Stage-specific Assays for Coated Pit Formation and Coated Vesicle Budding In Vitro

Sandra L. Schmid and Elizabeth Smythe

Departments of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

Abstract. Internalization of biotin-S-S-\(^{125}\)I-transferrin (\(^{125}\)I-BSST) into semiintact A431 cells were assessed by two different criteria which have allowed us to distinguish partial reactions in the complex overall process of receptor-mediated endocytosis. Early events resulting in the sequestration of ligand into deeply invaginated coated pits were measured by inaccessibility of \(^{125}\)I-BSST to exogenously added antibodies. Later events involving coated vesicle budding and membrane fission were measured by resistance of \(^{125}\)I-BSST to reduction by the membrane impermeant-reducing agent, MesNa. Acquisition of Ab inaccessibility occurred very efficiently in this cell-free system (\(~50\%\) of total cell-associated \(^{125}\)I-BSST became inaccessible) and could be inhibited by anti-clathrin mAbs and by antibodies directed against the cytoplasmic domain of the transferrin-receptor. In contrast, acquisition of MesNa resistance occurred less efficiently (\(~10-20\%\) of total cell-associated \(^{125}\)I-BSST) and showed differential sensitivity to inhibition by anti-clathrin and anti-transferrin receptor mAbs. Both partial reactions were stimulated by ATP and cytosol; indicating at least two ATP-requiring events in receptor-mediated endocytosis. The temperature dependence of both reactions was similar to that for \(^{125}\)I-BSST internalization in intact cells with no activity being observed below 10°C.

Morphological studies using gold-labeled ligands confirmed that internalization of transferrin receptors into semiintact A431 cell occurred via coated pits and coated vesicles and resulted in delivery of ligand to endosomal structures.

Receptor-mediated endocytosis is a complex process involving several biochemically distinct stages. These include: (a) recruitment of coat proteins to the plasma membrane and nucleation of coated pit formation; (b) assembly of coat constituents leading and coated pit growth; (c) specific sequestration of receptors into coated pits; (d) membrane invagination; and (e) coat closure and membrane fission leading to coated vesicle budding. These processes are well defined morphologically. Biochemically, many of the coat constituents have been identified, cloned, and the nature of their interactions in the coat structure have been defined (see reviews by Brodsky, 1988; Morris et al., 1989; Pearse and Robinson, 1990; Keen, 1990). Signals on the cytoplasmic tails of receptors which direct them into coated pits have been functionally and structurally mapped (Chen et al., 1990; Collawn et al., 1990). Despite this wealth of molecular and structural information, very little is known about what regulates coated pit assembly, growth, invagination, and coated vesicle budding.

An understanding of the dynamic processes involved in coated pit assembly and budding will require the use of cell-free assays which faithfully reconstitute these events. The development of such assays has recently begun (Moore et al., 1987; Smythe et al., 1989; Podbilewicz and Mellman, 1990). Using an assay that measured the sequestration of prebound \(^{125}\)I-transferrin (\(^{125}\)I-Tfn) from anti-transferrin antibodies, Smythe et al. (1989) demonstrated the receptor-mediated internalization of \(^{125}\)I-Tfn into semiintact A431 cells. Although relatively inefficient (\(~10-15\%\) of total bound \(^{125}\)I-Tfn became inaccessible) Tfn sequestration was stimulated by ATP and cytosol and required elevated temperatures. Detailed morphological analysis of these events using Tfn-HRP and serial-thin section analysis suggested that both ATP and cytosol were required at two distinct stages for Tfn-receptor-mediated endocytosis: de novo coated pit formation and coated vesicle budding.

Two other cell-free assays have been developed to measure coated pit formation and endocytosis. Anderson and colleagues (Moore et al., 1987; Mahaffery et al., 1989) have developed an assay that measures clathrin assembly onto the inner plasma membrane surface of cells from which endogenous coats had been stripped. Using this assay, clathrin from crude cytosolic fractions, or highly purified from bovine brain coated vesicles, assembled efficiently forming coated pits, which were indistinguishable from those on untreated membranes. Clathrin assembly appeared not to require ATP or other cytosolic factors and occurred equally well at 4°C or 37°C. Podbilewicz and Mellman (1990) have recently reported an assay measuring \(^{125}\)I-Tfn internalization into an acid-resistant compartment at the basolateral membrane of perforated MDCK cells grown on filters in which the apical plasma membrane has been disrupted. \(^{125}\)I-Tfn internaliza-
tion in this assay was also efficient (60–80% of intact cells) but did not require addition of either ATP or cytosol.

Results concerning the energy requirements for receptor-mediated endocytosis in vivo have also been inconsistent. Although several groups have reported that receptor-mediated endocytosis is blocked in ATP-depleted cells (Haigler et al., 1980; Ceichanover et al., 1983; Hertel et al., 1986), others have suggested that a single round of endocytosis can occur in ATP-depleted cells (Clarke and Weigel, 1985; Larkin et al., 1985). In an attempt to resolve this issue, we have recently used two assays for the receptor-mediated endocytosis of transferrin which distinguish between internalization into bona fide coated vesicles and sequestration of ligand in deeply invaginated coated pits (Schmid and Carter, 1990). We found that although the sequestration of ligand into deeply invaginated coated pits occurred efficiently in cells depleted of ATP, internalization of ligand into sealed coated vesicles was markedly inhibited. These results suggested an explanation for inconsistencies in the literature and demonstrated that ATP is in fact required for receptor-mediated endocytosis in intact cells.

The demonstration of an ATP requirement for receptor-mediated endocytosis in vivo, in turn suggests that faithful reconstitution of endocytic events in vitro should also be ATP dependent. To further explore in enzymological detail the energy requirements and molecular requirements for coated pit formation and coated vesicle budding we have modified the assay originally described by Smythe et al. (1989) to increase its efficiency. In addition we have used these two assays for transferrin internalization to measuring receptor-mediated endocytosis in seminfect Act A431 cells, allowing us to distinguish the requirements for coated pit invagination and coated vesicle budding.

Materials and Methods

Materials

Yeast hexokinase, rabbit creatine phosphokinase, ATP, 2-deoxyglucose, creatine phosphate and 2-mercaptoethanesulfonic acid (MesNa) were obtained from Sigma Chemicals (St. Louis, MO). NaI was obtained from Amersham Corp. (Arlington Heights, IL). Avidin-Sepharose was from Pierce Chemical Co. (Rockford, IL). Staph-A cells ("Pansorbin") were obtained from CalBiochem-Behring Corp. (La Jolla, CA). All other chemicals were reagent grade.

Cell Lines and Antibodies

A431 cells were obtained from G. Warren (Imperial Cancer Research Foundation, London) and were grown in DME media (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS and Pen/Strep. Cells were passaged every 2-3 d by trypsinization. K562 cells, also obtained from G. Warren, were maintained in suspension in αMEM containing 5% FCS and Pen/Strep at cell densities between 10⁴ and 10⁷ cells/ml.

