been frozen on dry ice immediately after slaughter and stored at -70°C . Coated vesicles were prepared by differential centrifugation, essentially as described by Campbell et al. (1984). Briefly, 1 kg of tissue was homogenized in an equal volume of 100 mM Mes (2-[N-morpholino]-ethansulfonic acid), pH 6.5, 0.5

mM MgCl_2 , 1 mM EGTA, 1 mM PMSF, 0.1 mM DTT (Buffer A). The homogenate was centrifuged in a Beckman JA-10 rotor (Beckman Instruments, Inc., Fullerton, CA) at 10,000 rpm for 30 min. The supernatant was removed and saved. The pellet was rehomogenized and centrifuged as above. The combined supernatants were centrifuged at 30,000 rpm in a Beckman Ti 45 rotor (Beckman Instruments, Inc.) for 1 h. The resulting microsomal pellet was resuspended in ~100 ml of buffer A and an equal volume of a Ficoll (Sigma Type 400-DL; Sigma Chemical Co., St. Louis, MO)/sucrose solution (both 12.5% wt/vol in buffer A) was added. After centrifugation in a Beckman JA20 rotor (Beckman Instruments, Inc.) at 18,500 rpm for 40 min, the supernatant was diluted fivefold with buffer A and centrifuged for 1 h in a Ti45 rotor at 33,000 rpm. The pellet containing the crude coated vesicles was resuspended in 100 ml of extraction buffer (3 vols 1 M Tris, pH 8:1 vol buffer A) and extraction allowed to proceed for 10 min at room temperature (Keen, 1987). After centrifugation for 60 min at 45,000 rpm in a Ti45 rotor, the supernatant was subjected to a 50% $(\text{NH}_4)_2\text{SO}_4$ precipitation and the pellet, collected by centrifugation at 10,000 rpm for 15 min in a JA-14 rotor. This fraction was resuspended in ~20 ml of column buffer (1 vol 1 M Tris, pH 7.0:1 vol buffer A containing 0.1 mM DTT) and loaded on a Sephacryl S-400 (Pharmacia Fine Chemicals, Piscataway, NJ) column (3.5 × 135 cm) equilibrated in column buffer. The column was eluted overnight at a flow rate of 60 ml/h. Clathrin- and adaptor-containing fractions, detected by SDS-PAGE, were pooled and precipitated with 50% $(\text{NH}_4)_2\text{SO}_4$. The clathrin pellet was resuspended and dialyzed against assay buffer (100 mM K-Acetate, 85 mM sucrose, 20 mM Hepes, 1 mM MgAcetate, pH 7.4, referred to as KSHM) and the adaptor pellet against 10 mM Tris, pH 8.5, containing 0.1 mM DTT. Both fractions were stored in small aliquots at -70°C without loss of activity. After thawing for use in the assay the aliquots were kept at 4°C where they remained active for 3–4 d. Clathrin isolated by this procedure efficiently (>70%) assembled into empty cages after dialysis against 20 mM Mes, 1 mM EDTA, 2 mM CaCl_2 , pH 6.3. Routinely, ~35 mg of purified clathrin and ~10 mg of purified adaptors were obtained from 1 kg of brain. No increases in either yield or specific activity were observed in adaptors purified from the brains of freshly slaughtered cattle.

Purification of AP1 and AP2 Adaptor Complexes

Separation of AP1 and AP2 adaptors was carried out on clathrin–Sephacryl exactly as described by Keen (1987). Briefly, APs obtained after chromatography on S400 were mixed with clathrin–Sephacryl in column buffer and then dialyzed overnight against Buffer D (0.1 M Mes, 1 mM EDTA, 0.1 mM DTT, 10% glycerol, 0.02% sodium azide, pH 6.5) with vigorous stirring. The suspension was transferred to a column and washed at room temperature with Buffer D. Fractions containing AP1, AP3, and contaminating proteins were eluted in Buffer D. AP2 complexes were retained and could be eluted with 0.4 M Tris in Buffer D. After elution from clathrin–Sephacryl the pooled fractions were concentrated using an Amicon Centriprep-10 concentrator (Amicon, Beverly, MA). They were then stored at 4°C . Before addition to the assay they were dialyzed against 10 mM Tris, pH 8.5. The purity of the adaptor complexes was assessed by SDS-PAGE using a gradient of 5–15% acrylamide (Keen, 1987) and by immunoblotting with mAb, 100/2, specific to the α subunit of AP2 (Schröder and Ungewickell, 1991).

Purification of Cytosolic Clathrin

For the isolation of cytosolic clathrin, 400 ml of the cytosol fraction from the coated vesicle preparation was precipitated with 30% $(\text{NH}_4)_2\text{SO}_4$. The pellet was resuspended in 40 ml buffer B (10 mM sodium phosphate, pH 7.2, 25 mM NaCl, 0.1 mM DTT and 0.1 mM PMSF) and dialyzed extensively against buffer B. The preparation was then applied to a DEAE cellulose column (2.5 × 10 cm) equilibrated in buffer B. After extensive washing with buffer B, bound proteins were eluted with a linear gradient of 0–1 M KCl in buffer B. Clathrin-containing fractions were detected by Western blotting using a polyclonal antiserum raised against purified clathrin which recognizes both heavy and light chains. These fractions were pooled and precipitated with 50% $(\text{NH}_4)_2\text{SO}_4$. The pellet was resuspended in 30 ml 0.2 M Tris-HCl, 1 mM EDTA, 0.1 mM DTT, 10% glycerol, pH 6.5, combined with 17 ml of clathrin–Sephacryl and dialyzed overnight against two changes of Buffer D. The clathrin–Sephacryl was transferred to a column, washed extensively with Buffer D and then eluted with 0.4 M Tris in Buffer D. The final fraction, enriched in cytosolic clathrin, was concentrated in an Amicon Centriprep-10 (Amicon, Beverly, MA) and stored at 4°C after dialysis against KSHM.

Preparation of Cytosol

Cytosol for assays was prepared from bovine brain homogenized in KSHM as previously described (Schmid and Smythe, 1991).

