Multiple GTP-binding Proteins Participate in Clathrin-coated Vesicle-mediated Endocytosis

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Abstract. We have examined the effects of various agonists and antagonists of GTP-binding proteins on receptor-mediated endocytosis in vitro. Stage-specific assays which distinguish coated pit assembly, invagination, and coat vesicle budding have been used to demonstrate requirements for GTP-binding protein(s) in each of these events. Coated pit invagination and coated vesicle budding are both stimulated by addition of GTP and inhibited by GDPβS. Although coated pit invagination is resistant to GTPγS, AlF_4^-, and mastoparan, late events involved in coated vesicle budding are inhibited by these antagonists of G protein function. Earlier events involved in coated pit assembly are also inhibited by GTPγS, AlF_4^-, and mastoparan. These results demonstrate that multiple GTP-binding proteins, including heterotrimeric G proteins, participate at discrete stages in receptor-mediated endocytosis via clathrin-coated pits.

INTRACELLULAR membrane trafficking is mediated by transport vesicles which bud from one organelle and then fuse with an appropriate target organelle. Vesicle formation occurs at specialized regions of the membrane distinguished by an underlying protein coat. There are now two recognized classes of coat structures which function in transport vesicle formation. The best studied of these are clathrin-coated pits and coated vesicles (CCV)[1] which are involved in receptor-mediated endocytosis and transport from the Golgi complex to lysosomes (reviewed by Brodsky, 1988; Pearse and Robinson, 1990). The major coat constituents of CCVs are clathrin triskelions and adaptors (reviewed by Pearse and Crowther, 1987). Clathrin triskelions are composed of three 180-kD heavy chains and three tightly associated ~30-kD light chains. Adaptor complexes are heterotetramers composed of two ~100–110-kD adaptin molecules and two smaller subunits of 47–50 and 17–19 kD (reviewed by Pearse and Robinson, 1990; Keen, 1990).

More recently a second class of transport vesicles, referred to as "nonclathrin-coated vesicles" or "COP-coated vesicles" has been shown to mediate vesicular traffic along the exocytic pathway (Orci et al., 1986). The coat constituents of nonclathrin-coated vesicles include polypeptides of 160 (α-cop), 110 (β-cop), 98 (γ-cop), and 68 kD (δ-cop), smaller subunits of 36 and 35 kD (Maholtra et al., 1989), as well as ADP-ribosylation factor (ARF), a 20-kD GTP-binding protein (Serafini et al., 1991). Sequence analysis has demonstrated that β-cop is distantly related to β-adaptin (17% homology in the NH_2-terminal half of the molecule) suggesting some functional relationship between these two coat proteins (Duden et al., 1991).

Both classes of coated pits assemble from a cytosolic pool of coat proteins. Clathrin and adaptors exist as distinct soluble pools which appear to assemble sequentially to form clathrin-coated pits (Mahaffey et al., 1990; Smythe et al., 1992*b*). In contrast, COPs are present in the cytosol as a large multimeric precursor termed a "coatomer" which presumably self-assembles onto membranes to form COP-coated pits (Waters et al., 1991). Whereas clathrin-coated pits act as selective membrane filters that concentrate specific receptor–ligand complexes for inclusion into a budding transport vesicle; COP-coated pits appear to be nonselective, mediating "bulk-flow" transport events.

A growing body of genetic and biochemical evidence has established that multiple classes of GTP-binding proteins participate in COP-CV–mediated membrane transport events. In addition to ARF (Serafini, 1991), several members of the rab family of *ras*-related small GTP-binding proteins also participate in vesicular transport events, although their exact function remains unknown (reviewed by Balch, 1990; Goud and McCaffrey, 1991). More recent evidence has suggested the involvement of heterotrimeric G proteins in vesicular transport along both the exocytic pathway and the endosome/lysosome pathways (Donaldson et al., 1991; Stow et al., 1991; Barr et al., 1991; Columbo et al., 1992; reviewed by Balch, 1992; Barr et al., 1992). For both the large and small G proteins, GTP is believed to act as a molecular switch such that the G protein is activated in the GTP-bound form and inactive when GDP is bound (reviewed by Bourne et al., 1990).

To date no evidence exists for the involvement of either of these classes of GTP-binding proteins in clathrin-coated vesicle formation. However, recent genetic evidence has

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[1] *Abbreviations used in this paper*: BFA, brefeldin A; CCV, clathrin-coated vesicle; COP-CV, nonclathrin-coated vesicle; GDPβS, guanosine-5′-*o*-(2-thiodiphosphate); GTPγS, guanosine-5′-*o*-(3-thiotriphosphate); MESNa, mercaptoethane sulfonic acid.

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implicated a role for dynamin, a microtubule-stimulated GTPase (Schpetner and Vallee, 1992; Collins, 1991) in this process. Dynamin has been identified as the mammalian homologue to the gene product responsible for the temperature-sensitive *shibire* mutation in Drosophila (van der Bliek and Meyerowitz, 1991; Chen et al., 1991). At the non-permissive temperature the *shibire* mutation results in a pleiotropic defect in endocytosis which leads to an accumulation of elongated coated pits at the cell surface (Kosaka and Ikeda, 1983). Biochemical evidence for dynamin function in endocytosis is lacking and paradoxically there exists no evidence for the involvement of microtubules in coated vesicle formation (Morgan and Iacopetta, 1987; Hunziker et al., 1990).