Sheep anti-transferrin antiserum was a gift from the Scottish Antibody Production Unit (Carlue, Scotland). Rabbit anti-mouse IgG was obtained from CalBiochem-Behring Corp. X19 and X22 anti-clathrin mAbs were generous gifts of Frances Brodsky (University of California, San Francisco, CA) and were supplied as purified IgG. mAbs by Donald Simonetti from Ian Trowbridge (Salk Institute, La Jolla, CA). These antibodies were further purified by affinity chromatography using the Immunopure (A/G) Immunoglobulin Purification Kit from Pierce Chemical Co.

Preparation of Cytosol

Cytosol fractions were prepared from either K562 cells or from bovine brain. K562 cells were washed in KSHM (100 mM KAcetate, 85 mM sucrose, 1 mM MgAcetate and 20 mM Heps-NaOH, pH 7.4) and homogenized in ~2 vol KSHM using a ball-bearing homogenizer (Balch and Rothman, 1985). Cytosol was collected from the postnuclear supernatant after centrifugation in an SW41 rotor (Beckman Instruments Inc., Palo Alto, CA) at ~200,000 gₑ for 1 h. Bovine brains were obtained from Federal Beef (Los Angeles, CA) and were frozen on dry ice immediately after slaughter and stored at −70°C until use. For cytosol preparations, ~10⁶ g of brain was thawed and washed in KSHM. Brain was homogenized in ~2 vol KSHM using a Dounce homogenizer with five passes each of the A and B pestles. The homogenate was centrifuged at 10,000 g for 15 min and the supernatant was subjected to second centrifugation in a Ti50 rotor (Beckman Instruments) at 40,000 rpm for 1 h. Cytosolic fractions from both K562 cells were stored, after rapid freezing, in aliquots at −70°C for use. Cytosolic fractions were prepared at high concentrations (~10 mg/ml) to maximize activity. Bovine brain cytosol fully substitutes for human K562 cytosol in both Ab-inaccessibility and MesNa-resistance assays (data not shown).

Preparation of Biotinylated Ligands

Human diferric transferrin (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was biotinylated (Pierce Chemical Co.) and iodinated exactly as previously described (Schmid and Carter, 1990). Biotinylated HTR-D65 was prepared as follows. Purified mAb HTR-D65 (240 μg) was incubated with 9.7 μg (~10-fold molar excess) NHS-SS-Biotin for 60 min at room temperature in 100 mM NaCl, 50 mM Na Pi, pH 7.2. Unreacted NHS-SS-Biotin was removed by a G25 spin-desalt column and the biotinylated mAb was iodinated in the presence of iodogen exactly as described for ¹²⁵I-BSST. ¹²⁵I-BSST-HTR-D65 was stored in aliquots at −70°C and was stable for at least 2 mo.

Assay for Internalization of ¹²⁵I-BSST into Semeintact A431 Cells

Assays were performed essentially as described by Smythe et al. (1989), with the following modifications to increase efficiency of internalization and consistency of results. 6.5 × 10⁶ cells were seeded 20-24 h before experiments onto 150 × 25-mm culture dishes (Falcon Labware, Becton Dickinson Co., Oxnard, CA) so that they were 80-90% confluent before scraping. Dishes were washed four times at 4°C with KSHM and seminfect A431 cells were removed by scraping with a rubber policeman. Cells were diluted to 15 ml in KSHM, incubated for ~5 min on ice and pelleted (800 g for 4 min). Scraped cells were >90-95% seminfect as assessed by Trypan blue permeability. Semeintact cells were then gently resuspended on ice into 250-300 μl KSHM/dish containing 40 μg/ml ¹²⁵I-BSST and 8 mg/ml BSA. Each 15-cm dish provided sufficient cells for 25-30 assays. 10 μg of BSA were then dispensed into Eppendorf tubes containing the washing assay reagents in 30 μl. The tubes were rapidly transferred to 37°C for the indicated incubation times. Complete assay mixtures (40 μl total volume in KSHM) contained ~2 × 10⁶ seminfect cells, 4 μg/ml ¹²⁵I-BSST, 2 mg/ml BSA, either an ATP-regenerating system (consisting of 1 mM ATP, 5 mM creatine phosphate and 0.2 IU creatine phosphokinase) or an ATP-depleting system (consisting of 5 mM 2-deoxyglucose and 30 U/ml hexokinase) and cytosol. Semeintact cells were incubated in the continuous presence of saturating concentrations of ligand to ensure that each endocytic event was scored. Following incubation at 37°C, tubes were returned to ice and the extent of ¹²⁵I-BSST internalization quantitated either by Ab-inaccessibility or MesNa-resistance assays. Both assays yield a significant signal over background (10-20:1 for Ab inaccessibility, ~3-5:1 for MesNa-resistance). Data is reproducible, with backgrounds of 2-5% for Ab inaccessibility, 5-10% for MesNa resistance and duplicates of ±5% of signal. Efficiency varies somewhat from preparation to preparation as indicated by error bars in many of the experiments.

Ab Inaccessibility Assay. This assay is a slight modification of that described by Smythe et al. (1989) and was performed exactly as described by Schmid and Carter (1990). Total cell-associated ¹²⁵I-BSST was determined by adding the counts in the Staph-A pellet and supernantant of samples incubated at 37°C in the absence of ATP. Data in general are expressed as the percentage of cell-associated ¹²⁵I-BSST, which is inaccessible to Ab pre-
cipitation (i.e., counts per minute in Staph-A supernatant/cpm in Staph-A pellet + counts per minute in Staph-A supernatant × 100%).

**MesNa Resistance Assay.** This assay was performed exactly as described by Schmid and Carter (1990), except that 10 mM MesNa was substituted for 50 mM glutathione in each wash step. Control experiments showed that some reducing reagents gave identical results. To determine total cell-associated $^{125}$I-BSST, samples incubated at 37°C in the absence of ATP were pelleted to remove unbound $^{125}$I-BSST and resuspended in 200 μl PBS containing 50 mM iodoacetamide and 1% TX-100. Total cell-associated $^{125}$I-BSST was assayed by washing repeatedly over 15 min with 50 mM NaAcetate, 100 mM NaCl, pH 4.5 containing 100 μg/ml desferoxamine (CIBA-Geigy, Basel, Switzerland), followed by repeated washes with PBS containing 100 μg/ml desferoxamine. Semintact cells were prepared and incubated exactly as described for the internalization assay. TfR-R recycling was assayed by the reappearance of Ab-accessible counts per minute in the media and on the cell surface.

**Test of TfR Recycling in Semintact A431 Cells**

The approach taken to measure TfR recycling in semintact A431 cells was essentially as described for MDBK cells by Podbielniwitz and Mellman (1990). Intact A431 cells were incubated for 30 min at 37°C in the presence of 4 μg/ml $^{125}$I-Tfn. The cells were washed at ice and surface bound $^{125}$I-BSST was removed by washing repeatedly over ~15 min with 50 mM NaAcetate, 100 mM NaCl, pH 4.5 containing 100 μg/ml desferoxamine (CIBA-Geigy, Basel, Switzerland), followed by repeated washes with PBS containing 100 μg/ml desferoxamine. Semintact cells were prepared and incubated exactly as described for the internalization assay. TfR-R recycling was assayed by the reappearance of Ab-accessible counts per minute in the media and on the cell surface.