Assay for ^{125}I -BSST Internalization in Perforated A431 Cells

Human diferric transferrin (Boehringer Mannheim Corp., Indianapolis, IN) biotinylated and iodinated (^{125}I -BSST) as described previously (Schmid and Smythe, 1991), was used as a ligand for internalization. Standard incubations contained an ATP regenerating system, 4 $\mu\text{g}/\text{ml}$ ^{125}I -BSST, bovine brain cytosol and coat proteins as indicated for each experiment. All components were mixed in 1.5 ml eppendorf tubes on ice in 30 μl KSHM before addition of $\sim 1\text{--}2 \times 10^5$ perforated A431 cells in 10 μl KSHM. Incubations were initiated by transfer to 37°C waterbaths and terminated by return to ice. The antibody inaccessibility and MesNa resistance assays were carried out exactly as previously described (Schmid and Smythe, 1991; Smythe et al., 1992). For each experiment the signal obtained in the absence of ATP (generally $\sim 7\text{--}12\%$ of total cell associated ^{125}I -BSST) has been subtracted as background so that the data shown represent ATP-dependent events. Since neither ATP γS nor AMPPNP will substitute for ATP in either of these reactions (Smythe et al., 1989; C. LaMaze, Carter and Schmid, unpublished results) the ATP-dependent events presented in the data shown represent energy-requiring events.

Other Procedures

Protein determination was carried out using the Pierce BCA assay (Pierce Chemical Co., Rockford, IL) or by measurement of the absorbance at 280 nm using an extinction coefficient of 11.0 for clathrin (Winkler and Stanley, 1983) and 8.7 for AP2 (Keen, 1987). SDS-PAGE was performed according to the method of Laemmli et al. (1970). For Western blotting, gels were electrotransferred to sheets of reinforced nitrocellulose (Schleicher and Schuell, Keene, NH) using a Bio-Rad (Richmond, CA) mini-gel apparatus. Transfer was for 1.5 h at 100 V in 49.6 mM Tris, 384 mM glycine, 20% (vol/vol) methanol, 0.01% SDS. Blots were blocked in TBS containing 3% milk protein. Blots were incubated overnight with anti-clathrin serum at 1:1,000 dilution and with anti-adaptin mAbs at 10 $\mu\text{g}/\text{ml}$. Clathrin or APs were visualized using alkaline phosphatase conjugated to secondary antibodies (Pierce Chemical Co.).

Results

Purified Adaptors Stimulate Sequestration of ^{125}I -BSST in Perforated A431 Cells

We have previously described stage-specific assays for receptor-mediated endocytosis into perforated A431 cells (Schmid and Smythe, 1991; Smythe et al., 1992). ^{125}I -Transferrin (Tfn) which is biotinylated via a cleavable disulphide bond (referred to as ^{125}I -BSST) is used as a ligand for internalization via the Tfn-receptor. The internalization of ^{125}I -BSST into sealed coated vesicles is measured by its acquisition of resistance to the low molecular weight membrane impermeant reducing agent, MesNa (sodium, β -mercaptoethane sulfonate). The inaccessibility of ^{125}I -BSST to exogenously added antitransferrin antibodies, termed "sequestration," occurs as a result of both its inclusion into deeply invaginated coated pits and its internalization into sealed coated vesicles (Schmid and Carter, 1990; Schmid and Smythe, 1991). By measuring separate but overlapping events in receptor-mediated endocytosis, these assays provide a means of dissecting three biochemically distinct events in coated vesicle formation: coated pit assembly, invagination, and vesicle budding.

Since receptor-mediated endocytosis in vitro is inhibited by the addition of anti-clathrin antibodies (Schmid and Smythe, 1991), this system should provide a means for ex-

amining the role of coat proteins in the functional context of ligand sequestration and internalization. We therefore tested the effect of purified clathrin and adaptors, the major coat proteins, on ^{125}I -BSST internalization. Perforated A431 cells were prepared and incubated under standard assay conditions, as described in Materials and Methods. Purified adaptors and/or clathrin were added to reaction mixtures in the absence of cytosol or in the presence of either low or high levels of cytosol, as indicated in Fig. 1. After incubation for 30 min at 37°C, the sequestration of ^{125}I -BSST in vitro was assessed using the antibody-inaccessibility assay. The data in Fig. 1 shows that in the absence of cytosol, the presence of purified clathrin and/or adaptors had no effect on the extent of Tfn sequestration in vitro. In contrast, in the presence of limiting concentrations of cytosol (0.5–1 mg/ml), the addition of adaptors purified from coated vesicles stimulated ^{125}I -BSST sequestration more than twofold, to a level greater than or equal to that seen in the presence of maximal (5–7 mg/ml) cytosol.

Clathrin purified from coated vesicles had no effect on ^{125}I -BSST sequestration either in the absence or presence of cytosol (Fig. 1). In addition, the presence of clathrin had no effect on adaptor-dependent stimulation of Tfn sequestration. As this result was unexpected, the effect of clathrin alone or in the presence of adaptors was extensively examined under a variety of conditions including a full range of cytosol concentrations, and a full range of ratios of clathrin to adaptors. Other sources of cytosol containing lower levels of clathrin, such as human K562 cell cytosol, were also

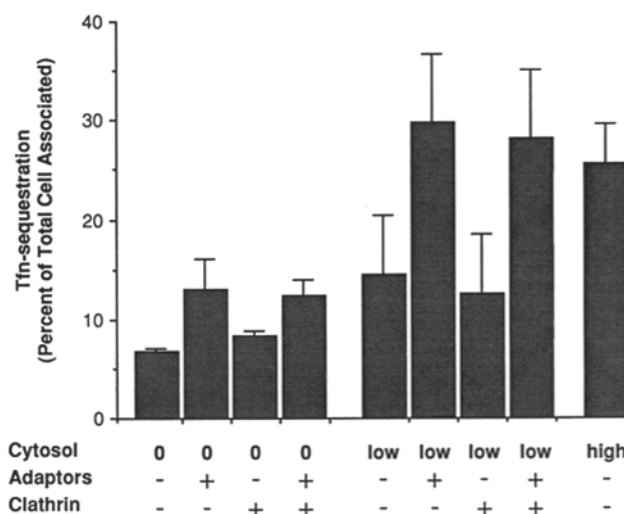


Figure 1. Purified adaptors stimulate ATP-dependent Tfn sequestration in vitro. Perforated A431 cells were prepared and incubated at 37°C as described in Materials and Methods. All incubations were in 40 μl of KSHM containing an ATP regenerating system and 4 $\mu\text{g}/\text{ml}$ ^{125}I -BSST and in addition, as indicated, bovine brain cytosol at low (0.75 mg/ml) or high (6.5 mg/ml) concentrations, 10 μg adaptors and/or 10 μg clathrin. The ATP-dependent sequestration of ^{125}I -BSST from exogenously added anti-Tfn antibodies is indicated and expressed as a percentage of the total cell associated ^{125}I -BSST after subtracting ATP-independent backgrounds, as described in Materials and Methods. The data shown are the average (\pm SD) of six independent experiments using at least three different preparations of APs and clathrin.

tested. In addition, we confirmed that the isolated clathrin was capable of self-assembly into cages (see Materials and Methods). However, we were unable to detect any effect of addition of coated vesicle-derived clathrin on Tfn sequestration *in vitro* (data not shown).

Purified Adaptors Stimulate Early but not Late Events in Tfn Internalization

Our earlier studies suggested that although newly formed coated pits could efficiently invaginate to sequester receptor-bound ^{125}I -BSST *in vitro*, they were unable to form sealed coated vesicles (Schmid and Smythe, 1991). Thus we suggested that coated vesicles which were formed *in vitro* were

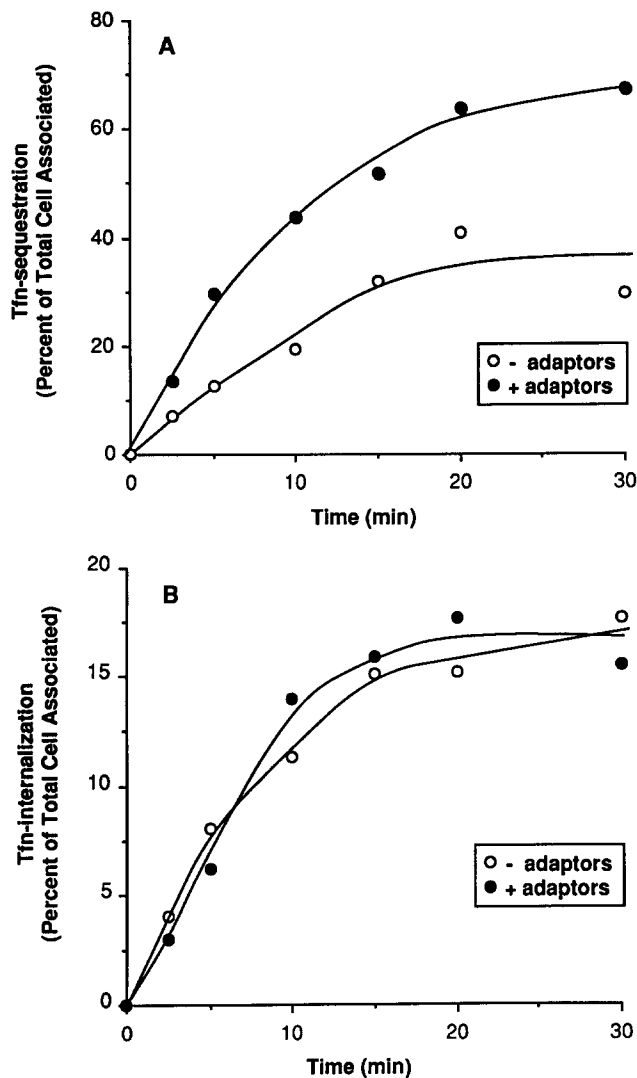


Figure 2. Purified adaptors stimulate sequestration into deeply invaginated pits but not coated vesicle budding. Perforated cells were incubated in a complete assay mixture containing 0.6 mg/ml bovine brain cytosol with (●) or without (○) 10 μg of adaptors for the indicated times at 37°C before returning to ice and analysis for either Ab inaccessibility (A) or MesNa-resistance (B) as described in Materials and Methods. The backgrounds obtained after a 30-min incubation on ice (4% for Ab inaccessibility, 11% for MesNa-resistance) have been subtracted from the results shown.

derived from a subpopulation of “activated” coated pits which had already been assembled *in vivo* before preparing perforated A431 cells. This hypothesis would predict that late events measured by acquisition of MesNa resistance would be unaffected by factors which enhance early events such as coated pit assembly. The latter would result only in enhanced sequestration of ligands into deeply invaginated coated pits. To test this hypothesis and to further explore the mechanism of adaptor stimulated ^{125}I -BSST internalization, we compared the effect of purified adaptors on the rate of ^{125}I -BSST internalization into perforated A431 cells in a standard incubation as assessed by either MesNa-resistance, which measures coated vesicle budding or Ab-inaccessibility, which measures coated pit invagination and coated vesicle budding. While purified adaptors caused a more than twofold increase in both the rate (i.e., the percent of Tfn sequestered/min) and extent of ^{125}I -BSST sequestration as assessed by Ab-inaccessibility (Fig. 2A), they had no effect on either the rate or extent of ^{125}I -BSST internalization as assessed by the acquisition of MesNa-resistance (Fig. 2B). This result is consistent with our previous model for receptor-mediated endocytosis *in vitro* (Schmid and Smythe, 1991) and indicates, as expected, a role for adaptors in early events leading to the formation of deeply invaginated coated pits.