**Morphological Characterization of TfR Internalization in Semintact A431 Cells**

Colloidal gold (5.9 nm, mean diameter) was purchased from Ted Pella, Inc. (Redding, CA). Initial attempts to use TfR-gold as a morphological marker proved unsuccessful, therefore the mAb HTR-D65 was conjugated to gold to follow TfR-R internalization. Gold conjugates were prepared and purified as described by Leunissen and DeMey (1987). HTR-D65-gold was stored at 4°C in PBS containing <20% sucrose and was used within 2 wk of preparation. Before use, the gold-labeled antibody was pelleted (T150 rotor; Beckman Instruments) at 40,000 rpm for 60 min and resuspended in KSHM.

Incubations for morphological studies were exactly as described for biochemical studies except that they were scaled up sixfold (i.e., 2.5 μl total volume, ~1 × 10⁶ cells/incubation) and cells were incubated in the presence of HTR-D65-gold. After incubation, the cells were returned to ice, pelleted to remove unbound HTR-D65-gold and resuspended in 1% glutaraldehyde, 3% paraformaldehyde, 0.1 M cacodylate buffer, pH 7.2. Pellets were postfixed with 1% OsO4 in 0.1 M cacodylate buffer, pH 7.2, embedded in epon and sectioned for microscopy by Dr. Cheng-Ming Chang of the Research Institute of Scripps Clinic EM laboratory. Quantitation of gold particles was performed at the microscope by random examination of complete semintact cell profiles at a magnification of 23,500.

**Results**

**Stage-specific Cell-free Assays for Receptor-mediated Endocytosis of $^{125}$I-BSST**

To begin to characterize the molecular requirements and mechanisms for receptor-mediated endocytosis we have modified a cell-free assay for endocytosis of transferrin originally described by Smythe et al. (1989) to increase both efficiency and consistency of results. In addition, $^{125}$I-Tfn was biotinylated via a cleavable disulfide bond (referred to as $^{125}$I-BSST) and was used as a ligand, so that two distinct assays for transferrin internalization could be used (Schmid and Carter, 1990). In the first assay, Ab inaccessibility (Smythe et al., 1989), internalization of $^{125}$I-BSST was measured after allowing anti-transferrin antibodies to bind to surface-bound $^{125}$I-BSST. Following addition of detergent and excess unlabelled transferrin, surface bound $^{125}$I-BSST-IgG complexes were adsorbed to Staph A cells (30 μl of a 15% suspension) and Ab-inaccessible $^{125}$I-BSST recovered in the Staph A-supernatant was counted on a gamma counter (Beckman Instruments). For MesNa resistance, plates were washed at 4°C with 10 mM MesNa in 50 mM Tris pH 8.6, 100 mM NaCl and incubated in this solution for 30 min. These washing and incubation steps were repeated twice, followed by a final wash in PBS containing 50 mM iodoacetamide. The cells were lysed in 300 μl PBS containing 1% TX-100, 0.2% BSA, and 5 μg/ml unlabelled transferrin. Transferrin-anti-transferrin immune complexes were adsorbed to Staph A cells (30 μl of a 15% suspension) and Ab-inaccessible $^{125}$I-BSST recovered in the Staph A-supernatant was counted on a gamma counter (Beckman Instruments). For MesNa resistance, plates were incubated in this solution for 30 min. These washing and incubation steps were repeated twice, followed by a final wash in PBS containing 50 mM iodoacetamide. The cells were lysed in 300 μl PBS containing 1% TX-100, 0.2% BSA, and 50 mM iodoacetamide. MesNa-resistant $^{125}$I-BSST was quantitated after adsorption to avidin-Sepharose beads (50 μl of a 50% suspension).

**Figure 1.** Ab inaccessibility and MesNa-resistance assays give the same kinetics and extent for internalization of $^{125}$I-BSST into intact A431 cells. A431 cells (4 × 10⁶) were seeded onto 35-mm dishes 1 d before use. Plates were preincubated at 37°C in serum-free media to remove endogenous transferrin 30 min before use. Duplicate plates were incubated for the indicated times at 37°C in the presence of 4 μg/ml $^{125}$I-BSST in serum-free media. Plates were then transferred to ice and internalized $^{125}$I-BSST was determined either by Ab inaccessibility (●) or MesNa resistance (○) as follows. For Ab inaccessibility, plates were incubated with 500 μl PBS containing 3 μl of anti-transferrin rabbit serum for 90 min at 4°C. The antibody solution was removed and cells were solubilized by addition of 300 μl PBS containing 1% TX-100, 1 mM MgCl₂, 0.2% BSA, and 5 μg/ml unlabelled transferrin. Transferrin-anti-transferrin immune complexes were adsorbed to Staph A cells (30 μl of a 10% suspension) and Ab-inaccessible $^{125}$I-BSST recovered in the Staph A-supernatant was counted on a gamma counter (Beckman Instruments). For MesNa resistance, plates were washed at 4°C with 10 mM MesNa in 50 mM Tris pH 8.6, 100 mM NaCl and incubated in this solution for 30 min. These washing and incubation steps were repeated twice, followed by a final wash in PBS containing 50 mM iodoacetamide. The cells were lysed in 300 μl PBS containing 1% TX-100, 0.2% BSA, and 50 mM iodoacetamide. MesNa-resistant $^{125}$I-BSST was quantitated after adsorption to avidin-Sepharose beads (50 μl of a 50% suspension).
provides a basis for measurement of partial reactions and for interpretation, the additional signal obtained with the Abinac- transfery in internalization into sealed coated vesicles, Abin- occurred with lower efficiency (∼10–20% of total bound) and 125I-BSST at 37°C for the indicated times. The cells were returned to ice and the extent of internalization of 125I-BSST was determined by either Ab inaccessibility (○) or MesNa resistance (○), exactly as described in Materials and Methods. Data, which represent the average of five experiments, are expressed as the percentage of total cell-associated 125I-BSST. Backgrounds (t = 0 min) which ranged from 2–4% for Ab inaccessibility and 4–8% for MesNa resistance, have been subtracted. (Inset) For comparative purposes, representative results from a single experiment are plotted as raw data (i.e., counts per minute Ab inac- cessible or MesNa resistant, 0 min backgrounds not subtracted).

125I-BSST Acquires Ab Inaccessibility More Efficiently than MesNa Resistance in Semiintact A431 Cells

In contrast to the results obtained in intact cells (Fig. 1), the data in Fig. 2 suggest that Ab inaccessibility and MesNa resistance measure distinct steps in 125I-BSST internalization into semiintact cells. Acquisition of Ab inaccessibility was efficient (∼50% of total bound internalized) and occurred with kinetics similar to Tfn uptake in intact A431 cells (t1/2 ∼ 7.5 min). In contrast, acquisition of MesNa resistance occurred with lower efficiency (∼10–20% of total bound) and with more rapid kinetics (t1/2 ∼ 2.5 min). Similar results obtained in previous studies measuring the internalization of 125I-BSST into intact cells depleted of ATP (Schmid and Carter, 1990) suggested that although both assays measure transferrin internalization into sealed coated vesicles, Ab inaccessibility can, in addition, score the sequestration of transferrin into deeply invaginated coated pits. In this interpretation, the additional signal obtained with the Ab inaccessibility assay (Fig. 2) would be due to early events leading to the formation of deeply invaginated pits that remain accessible to MesNa. The use of these two assays for measuring 125I-BSST internalization into semiintact A431 cells thus provides a basis for measurement of partial reactions and for biochemical dissection of the complex series of events leading to receptor-mediated endocytosis of transferrin.