Adaptor-Dependent Stimulation of ^{125}I -BSST Sequestration Requires Cytosolic Factors

Efficient receptor-mediated endocytosis into perforated A431 cells requires high concentrations of cytosol which could reflect the need for stoichiometric amounts of soluble coat constituents. To test whether adaptors were indeed a limiting

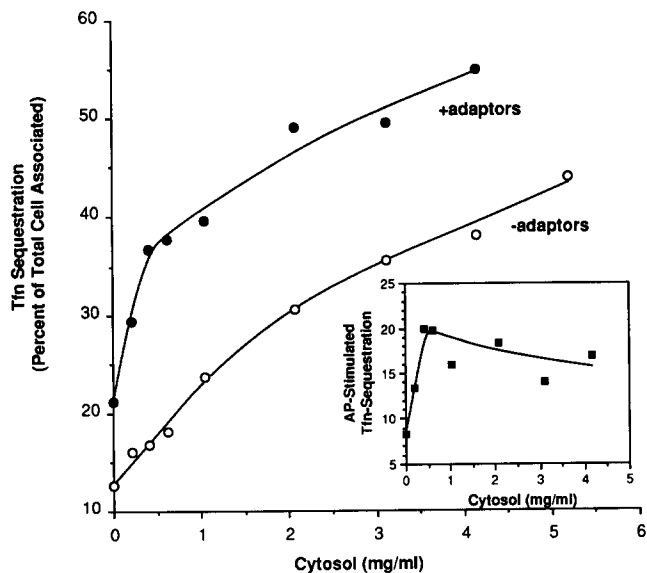


Figure 3. AP-stimulated Tfn sequestration is cytosol dependent. Perforated A431 cells were incubated for 30 min at 37°C with increasing concentrations of bovine brain cytosol in the presence (●) or absence (○) of 10 μg AP and then returned to ice for determination of the extent of Tfn sequestration by Ab inaccessibility. The calculated difference between each set of points, which reflects the cytosol dependence of AP-stimulated sequestration, is shown in the inset.

cytosolic factor and to determine whether other cytosolic factors were required for adaptor-stimulated sequestration we titrated the cytosolic requirement for ^{125}I -BSST sequestration in the presence and absence of adaptors. The data in Fig. 3 compares the effect of increasing concentrations of cytosol on the extent of ^{125}I -BSST sequestration into perforated A431 cells in the presence or absence of adaptors. The cytosol dependence in the absence of adaptors was subtracted from that in the presence of adaptors to generate the data shown in the inset which reflects the cytosol dependence of adaptor-stimulated ^{125}I -BSST sequestration. In the absence of adaptors, half-maximal activity requires >2 mg/ml cytosol. This cytosol requirement is reduced by almost 10-fold in the presence of purified adaptors (half-maximal adaptor-dependent stimulation required ~ 0.2 mg/ml cytosol, see *inset* Fig. 3). This result suggests that adaptors are a limiting component in the cytosolic fraction *in vitro* and that other cytosolic factors are required for coated pit growth and invagination.

AP2 Specifically Stimulates Tfn Sequestration

Two classes of adaptors exist in all cell types: AP1 which localizes to the Golgi complex and AP2 which is found at the plasma membrane (Robinson and Pearse, 1986; Robinson, 1987). Both AP1 and AP2 are present in the adaptor fraction isolated chromatographically after extraction of bovine brain coated vesicles (Fig. 4, lane 3). Also present in this fraction is AP3, an ~ 155 kD neuron-specific adaptin-

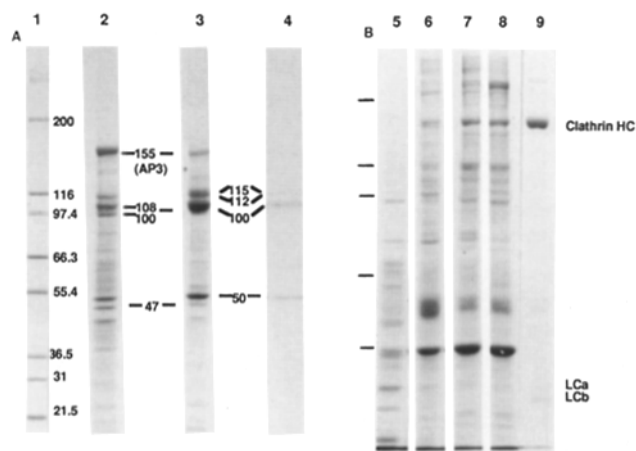


Figure 4. SDS-PAGE of coat protein preparations. (A) SDS-PAGE (5–15% acrylamide) of purified AP complexes stained with Coomassie blue. (Lane 1) Molecular weight markers (Novex); (lane 2) clathrin-Sepharose flow-through containing AP1 polypeptides (108, 100 and 47 kD, as indicated), AP3 (155 kD) and contaminating bands; (lane 3) Sephacryl S400-purified APs; and (lane 4) fraction eluted from clathrin-Sepharose with 0.4 M Tris containing AP2 polypeptides (115, 112, 100, and 50 kD, as indicated). (Note the absence of the 47-kD subunit most diagnostic of AP1). (B) SDS-PAGE (8% acrylamide) stained with Coomassie blue (20 μg of each fraction was loaded per lane). (Lane 5) Bovine brain cytosol; (lane 6) 30% ammonium sulfate fraction; (lane 7) clathrin-containing pool from DEAE-cellulose; (lane 8) bound and eluted fraction from clathrin-Sepharose; and (lane 9) 5 μg of clathrin purified from coated vesicles. Mol. wt. markers indicated at left were 200, 116, 97.4, 66, and 43 kD.

related molecule of unknown function (Murphy et al., 1991). If the observed stimulation of Tfn sequestration by adaptors represents their physiological role either in de novo-coated pit formation or growth at the cell surface, then only AP2 should be active. To test this expectation, the adaptor complexes were separated from each other by affinity chromatography on clathrin-Sepharose (Keen, 1987). In this procedure, AP1, AP3, and contaminating proteins fail to bind clathrin-Sepharose and are eluted in the column washes (Fig. 4, lane 2) whereas AP2 is selectively retained and eluted with 0.4 M Tris (Fig. 4, lane 4). The absence of contaminating amounts of AP2 in the flow through fractions was verified by immunoblotting with mAbs specific for AP2 (Schröder and Ungewickell, 1991, data not shown) and by gradient gel analysis which allows AP1 and AP2 to be distinguished based on subunit composition (note the absence of the AP1-specific 47-kD subunit in AP2 fractions, Fig. 4, lane 4).

Purified AP1 or AP2 complexes were added to perforated A431 cells in the presence of a limiting amount of cytosol and the extent of Tfn sequestration was measured by Ab-inaccessibility after 30 min (Fig. 5 A). Addition of equal amounts of purified AP1 and AP2 complexes showed that only AP2 adaptors were capable of stimulating Tfn sequestration. AP1 (and AP3) complexes were inactive, or slightly inhibitory. This demonstrates the specificity of the adaptor-dependent stimulation since only those adaptors which are found in coated pits at the cell surface increased the extent of Tfn sequestration.

The concentration dependence for AP stimulation of Tfn sequestration is shown in Fig. 5 B. Half-maximal stimulation of Tfn-sequestration was obtained in the presence of ~ 0.2 μM of S400 purified APs. Further purification of this mixed AP fraction by affinity chromatography on clathrin-Sepharose resulted in an ~ 1.5 –2-fold increase in specific activity (data not shown). These values are consistent with previous K_d values obtained for AP-membrane associations (Virshup and Bennett, 1988; Mahaffey et al., 1990) and AP-clathrin interactions *in vitro* (Schröder and Ungewickell, 1991).

A Role for Clathrin in Adaptor-stimulated Tfn Sequestration

As indicated, adaptor-stimulated sequestration was independent of the addition of exogenous purified clathrin (Fig. 1). However, since it was likely that the added adaptors were involved in either de novo-coated pit assembly or coated pit growth, it would be expected that clathrin would also be involved in this process. To further investigate a role for clathrin we examined the effect of the anticlathrin antibody, X22, on AP-stimulated ^{125}I -BSST sequestration. X22 is a well-characterized antibody which inhibits clathrin assembly *in vitro* (Blank and Brodsky, 1986) and receptor-mediated endocytosis both *in vivo* (Doxsey et al., 1987; Chin et al., 1989) and *in vitro* (Schmid and Smythe, 1991). Cytosol (3.5 mg/ml) was preincubated for 30 min on ice with X22 in the presence or absence of excess clathrin or with a nonspecific IgG of the same subclass. The cytosol was then diluted fourfold into a standard reaction mixture and assayed for its ability to support adaptor stimulated sequestration (Fig. 6). Pretreatment of cytosol with X22 reduced AP stimulated Tfn sequestration seen in the presence of low cytosol from 16 to 3%, corresponding to an $\sim 80\%$ inhibition. Purified clathrin

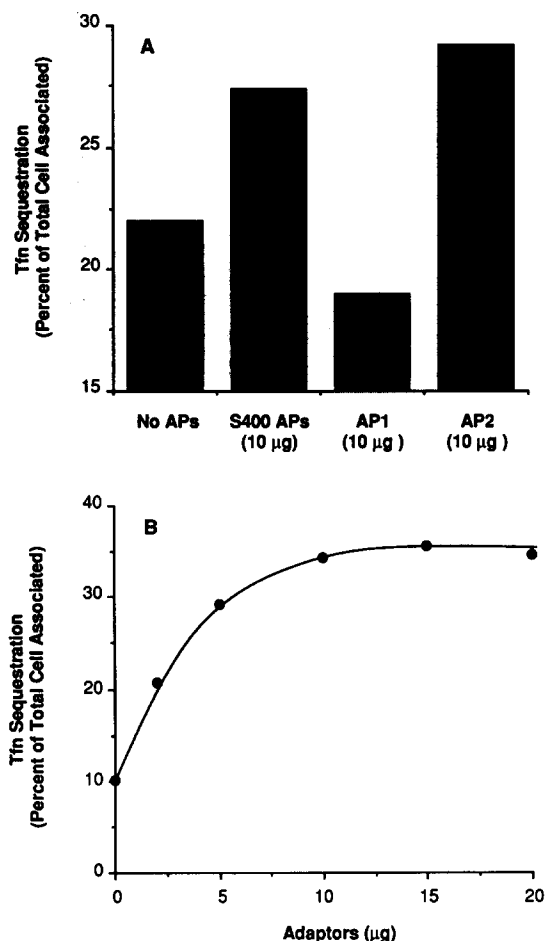


Figure 5. AP2 but not AP1 complexes specifically stimulate ATP-dependent TfN sequestration. (A) Perforated A431 cells in a complete assay mixture containing 1 mg/ml cytosol were incubated for 30 min at 37°C in the presence of 10 µg of S400 APs, AP1, or AP2 fractions as shown and then returned to ice for determination of the extent of TfN sequestration by Ab inaccessibility. (B) Concentration dependence of AP stimulation. Incubations were performed as in A in the presence of increasing amounts of S400-purified APs.

could protect against this inhibition and nonspecific IgG was only slightly inhibitory. The sequestration of ¹²⁵I-BSST seen at limiting cytosol concentrations in the absence of adaptors (Fig. 6, **■**) was unaffected, indicating that internalization of TfN from preformed coated pits was not inhibited by the diluted antibody under these conditions. This result was consistent with our previous findings that inhibition of events involving membrane-associated clathrin by X22 requires preincubation of perforated A431 cells with the antibody (Schmid and Smythe, 1991). These data indicate that the cytosolic fraction provides a soluble pool of clathrin which is required for adaptor-stimulated ¹²⁵I-BSST sequestration.