Ab Inaccessibility and MesNa Resistance Measure Biochemically Distinct Events in Tfn Internalization

To test whether these two assays were in fact measuring dis- tinct events involved in Tfn internalization, we first com- pared their energy and cytosolic requirements. The data in Fig. 3 show the cumulative results from a number of experi- ments in which semiintact cells were allowed to internalize 125I-BSST for 30 min at 37°C in the presence or absence of added cytosol, and in the presence of either an ATP-regener- ating system or an ATP-depleting system. Both the acquisi- tion of Ab inaccessibility and of MesNa resistance were stimulated by ATP. Although there was significant acquisition of Ab inaccessibility in the absence of ATP (10–15% over 4°C backgrounds), there is no significant ATP-independent signal for acquisition of MesNa resistance. Sequestration of ligands into deeply invaginated pits was however stimulated approximately four to fivefold in the presence of ATP. A comparison between the ATP-dependent signal for acquisition of MesNa resistance and Ab inaccessibility shows that a significant portion of the ATP-dependent Ab inaccessibility signal (30–40% of total) cannot be ac- counted for by the ATP-dependent MesNa-resistant signal (∼15–20% of total). This additional signal (accounting for 15–20% of total cell-associated 125I-BSST) presumably arises from another earlier ATP-requiring step in receptor- mediated endocytosis that leads to enhanced sequestration of ligand into deeply invaginated coated pits.

Cytosolic factors appear to be required for acquisition of both Ab inaccessibility and MesNa resistance although both assays showed a significant signal in the absence of added cytosol (Fig. 3). The cytosol-independent signal obtained using the MesNa-resistance assay corresponded to ∼50% of

Figure 2. Internalization of 125I-BSST into semi-intact A431 cells: acquisition of Ab inaccessibility is more efficient than acquisition of MesNa resistance. Semiintact A431 cells were incubated in the presence of 4 μg/ml 125I-BSST at 37°C for the indicated times.

Figure 3. ATP and cytosol stimulate acquisition of both Ab inaccessibility and MesNa resistance. Incubations were for 30 min at 37°C and were performed as described in Materials and Methods. Internal- ization of 125I-BSST was assessed by either Ab inaccessibility (solid bars) or MesNa resistance (striped bars). (4°C) Complete incubation performed at 4°C for 30 min; (Complete) incubation in the presence of 5 mg/ml K562 cytosol and an ATP-regenerating system; (−cytosol) incubation in the absence of added cytosol; (−ATP) incubation in the presence of 5 mg/ml K562 cytosol and an ATP-depleting system; (ATP-dependent) the difference between the sig- nal obtained after complete incubation and that obtained in the absence of ATP. Data shown are the average of seven experiments.
Multiple Events in Transferrin Internalization Are Temperature Dependent

Receptor-mediated endocytosis in intact cells is temperature dependent with no detectable internalization occurring at temperatures below 10°C (Marsh et al., 1980; Fig. 4, closed circles). In contrast, clathrin coat assembly is spontaneous and occurs efficiently at 4°C. Indeed, Anderson and colleagues (Moore et al., 1987; Mahaffey et al., 1989) have demonstrated that the assembly of coated pits onto stripped plasma membranes also occurs in a temperature-independent manner. We therefore examined the temperature dependence of both acquisition of Ab inaccessibility and MesNa resistance in order to investigate whether spontaneous coat assembly could lead to the productive sequestration of ligand.

The maximum signal that was obtained at cytosol concentrations of ~2.5–3 mg/ml brain cytosol. The cytosol-independent signal obtained using the Ab-inaccessibility assay corresponded to ~30–40% of the maximum signal that required 4–5 mg/ml bovine brain cytosol (data not shown). These results suggested a higher cytosol requirement for acquisition of Ab inaccessibility than for MesNa resistance.

**Figure 4.** 125I-BSST internalization both in vivo and in vitro is strongly temperature dependent. Internalization assays using semi-intact or intact cells were performed as described in Fig. 3 or Fig. 1 and incubated at the indicated temperatures for 30 min. The extent of internalization of 125I-BSST was assessed either by Ab inaccessibility (top) or by MesNa resistance (bottom). The data are expressed as the percent of maximum signal obtained at 37°C to allow comparison between different assay conditions. Actual values for the percent of total cell-associated 125I-BSST which was internalized under the various assay conditions and assessed by either Ab inaccessibility or MesNa resistance, respectively, were as follows: intact cells (●), 202%, 195%; semiintact cells, complete incubation (△), 43.7%, 21%; semiintact cells–cytosol (○), 19.6%, 14%; semiintact cells–ATP (□), 12%, 2% (not shown).

**Figure 5.** Anti-clathrin mAb X22 inhibits 125I-BSST acquisition of Ab inaccessibility in semiintact cells. Semiintact cells were preincubated at 4°C in the presence of 5 mg/ml bovine brain cytosol, an ATP-regenerating system and the indicated concentration of anti-clathrin mAb X22 for 30 min. 125I-BSST and BSA were added from a 10X stock for final concentrations of 4 and 2 mg/ml, respectively and the cells were transferred to 37°C for 30 min. A shows the raw data from one representative experiments. B shows data averaged from four independent experiments. The data is expressed as a percent of the cytosol-dependent levels (i.e., the difference between the complete incubation and incubation in the absence of cytosol) obtained in the absence of mAb.
Incubations and assays were performed in the presence of antibody or MesNa in a semiintact A431 cell system. The presence of these reagents resulted in Ab inaccessibility at temperatures below 10°C. Acquisition of MesNa was obtained in intact cells. "I-BSST did not acquire Ab inaccessibility at temperatures below 10°C. Acquisition of MesNa in vitro appeared to require temperatures over 15°C. This did not appear to be the case in intact cells where Ab inaccessibility and MesNa resistance showed the same temperature dependencies (Fig. 5, a and b, closed circles). Surprisingly, even the partial cytosol and ATP-independent reactions detected using both assays showed temperature dependencies comparable to intact cells. These results suggest that although the spontaneous assembly of coated pits might be occurring in these semiintact A431 cells, it does not lead to productive sequestration of ligand either from antibody or MesNa.

Table I. Comparison of Inhibitory Effects of mAbs X22 and HTR-H68 on 125I-BSST Internalization as Assessed by Either Ab Inaccessibility or MesNa Resistance

| Incubation conditions                  | No antibody | X22 (0.5 mg/ml) | HTR-H68 (0.4 mg/ml) |
|----------------------------------------|-------------|----------------|-------------------|
| Ab inaccessibility assay               | 100%        | 51 ± 8%        | 53 ± 13%          |
| MesNa resistance assay                  | 100%        | 56 ± 14%       | 84 ± 12%          |

Incubations and assays were performed in the presence of either X22 or HTR-H68 as described in Figs. 5 and 6. Results are expressed as the percent of total 125I-BSST internalized after 30 min at 37°C relative to control (no antibody) incubations.