A Cytosolic Fraction Enriched in Clathrin Supports Adaptor-Stimulated Sequestration

To further explore the role of cytosolic clathrin in adaptor-stimulated sequestration, fractions enriched in soluble clathrin were prepared from bovine brain cytosol by (NH₄)₂SO₄ precipitation followed by DEAE cellulose and clathrin-

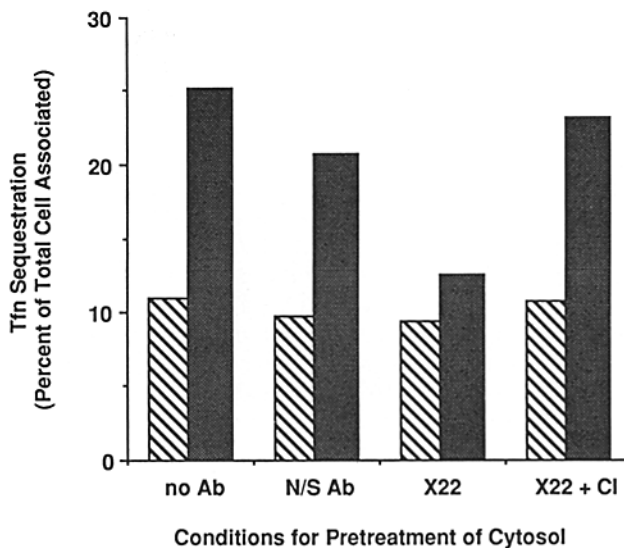


Figure 6. Inhibitory antibodies reveal a requirement for cytosolic clathrin in AP-stimulated TfN sequestration. Cytosol (10 µl at 3.5 mg/ml) was pretreated at 4°C for 30 min with 4 µg of either mAb X22 in the presence or absence of 10 µg of purified clathrin or with a nonspecific IgG of the same subclass. After preincubation the cytosol was diluted 1 in 4 into an assay mixture containing perforated A431 cells, ¹²⁵I-BSST and an ATP-regenerating system with or without 10 µg of purified APs. Incubations were for 30 min at 37°C before return to ice for determination of the extent of TfN sequestration by Ab inaccessibility. -AP (**▨**); +AP (**■**).

Sepharose chromatography (Fig. 4 B). These fractions were tested alone or in combination with purified APs and cytosol for their ability to stimulate ¹²⁵I-BSST sequestration in perforated A431 cells. The data in Fig. 7 shows that in the absence of cytosol neither purified APs nor the cytosolic fraction enriched in clathrin were active alone. However, when present together, the enriched cytosolic fraction and purified adaptors enhance ¹²⁵I-BSST sequestration by threefold. This result contrasts with that obtained using clathrin isolated from coated vesicles which was inactive when assayed under identical conditions (see Fig. 1, incubations without added cytosol). The clathrin-enriched fraction partially stimulated sequestration in the presence of limiting concentrations of cytosol, suggesting that clathrin may also be limiting under these conditions. Again, further stimulation was obtained upon addition of adaptors. In the absence of crude cytosol, maximal AP stimulation was obtained at <25 µg/ml of the enriched clathrin fraction, corresponding to an ~40-fold greater specific activity over crude cytosol in mediating AP-stimulated TfN sequestration (i.e., maximum AP-dependent stimulation requires ~0.5 mg/ml of crude cytosol). Thus the further stimulation obtained in the presence of limiting cytosol suggests that although the enriched clathrin fraction satisfies much of the cytosolic requirement for AP stimulation, other factor(s) may also be required for maximum activity.

Perforated Cells May Be "Primed" for Adaptor Stimulation of TfN Sequestration

Our results suggest that APs act at an early step to stimulate TfN sequestration into deeply invaginated pits. This was ex-

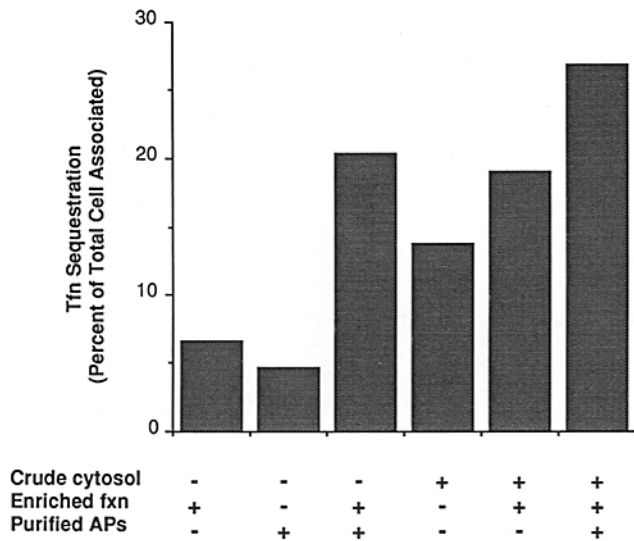


Figure 7. Cytosolic fraction enriched in clathrin supports ATP-dependent AP stimulation of Tfn sequestration. Perforated A431 cells were incubated in the presence or absence of crude cytosol (0.9 mg/ml), a cytosolic fraction enriched in clathrin (25 μ g/ml), and/or purified APs (250 μ g/ml) for 30 min at 37°C and Tfn sequestration was determined using the Ab inaccessibility assay. The data shown for ATP-dependent Tfn sequestration are the average results from two assays using independent preparations of the enriched cytosolic fraction.

aminated directly by testing whether preincubation of perforated cell preparations with APs could “prime” these membranes for AP-stimulated Tfn sequestration in a subsequent incubation. Perforated cells were preincubated at 30°C for 3 min in the absence of 125 I-BSST under various conditions and then briefly (5 s) centrifuged. The supernatants were removed and the cell pellets were then resuspended with cytosol and/or ATP and/or adaptors and reincubated in the presence of 125 I-BSST at 37°C for 30 min. The extent of Tfn sequestration as assessed by Ab inaccessibility was then determined. The presence of adaptors in the preincubation step obviated the requirement for adaptors in the subsequent incubation (compare set B with set C in Fig. 8 A). This suggests that the addition of adaptors during preincubation “primes” the membranes so that adaptors are no longer limiting for Tfn sequestration in the second incubation. This two-step incubation protocol enabled further examination of the requirements for the AP-dependent “priming” reaction. As seen in Fig. 8 A, the adaptor-dependent “priming” effect was independent of added ATP and cytosol (set D) and occurred on ice (set E). It is important to note, however, that ATP, cytosol and incubation at elevated temperatures were all essential in the second stage incubation leading to 125 I-BSST sequestration.

Analysis of the adaptor titration for the preincubation effect showed that it was identical to that observed in a direct assay of adaptor stimulated sequestration (compare Fig. 8 B with Fig. 5 B). This argues that the preincubation effect is truly a “priming” reaction for events which normally occur during the adaptor-dependent stimulation at elevated temperatures. That the membranes may be “primed” in this way indicates that the adaptors act very early, probably by assem-

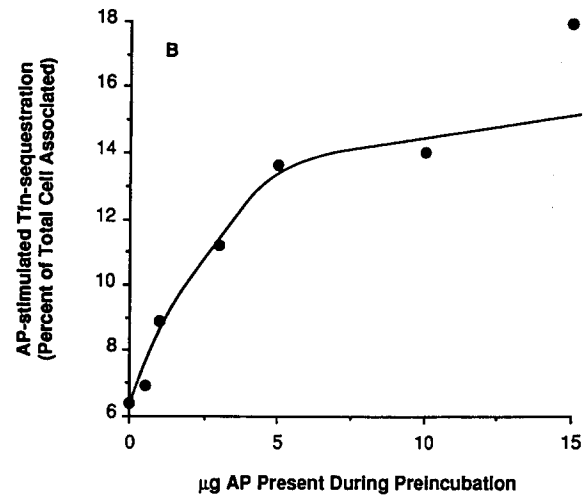
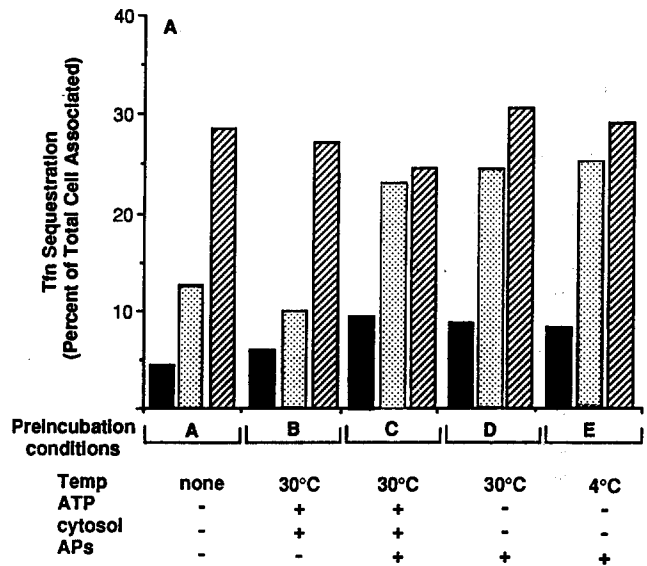


Figure 8. Preincubation with APs allows cell membranes to be “primed” for AP-independent sequestration in a two-step incubation. (A) Perforated A431 cells were preincubated in 40 μ l final volume of KSHM containing 1 mg/ml BSA with or without adaptors (10 μ g/assay), ATP and cytosol (0.6 mg/ml) as indicated for 3 min at 30°C or for 5 min at 4°C. These times of preincubation were optimized to give the best signal in the subsequent assay without significant increase in ATP or temperature-independent backgrounds. The cells were then centrifuged at full speed for 5 s in an Eppendorf refrigerated centrifuge, model 5402. The supernatant was aspirated and the membrane pellet resuspended in the assay mix containing 4 μ g/ml 125 I-BSST, with or without 0.8 mg/ml cytosol and with or without 10 μ g of APs. After 30 min at 37°C the amount of 125 I-BSST inaccessible to antibody was determined. (⊗) +cytosol/+AP; (⊠) +cytosol/-AP; (■) -cytosol/-AP. (B) Preincubation was performed at 4°C as described in A in the presence of increasing concentrations of APs. After pelleting to remove unbound APs, assays were performed as described for A in the presence of 0.6 mg/ml cytosol and in the absence of APs. The data plotted are the extent of cytosol-dependent Tfn sequestration obtained in the second incubation.

bly onto the membranes, and that cytosol and ATP are required after this initial step leading to productive sequestration of Tfn into deeply invaginated pits.

Discussion

We have examined the role of purified coat proteins in receptor-mediated endocytosis *in vitro* using stage-specific assays which differentiate between coated pit assembly, invagination and coated-vesicle budding (Smythe et al., 1989; Schmid and Smythe, 1991). Purified adaptors were found to markedly stimulate both the rate and extent of early events leading to the sequestration of ligand into deeply invaginated coated pits. In contrast, they had no effect on the late events involved in membrane fission and coated vesicle budding. Consistent with current models for adaptor function, these results suggest that APs participate in the initiation of a new pit and/or during the growth of existing pits.

Further evidence that the adaptors are acting at an early stage was provided by the observation that it was possible to "prime" the perforated cell membranes with adaptors during a preincubation. Our observation that "priming" was independent of ATP and cytosol and occurs at 4°C is consistent with previous findings on AP-membrane interactions (Mahaffey et al., 1990). In addition, we demonstrated that after attachment to the membranes, the APs were functionally active and stimulated productive ligand sequestration. After "priming" of membranes with adaptors, productive ligand sequestration in the subsequent warm-up reaction demonstrated an absolute requirement for cytosol, ATP, and elevated temperature. This result suggests that other cytosolic factors act after AP binding, perhaps to mediate lattice growth and invagination. Based on the ATP dependence of coated pit invagination, in addition to clathrin, these cytosolic factors presumably include at least one as yet unidentified ATPase.

The addition of APs increased both the rate and extent of ligand sequestration which occurred in the presence of limiting amounts of cytosol. No further increase in rate occurred as a result of priming the membranes with APs (data not shown). This result suggests that events subsequent to AP binding but nonetheless dependent on APs may be rate limiting. This AP-dependent increase in the rate of ligand sequestration could be due to an increased number of coated pits and/or an increase in the efficiency of Tfn-receptor sequestration into deeply invaginated coated pits.