Anti-clathrin Antibodies Inhibit Transferrin Internalization In Vitro

Although spontaneous clathrin and coat protein assembly cannot account for the sequestration of ligand we detect, it is clear that if our assay is a valid reflection of the events involved in TfR receptor mediated endocytosis, directed clathrin assembly should play a role in the early events leading to acquisition of Ab-inaccessible 125I-BSST. To test this we examined the effect of mAbs which inhibit clathrin assembly on 125I-BSST internalization using the Ab inaccessibility assay. As indicated in Fig. 5 (A), incubation of semiintact cells in the presence of both cytosol and ATP results in efficient sequestration of bound 125I-BSST from exogenously added antibodies. This signal is reduced approximately fourfold in the absence of ATP and approximately twofold in the absence of cytosol. Preincubation of bovine brain cytosol with the anti-clathrin mAb X22 (Brodsky, 1985; Blank and Brodsky, 1986) before addition of cells and incubation at 37°C to allow for 125I-BSST internalization resulted in an ~60% inhibition of the extent of inaccessibility of 125I-BSST to antibody. This level of residual activity which could not be inhibited even in the presence of an ~50-fold excess of X22 to clathrin was comparable to the cytosol-independent signal we obtained and corresponded to the level of internalization obtained in the absence of cytosol. The cytosol-independent signal was not affected by anti-clathrin antibodies and effective inhibition required preincubation of the cytosolic fraction, suggesting that anti-clathrin mAbs efficiently inhibit only the cytosol-dependent internalization of 125I-BSST. For this reason, the cumulative data in Fig. 5 B has been expressed as the percent of the cytosol-dependent Ab-inaccessibility signal inhibited by X22. 90% of the cytosol-dependent sequestration of 125I-BSST from antibody can be inhibited by X22. In the presence of 5 mg/ml bovine brain cytosol, half-maximal inhibition occurred at ~150 μg/ml X22, which represents an ~10-15-fold molar excess of mAb (assuming that clathrin represents ~0.1% of total cellular protein in most cell types and 0.1% of cytosolic protein in bovine brain [Goud et al., 1985]). The anti-clathrin mAb X19 (Brodsky, 1985; Blank and Brodsky, 1986) gave similar results although it appeared in initial studies to require slightly higher concentrations (data not shown). Nonspecific IgG of the same subclass had little or no effect. Inhibition by X22 (at 500 μg/ml) could be fully protected by the addition of a 1.5-fold molar excess of bovine brain clathrin, demonstrating the specificity of the inhibition despite the high concentrations of antibody required. The ability of X22 to inhibit acquisition of Ab inaccessibility during internalization of 125I-BSST into semiintact A431 cells is consistent with a role for cytosolic clathrin in the process and supports the validity of this assay system.
The cytoplasmic tail of the transferrin receptor is essential for efficient internalization via coated pits (Iacopetta et al., 1988; Jing et al., 1990) and recently an "endocytosis" signal (YXRF) in the cytoplasmic domain of the transferrin receptor has been functionally and structurally defined (Collawn et al., 1990). Therefore to further test the validity of this cell-free assay for Tf internalization, we explored the effect of mAbs directed toward the cytoplasmic domain of the transferrin receptor on \(^{125}\)I-BSST internalization. Seminintact A431 cells, cytosol and ATP were preincubated at 4°C for 15–30 min in the presence of mAb HTR-H68, which recognizes the cytoplasmic domain of the transferrin receptor. As a control we used mAb HTR-D65, which recognizes the ectodomain of the transferrin receptor. After addition of \(^{125}\)I-BSST, the cells were transferred to 37°C for 30 min to allow internalization, removed to ice and analyzed for Ab-inaccessible \(^{125}\)I-BSST. As seen in Fig. 6, HTR-H68, the cytoplasmic domain–specific mAb, completely inhibited the cytosol-dependent sequestration of \(^{125}\)I-BSST into an Ab-inaccessible compartment in seminintact cells. Half-maximal inhibition was obtained at \(\sim 50 \mu g/ml\) antibody. In contrast, at lower concentrations HTR-D65, the ectodomain–specific mAb stimulated \(^{125}\)I-BSST sequestration into an Ab-inaccessible compartment. This stimulation could be due to micro-clustering of receptors by the multivalent IgG leading to more efficient inclusion into coated pits. Higher concentrations of HTR-D65 had a slight inhibitory effect, perhaps due to the formation of larger aggregates which might be less efficiently internalized. Consistent with in vivo findings, these results suggest that the mechanisms involved in vitro in efficient internalization of \(^{125}\)I-BSST require specific interactions with cytoplasmic tails of Tfn-Rs.

We next compared the effects of both X22 and HTR-H68 on the acquisition of MesNa resistance with their effects on the acquisition of Ab inaccessibility. Seminintact A431 cells and cytosol were preincubated at 4°C with either X22 or HTR-H68 for 30 min before addition of \(^{125}\)I-BSST and incubation for 30 min at 37°C. After internalization of \(^{125}\)I-BSST, the cells were returned to ice and assayed by either Ab inaccessibility or MesNa resistance. The results appear in Table I. Somewhat unexpectedly the anti-clathrin mAb, X22, significantly inhibited the ATP-dependent acquisition of MesNa resistance. The extent of inhibition was below cytosol-independent levels and was at least comparable to that seen for Ab inaccessibility (see Table I). These results suggest either that clathrin assembly is required for a significant portion of the MesNa-resistant signal, or that these antibodies also inhibit the function of assembled clathrin structures.

When the effects of the anti-transferrin receptor mAb, HTR-H68 were examined, a quantitatively different result was obtained. Although preincubation of cells with HTR-H68 significantly inhibited the overall acquisition of Ab inaccessibility (\(\sim 65\%\)), overall acquisition of MesNa resistance was much less sensitive (\(\sim 25\%\) inhibition). It is important to consider these results in terms of the internalization of the total cell-associated \(^{125}\)I-BSST. Thus, 65% inhibition of Ab inaccessibility corresponds to a 25% inhibition in internalization of total cell-associated \(^{125}\)I-BSST, compared with a 25% inhibition of MesNa resistance which accounts for <5% inhibition. These results are consistent with the interpretation that most of the transferrin receptors involved in internalization of \(^{125}\)I-BSST into a MesNa resistant compartment were resistant to anti-transferrin antibodies, and perhaps already localized in coated pits.

### Table II. Internalization of \(^{125}\)I-HTR-D65 and Gold-HTR-D65 into Seminintact A431 Cells

|               | \(^{125}\)I-HTR-D65 (% of total) | MesNa resistance \(^{125}\)I-HTR-D65 (% of total) |
|---------------|---------------------------------|-----------------------------------------------|
| (a) 37°C incubation |                                 |                                               |
| Complete      | 62                              | 32                                            |
| -ATP          | 26                              | 3                                             |
| -cytosol      | 15                              | 12                                            |
| Total gold counted | (No. of cell profiles) |                                  | No. gold/ cell profile | PM | CP | CV | SV |
| (b) 4°C incubation |                                 |                                               |
| Bind/then fix | 344(8)                          | 43                                            | 67.4                      | 32.6 | 0 | 0 | 0 |
| Fixed/then bind | 314(9)                          | 35                                            | 72.6                      | 27.4 | 0 | 0 | 0 |
| 37°C incubation |                                 |                                               |
| Complete      | 407(5)                          | 81                                            | 63                        | 17 | 1.5 | 18 | 18 |
| -ATP          | 455(5)                          | 91                                            | 89                        | 8.6 | 0 | 2.4 | 18 |
| -cytosol      | 471(3)                          | 157                                           | 83                        | 10.6 | .2 | 6.2 | 18 |

(a) \(^{125}\)I-BSS-HTR-D65, the anti-transferrin receptor ectodomain antibody, was biotinylated and iodinated as described in Materials and Methods. Seminintact A431 cells were incubated for 30 min at 37°C in the presence of 4 \(\mu g/ml\) \(^{125}\)I-BSS-HTR-D65 and in the presence or absence of ATP and bovine brain cytosol, as indicated. After incubation, the extent of \(^{125}\)I-BSS-HTR-D65 internalization was assessed by either Ab-inaccessibility (solid bars) exactly as described for \(^{125}\)I-BSST except that rabbit anti-mouse IgG (1 \(\mu g/assay\)) was used as a second antibody and a nonspecific unlabeled mouse IgG, (0.5 \(\mu g/ml\)) was used in the lysis buffer; or MesNa resistance (striped bars), exactly as described for \(^{125}\)I-BSST. (b) parallel samples were incubated with 4 \(\mu g/ml\) HTR-D65 labeled with 6 nm gold particles as described in Materials and Methods. Quantitation of gold particles was performed at the microscope by random examination of cell profiles at a magnification of 23,500. Each seminintact cell profile was completely examined and all detectable gold particles were counted and found to be distributed amongst these four classes of structures: non-coated regions of the cell surface; coated structures either open to the cell surface or within two vesicle diameters of the cell surface; closed coated structures more than two vesicle diameters from the cell surface; and in large uncoated vesicles within the cytosol. No other structures (i.e., Golgi apparatus or mitochondria) were seen to contain gold particles. CP, coated pits; CV, coated vesicles; PM, plasma membrane; SV, smooth vesicles.

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Morphological Examination of Tfn Receptor Internalization in Semiintact A431 Cells

Processes involved in receptor-mediated endocytosis have been well defined morphologically. To establish a morphological basis for the events occurring in vitro, we conjugated mAb HTR-D65 to 5 nm gold particles to follow transferrin receptor internalization in the semiintact cell preparations.

As a control, and to provide a biochemical correlate to our morphological studies, we prepared 125I-BSS-D65 in an analogous manner to 125I-BSST as a ligand for biochemical studies. Semiintact A431 cells were incubated in the presence or absence of cytosol, ATP and 125I-BSS-D65 mAb for 30 min at 37°C. After return to ice, acquisition of Ab inaccessibility was assessed using rabbit-anti-mouse IgG to precipitate surface-bound 125I-BSS-D65 mAb. MesNa resis-
tance was measured exactly as described for $^{125}$I-BSST. As can be seen (Table II a) internalization of $^{125}$I-BSS-D65 occurred with identical properties to $^{125}$I-BSST. The acquisition of Ab inaccessibility occurred with greater efficiency than the acquisition of MesNa resistance. Internalization as assessed by both assays displayed the same cytosol and ATP dependencies observed for internalization of $^{125}$I-BSST.

Parallel samples of semintact A431 cells were incubated in the presence of 5 nm gold-labeled HTR-D65, fixed and prepared for EM. The structures involved in internalization of gold-labeled HTR-D65 mAb into semintact A431 cells appeared indistinguishable from those involved in intact A431 cells (data not shown). Gold particles were observed on the cell surface, in coated pits, coated vesicles and in smooth membrane structures (presumably endosomes) deep within the cell (Fig. 7 a-c). In contrast, when cells were incubated in the absence of ATP, gold particles were not observed in intracellular compartments. In many cases, coated pits on semintact cells incubated in the absence of either ATP or cytosol appeared very heavily labeled with gold particles (Fig. 7 d and e). Heavily labeled coated pits were not detected in A431 cells incubated in the presence of cytosol and ATP. Although not quantitated, these observations suggested that when coated vesicle budding is inhibited, transferrin receptors might accumulate in this intermediate. The results from the morphological studies are quantitated in Table II b. Consistent with the biochemical data, we observed a twofold decrease in the fraction of gold-D65 present in coated pits and a seven-fold decrease in the fraction of gold particles in internal smooth and coated vesicles when semintact cells were incubated in the presence of cytosol, but absence of ATP. Similarly, in the absence of cytosol but presence of ATP, we observed a twofold decrease in coated pit labeling and a threefold decrease in smooth vesicle labeling. The lower apparent efficiencies for coated pit localization (17%) and internalization (20%) determined morphologically as compared with the biochemical determinations (60% Ab inaccessible, 30% MesNa resistance) could be due in part to detrimental effects of the gold conjugation on the efficiency of mAb internalization and in part to non-specific and/or subsaturating levels of gold-mAb binding. Clusters of gold particles outside of coated pits were often observed (see for example Fig. 7 f). These morphological studies confirm that internalization into semintact A431 cells occurred via the normal cellular pathway of coated pits, coated vesicles, and endosomes.

Discussion

We have developed stage-specific cell-free assays for the receptor-mediated endocytosis of transferrin which should enable detailed biochemical dissection of the molecular requirements and mechanisms involved in this complex cellular process. Several criteria support the conclusion that the receptor-mediated endocytosis of transferrin into semintact cells is a valid reflection of events occurring in intact cells. (a) Internalization of Tfn in vitro is ATP dependent. In contrast, sequestration of Tfn into deeply invaginated pits can occur in the absence of ATP, consistent with results in intact cells (Schmid and Carter, 1990). (b) Tfn internalization in vitro requires cytosol as would be expected since clathrin and other coat proteins are thought to assemble from cytosol pools. (c) The temperature dependence of Tfn-internalization in vitro is identical to the temperature dependence of internalization into intact A431 cells. (d) Tfn internalization in vitro is efficiently inhibited by anti-clathrin antibodies and by antibodies directed towards the cytoplasmic domain of the Tfn receptor. (e) Morphological analysis of the internalization of gold-labeled anti-Tfn-R mAbs into semintact cells demonstrated that internalization occurs via coated pits and coated vesicles and results in delivery of gold-labeled antibodies to endosomal structures.