There are two populations of adaptors in all cell types, AP1 associated with coated pits in the Golgi region and AP2 associated with plasma membrane coated pits (Robinson and Pearse, 1986). The adaptor-dependent stimulation of endocytic uptake observed *in vitro* demonstrated the expected specificity since only AP2 complexes were active. Inhibition by AP1 complexes may reflect competition with limiting concentrations of cytosolic clathrin. The titration curve for APs revealed that half-maximal stimulation of internalization occurred at <0.2 μ M. This value is consistent with the previously reported K_d values for clathrin-adaptor interactions (Schröder and Ungewickell, 1991) and for adaptor binding to stripped membranes (Virshup and Bennett, 1988; Mahaffey et al., 1990). However, under conditions of maximal AP stimulation, adaptors are present at \sim 10-fold molar excess to clathrin. This observation most likely reflects the affinity of adaptors for membrane binding or for clathrin interactions as opposed to the absolute amount of adaptors present.

An alternative explanation could be that only a minor population of the purified adaptors used in these assays is active in ligand sequestration. However, this is less likely since "priming" membranes with adaptors during preincubation at 4°C did not reduce the specific activity of the remaining adaptors when used in a subsequent incubation with fresh perforated cells (data not shown). The cellular concentration of adaptors in bovine brain has not been directly measured, however data is available on the concentration of clathrin (Goud et al., 1985). From these values, estimates of adaptor concentrations (\sim 80–160 nM) can be made based on the assumption that the ratio of clathrin to adaptors is the same as that found in isolated coated vesicles (Beck and Keen, 1991). These estimated values for AP2 concentrations *in vivo* are consistent with the concentration dependence for adaptor activity we have observed *in vitro*.

Clathrin extracted and purified from coated vesicles was inactive in ligand sequestration, even though clathrin prepared in this way is fully competent in assembly both into cages and onto membranes. However, we could demonstrate a role for a soluble pool of clathrin in this process. Preincubation of cytosol with the anticlathrin mAb X22 resulted in almost complete inhibition of the adaptor-dependent sequestration. Sequestration seen in the presence of cytosol alone and due presumably to preformed coated pits was unaffected. This inhibition was specific in that clathrin could protect against it and, in addition, nonspecific IgG of the same subclass had no effect. This novel finding suggests that the soluble pool of clathrin may be functionally distinct from the assembled pool. Further support for this suggestion was obtained by demonstrating that a partially purified cytosolic fraction, enriched for clathrin, could substantially substitute for cytosol in mediating adaptor-stimulated sequestration.

This observed functional distinction between soluble and assembled clathrin pools may explain results obtained in other studies on the rebinding of clathrin to membranes which had been stripped under conditions of either high pH, to remove the bulk of endogenous clathrin, or high Tris, to remove both APs and clathrin. In these experiments, while clathrin (isolated from coated vesicles) was able to bind to pH-stripped membranes, clathrin did not bind to Tris-stripped membranes even after rebinding adaptors (Mahaffey et al., 1990). One interpretation suggested by the authors was that this result reflected the need for activation of adaptors for clathrin binding. Given the results presented here, an alternative explanation is that clathrin isolated from the assembled pool requires some form of activation itself before assembly into functional coated pits. It is possible that although pre-existing or residual lattices may act as nucleation sites for the assembly of coated vesicle-derived clathrin these growing lattices may not be able to invaginate leading to productive sequestration of ligand. This possibility is consistent with the observation that lattice formation and invagination are two discrete events (Miller et al., 1991).

In intact cells, clathrin may be enzymatically released from coated vesicles by the action of hsc70 which was identified as an uncoating ATPase in *in vitro* reactions (Schlossman et al., 1984; Rothman and Schmid, 1986). In these reactions clathrin is released as a complex with the uncoating ATPase and is unable to self-assemble into cage structures (Schlossman et al., 1984). It is possible that the clathrin present in the partially purified cytosolic fraction which is active in coated pit assembly and invagination may be associated with accessory proteins, like the uncoating ATPase, which modu-

lates its assembly activity. Although ~40-fold enriched over crude cytosol by activity and substantially enriched in clathrin content, there are several other components present in this enriched fraction, some of which appear to be enriched after chromatography on clathrin-Sepharose column. Therefore it remains a strong possibility that other, unidentified factors which have alternative/additional roles in the sequestration of ligand contribute to the ability of this fraction to mediate AP-stimulated Tfn sequestration. Further purification and analysis of the soluble clathrin pool and isolation of the factor(s) responsible for stimulation of ligand sequestration will be required to address these issues.

The Tfn-receptor is constitutively internalized and therefore occupies all intermediates in coated pit and coated vesicle formation at steady state. As a consequence, a number of partial reactions which occur during receptor-mediated endocytosis can contribute to the sequestration of ligand measured by the antibody inaccessibility assay. These include de novo-coated pit assembly and growth, invagination, and membrane fission leading to coated vesicle formation. Thus an increase in any one or in all of these partial reactions would result in an increase in the extent of ligand sequestration as measured by Ab inaccessibility. The extent of adaptor stimulated ¹²⁵I-BSSST sequestration is usually greater than or equal to that observed in the presence of maximal cytosol, even though the extent of late membrane fission events is unchanged. In addition to demonstrating that adaptors are a limiting component in cytosol, this result suggests that the presence of excess adaptors acts to increase the relative contribution of de novo-coated pit assembly and/or coated pit growth to the overall extent of ligand sequestration. These partial reactions may only occur to a limited extent even in the presence of maximal cytosol since adaptor concentration may still be limiting. Consistent with this notion is our inability to prime membranes for adaptor-stimulated ligand sequestration even with high concentrations of cytosol (data not shown). Similarly the extent of ligand sequestration observed in the presence of adaptors and cytosolic clathrin but in the absence of crude cytosol may reflect an increased contribution of de novo-coated pit formation to the Ab-inaccessible signal. Confirmation of these hypotheses will await detailed and quantitative morphological analysis of the events being detected biochemically.

In conclusion, analysis of coated pit assembly in vitro in the functional context of receptor-mediated endocytosis has provided new insight into the requirements for and mechanism of coat protein assembly at the cell surface. Continuation of these studies using highly purified or enriched coat proteins should enable identification of other required factor(s) and further understanding of the mechanisms which might regulate coated pit assembly and invagination.

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