Our data also support the interpretation that partial reactions along the complex overall pathway of coated pit assembly, invagination and budding are occurring during Tfn uptake into semintact A431 cells. Since Tfn receptors are constitutively internalized (Hopkins and Trowbridge, 1983; Watts, 1985), at any time Tfn receptors on the cell surface will be heterogeneously distributed at various stages of endocytosis. This heterogeneous distribution would contribute to our ability to detect partial reactions using the Ab inaccessibility and MesNa resistance assays. These partial reactions can be biochemically distinguished on the basis of their cytosol dependence, ATP dependence, kinetics, temperature dependence, and sensitivity to anti-clathrin and anti-transferrin receptor antibodies.

One model that summarizes these partial reactions and their distinguishing properties is shown in Fig. 8. The complex overall process of Tfn-receptor endocytosis could be described as occurring in the following biochemically distinct stages: initiation/nucleation of coat assembly, coated pit growth, invagination, and budding/membrane fission. We propose that the earliest event, initiation/nucleation is slow based on the differences between the kinetics of acquisition of Ab inaccessibility and of acquisition of MesNa resistance and on previous morphological studies that demonstrated very rapid conversion of shallow coated pits to deep coated pits (Smythe et al., 1989). We further suggest that this step accounts for the additional ATP-dependent signal we obtain using the Ab-inaccessible assay. This conclusion is based on the following evidence. First, previous morphological studies demonstrated that the conversion of shallow coated pits to deeply invaginated pits occurred efficiently in the absence of both ATP and cytosol but that an increase in the total number of coated pits required both (Smythe et al., 1989). It should be noted that the morphological criteria used to distinguish shallow pits from deeply invaginated pits in these studies did not correspond to the ability of these pits to sequester ligand from Ab. Although the latter reaction was 80-90% efficient and occurred within 5 min, Ab inaccessibility was only 10-15% efficient and required 10-15 min in these experiments. Secondly, previous biochemical studies (Moore et al., 1987; Mahaffey et al., 1989) have demonstrated that clathrin assembly into coated pits is ATP-independent. As indicated by the authors, since only 70-80% of plasma membrane-associated clathrin was removed in these studies, this ATP-independent clathrin assembly was likely to be occurring at pre-existing nucleated sites and therefore would, in our model, constitute coated pit growth. The ATP-independent Ab inaccessibility signal we observe was not dependent on cytosol (data not shown), nor can it be inhibited by anti-clathrin antibodies, therefore we propose that it results from invagination of fully grown coated pits lead-
Figure 8. Model for events involved in coated pit formation and coated vesicle budding and a summary of properties which distinguish these events. Receptor-mediated endocytosis can be described as occurring via at least four biochemically distinct events: initiation or nucleation of de novo coated pit assembly, growth of preexisting coated pits, invagination of fully formed coated pits and the budding/membrane fusion event leading to coated vesicle formation. Since transferrin receptors are constitutively internalized, at any time they would be expected to populate each of these proposed intermediate stages. Furthermore, we have used two assays which enable detection of not only the final product, coated vesicle budding, but also partial reactions leading the formation of deeply invaginated coated pits. Taking advantage of these features, we have begun to biochemically dissect this complex overall process. The results supporting this working model are discussed in the text.

The results obtained using anti-clathrin and anti-transferrin receptor antibodies are also consistent with this model. Both X22 and HTR-H68 totally inhibit the cytosol-dependent acquisition of Ab inaccessibility. This suggests that both the initiation/nucleation and growth stages are inhibited, consistent with their predicted requirements for assembly of cytosolic clathrin and for interactions with the cytoplasmic domains of Tf-Rs. In contrast, neither X22 nor HTR-H68 appears to inhibit the cytosol-independent acquisition of Ab inaccessibility. These results suggest that invagination of completed coated pits is not sensitive to these antibodies, although detailed morphological studies would be needed to confirm this interpretation. In contrast, the anti-clathrin antibody severely inhibited the acquisition of MesNa resistance suggesting that although this antibody does not affect invagination, it might inhibit budding of preformed coated pits. This possibility would not be unexpected since X22 binds to and cross-links clathrin cages (Blank and Brodsky, 1986). Acquisition of MesNa resistance was also partially inhibited by HTR-H68. Inhibition at this late stage could be due to mAb binding to the cytoplasmic domains of Tf-Rs so as to prevent their migration into preformed, budding coated pits. Alternatively, either the antibody might interact weakly with Tf-R in coated pits to disrupt budding or a small fraction of the overall MesNa resistance signal might be due to budding of newly formed coated pits. We cannot distinguish between these possibilities.

Our ability to inhibit Tf internalization into semiintact cells using anti-clathrin antibodies confirms and extends previous studies by Draper et al. (1990) which demonstrated a 40-50% inhibition by the anti-clathrin antibody X19 using the assay originally developed by Smythe et al. (1989). Although these investigators did not assess the ATP or cytosol...
dependence of Ab inaccessibility in A431 cells, they suggested that the residual activity obtained in the presence of excess X19 mAb might be due to internalization of ligand already present in fully formed coated pits. Our ability to inhibit 90% of the cytosol-dependent acquisition of Ab inaccessibility is consistent with this interpretation. High ratios of mAb to clathrin were also required for inhibition in this previous study (half-maximal inhibition required an ~50-fold molar excess of X19:clathrin). These relatively high ratios of mAb:clathrin required for inhibition in vitro are, however, consistent with two previous results involving inhibition of endocytosis in intact cells following delivery of a 5-30-fold molar excess of these antibodies (Doxsey et al., 1987; Chin et al., 1989).

Both Ab inaccessibility and MesNa resistance were stimulated by added cytosol. However, we were unable to demonstrate absolute cytosol dependence using either assay. These cytosol-independent signals might be due to one or a combination of the following factors: (a) Incomplete wash out of A431 cytosol from semintact cells. This possibility is supported by the day to day variability in cytosol dependence we observe and by our morphological data in which reduced, but not abolished, delivery of internalized ligand to endosomes was detected in the absence of added cytosol. Endosome fusion in vitro in a variety of systems is cytosol dependent (reviewed by Gruenberg and Howell, 1989). (b) Detection of partial reactions which might be cytosol independent. This possibility is supported by previous morphological studies that demonstrated that the invagination of shallow pits occurred efficiently in the absence of cytosol (Smythe et al., 1989). (c) The activity of cytosolic factors previously recruited to the membrane. This possibility would be similar to results obtained in vitro for vesicular intragolgi transport in which late membrane fusion events which appear cytosol independent are, in fact, driven by cytosolic factors which have assembled onto the vesicle and target membranes (Rothman and Orci, 1989).

Why is coated vesicle budding so inefficient in these semintact cell preparations? One possibility could be our inability to reconstitute the different ion conditions that normally exist on either side of the plasma membrane. Likewise, if ionics transients of any kind are required, these might be difficult to reconstitute in the open system used here. The low extent of coated vesicle budding is consistent with this signal being derived from that population of Tfn receptors already in a late stage of coated pit formation. This speculation is supported by the observation that antibodies that recognize the cytoplasmic domain of Tfn-R only partially inhibit the acquisition of MesNa resistance under conditions in which there is substantial inhibition of acquisition of Ab inaccessibility. This result would be predicted if Tfn-Rs already in coated pits were less accessible to these cytoplasmic domain antibodies than those outside of coated pits. Thus, we propose that an additional step in coated vesicle budding, referred to in Fig. 8 as 'activation' might be required before coated vesicles can bud from the cell surface. Only coated pits which have undergone this activation step in vivo will successfully bud in vitro. More work will be required to clearly demonstrate the existence of this step and to determine its molecular requirements.

Every step which we are able to distinguish biochemically leading to productive sequestration of $^{3}$P-BSST from either Ab or MesNa displayed temperature dependence similar to that for receptor-mediated endocytosis in intact A431 cells. This striking result suggests that the spontaneous assembly of coat proteins observed in other systems (Moore et al., 1987; Mahaffey et al., 1989) and which probably occurs in our system, does not lead to the productive sequestration of ligands. These findings, as well as the apparent ATP-dependence of both early and late events involved in coated pit and coated vesicle formation, suggest a catalytic role for as yet, unidentified noncoat protein(s) in mediating and/or regulating coat assembly and coated vesicle budding in intact cells. We are currently using these assays to identify these factors from bovine brain cytosol.

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References

Balch, W. E., and J. E. Rothman. 1985. Characterization of protein transport between successive compartments of the Golgi apparatus: asymmetric properties of the donor and acceptor activities in a cell-free system. Arch. Biochem. Biophys. 240:413-425.

Blank, G. S., and F. M. Brodsky. 1986. Site-specific disruption of clathrin assembly produces novel structures. EMBO (Eur. Mol. Biol. Organ.) J. 5:2087-2095.

Brodsky, F. M. 1985. Clathrin structure characterized with monoclonal antibodies. I. Analysis of multiple antigenic sites. J. Cell Biol. 101:2047-2054.

Brodsky, F. M. 1988. Living with clathrin: its role in intracellular membrane traffic. Science (Wash. DC). 242:1396-1402.

Chen, W.-J., J. L. Goldstein, and M. S. Brown. 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. J. Biol. Chem. 265:3116-3123.

Chin, D. J., R. M. Straubinger, S. Acton, I. Nithke, and F. M. Brodsky. 1989. 100-kDa polypeptides in peripheral clathrin-coated vesicles are required for receptor-mediated endocytosis. Proc. Natl. Acad. Sci. USA. 86:9289-9293.

Ciechanover, A., A. L. Schwartz, A. Dautry-Varsat, and H. F. Lodish. 1983. Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. J. Biol. Chem. 258:9681-9689.

Clarke, B. L., and P. H. Weigel. 1985. Recycling of the asialoglycoprotein receptor in isolated rat hepatocytes. J. Biol. Chem. 260:128-133.

Collawn, J. F., M. Stangel, L. A. Kuhn, W. Eseskoglu, S. Jing, I. S. Trowbridge, and J. A. Tainer. 1990. Transferrin receptor internalization sequence YXF$^\alpha$ implicates a tight turn as the structural recognition motif for endocytosis. Cell. 63:1061-1072.

Doxsey, S. J., F. M. Brodsky, G. S. Blank, and A. Hellenius. 1987. Inhibition of endocytosis by anti-clathrin antibodies. J. Cell Biol. 100:543-462.

Draper, R. K., Y. Goda, F. M. Brodsky, and S. R. Pfeffer. 1990. Antibodies to clathrin inhibit endocytosis but not recycling to the trans Golgi network in vitro. Science (Wash. DC). 248:1539-1541.

Goud, B., C. Huet, and D. Lowend. 1985. Assembled and unassembled pools of clathrin: a quantitative study using enzyme immunoassay. J. Cell Biol. 100:521-527.

Gruenberg, J., and K. E. Howell. 1989. Membrane traffic in endocytosis: insights from cell-free assays. Annu. Rev. Cell Biol. 5:453-482.

Haigler, H. T., F. R. Maxfield, M. C. Willingham, and I. Pastan. 1980. Danysolcadaverine inhibits internalization of $^{3}$P-Lipid growth factor in BALB 3T3 cells. J. Biol. Chem. 255:1290-1291.

Hartel, C., S. J. Coulter, and J. P. Perkins. 1986. The involvement of cellular ATP in receptor-mediated internalization of epidermal growth factor and hormone-induced internalization of $\beta$-adrenergic receptors. J. Biol. Chem. 261:5974-5980.

Hopkins, C. R., and I. S. Trowbridge. 1983. Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. J. Cell Biol. 97:508-521.
Iacopetta, B. J., S. Rothenberger, and L. C. Kuhn. 1988. A role for the cytoplasmic domain in transferrin receptor sorting and coated pit formation during endocytosis. Cell. 54:485–489.

Jing, S., T. Spencer, K. Miller, C. Hopkins, and I. S. Trowbridge. 1990. Role of the human transferrin receptor cytoplasmic domain in endocytosis: localization of a specific signal sequence for internalization. J. Cell. Biol. 110:283–294.

Keen, J. H. 1990. Clathrin and associated assembly and disassembly proteins. Annu. Rev. Biochem. 59:415–438.

Larkin, J. M., W. C. Donzell, and R. G. W. Anderson. 1985. Modulation of intracellular potassium and ATP: effects on coated pit function in fibroblasts and hepatocytes. J. Cell. Physiol. 124:372–378.

Leunissen, J. L. M., and J. R. DeMey. 1987. Preparation of gold probes. In Immuno-Gold Labeling in Cell Biology. A. J. Verkleij and J. L. M. Leunissen, editors. CRC Press Inc., Boca Raton, FL. 3–16.

Mahaffey, D. T., M. S. Moore, and R. G. W. Anderson. 1989. Coat proteins isolated from clathrin coated vesicles can assemble into coated pits. J. Cell Biol. 108:1615–1624.

Marsh, M., and A. Helenius. 1983. Penetration of Semliki Forest virus form acidic prelysosomal vacuoles. Cell. 32:931–940.

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.

Morris, S. A., S. Ahle, and E. Ungewickell. 1989. Clathrin-coated vesicles. Curr. Op. Cell Biol. 1:684–690.

Pearse, B. M. F., and M. S. Robinson. 1990. Clathrin: adaptors and sorting. Annu. Rev. Cell Biol. 6:151–171.

Podbilewicz, B., and I. Mellman. 1990. ATP and cytosol requirements for transferrin recycling in intact and disrupted MDCK cells. EMBO (Eur. Mol. Biol. Organ.) J. 9:3477–3487.

Rothman, J. E., and L. Orci. 1989. Movements of proteins through the Golgi stack: a molecular dissection of vesicular transport. FASEB (Fed. Am. Soc. Exp. Biol.) J. 4:1460–1465.

Schmid, S. L., and L. L. Carter. 1990. ATP is required for receptor-mediated endocytosis in intact cells. J. Cell Biol. 111:2307–2318.

Smythe, E., M. Pypaert, J. Lucocq, and G. Warren. 1989. Formation of coated vesicles from coated pits in broken A431 cells. J. Cell Biol. 108:843–853.

Watts, C. 1985. Rapid endocytosis of the transferrin receptor in the absence of bound transferrin. J. Cell Biol. 100:633–637.