Ironically, although there exists a considerably greater amount of structural and biochemical information on the protein constituents of the clathrin coat as compared to the recently identified COP-CV constituents, mechanistic studies on CCV-mediated transport have lagged behind (reviewed by Schmid, 1992). Much of our understanding of COP-CV–mediated transport events has been derived from biochemical studies of cell-free assay systems which reconstitute these processes (reviewed by Rothman and Orci, 1992). Therefore, to begin to dissect the molecular mechanisms of CCV-mediated endocytosis, we have developed stage-specific assays which biochemically distinguish three sequential events leading to coated vesicle formation. These sequential stages are coated pit assembly, coated pit invagination, and coated vesicle budding (Schmid and Smythe, 1991; Smythe et al., 1992*a*,*b*). Using these assays we have begun biochemical studies on the mechanism of CCV-mediated endocytosis. Here we present evidence that multiple GTP-binding proteins participate in CCV-mediated endocytosis and that distinct classes of GTP-binding proteins are differentially involved in coated pit assembly, invagination, and coated vesicle budding. The ability to measure discrete events in the process of coated vesicle formation provides a unique and powerful tool for identifying and functionally characterizing the GTP-binding proteins involved.

Materials and Methods

Cells and Reagents

A431 cells were cultured as previously described (Schmid and Smythe, 1991). Nucleotides and analogs were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Stock solutions (10 mM nucleotide in 50 mM Hepes, pH 7.5) were aliquoted, stored at −70°C and were used within 2 wk. A stock solution of mastoparan (1 mg/ml in H_2O; Sigma Chemical Co., St. Louis, MO) was stored for several weeks at −20°C. For use in the assay, mastoparan was diluted in KSHM buffer (100 mM K-Acetate, 85 mM sucrose, 20 mM Hepes, 1 mM MgAcetate, pH 7.4) containing 0.2% BSA. Peptide analogues of mastoparan were generously provided by T. Higashijima (University of Texas Southwestern Medical Center, Dallas, TX), arf 26 was from R. Kahn (National Cancer Institute, Bethesda, MD) and 1CS4 was from R. Ghadiri (The Scripps Research Institute, La Jolla, CA). Bovine brain and transducin $\beta\gamma$ subunits were generous gifts of G. Bokoch (The Scripps Research Institute) and Y.-K. Ho (University of Chicago, Chicago, IL). All other chemicals were reagent grade.

Preparation of ELISA Plates

ELISA plates were prepared as described elsewhere (Smythe et al., 1992*a*). Briefly, rabbit anti–human transferrin IgG (Boehringer Mannheim Biochemicals) was plated onto Maxisorb Immuno-module strips (Nunc InterLab, Thousand Oaks, CA) at a 1/1,000 dilution in 50 mM Na_2HCO_3, pH 9.6. Plates were incubated for 3 h at 37°C or overnight at 4°C, and then washed three times in PBS and incubated for 30 min at 37°C in blocking buffer (1% Triton X-100, 0.1% SDS, 0.2% BSA, 50 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.4). Plates were stored in blocking buffer at 4°C and used within 2 wk. Immediately before using, the strips were washed three times in PBS, incubated for 5 min in blocking buffer and washed three times in PBS (this is referred to as a wash cycle). 0.1 ml of blocking buffer was then added to each well.

Assays for Ligand Sequestration and Internalization

The assays are shown schematically in Fig. 1. Perforated A431 cells were prepared essentially as described with the exception that the perforated cell pellet was resuspended in ~0.5 ml KSHM for each 15-cm plate scraped (Schmid and Smythe, 1991; Smythe et al., 1992*a*). Human diferric transferrin (Boehringer-Mannheim Biochemicals) was biotinylated via a cleavable disulphide bond using NHS-SS-biotin (Pierce Chemical Co., Rockford, IL) as previously described except that it was not radiolabeled (Schmid and Smythe, 1991; Smythe et al., 1992*a*). This reagent, referred to as "BSST," served as the ligand for following transferrin receptor-mediated endocytosis. Gel-filtered cytosol was used in all experiments. The cytosol was prepared from bovine brain as previously described (Schmid and Smythe, 1992) except that it was immediately gel filtered by chromatography using a Sephadex G25 column equilibrated with KSHM. Gel-filtered cytosol preparations were rapidly frozen in liquid nitrogen and stored in aliquots at −70°C. Bovine brain adaptors were prepared exactly as described by Smythe et al. (1992*b*). Internalization and sequestration assays were performed exactly as previously described (Schmid and Smythe, 1991; Smythe et al., 1992*a*). Duplicate samples were run for each experimental point. Briefly, assay components including KSHM, an ATP-regenerating (containing 800 μM ATP, creatine phosphokinase and 5 mM creatine phosphate) or an ATP-depleting system (containing hexokinase and 5 mM glucose), gel-filtered cytosol and the reagent(s) of interest were added to 1.5-ml Eppendorf tubes (Brinkman Instruments Inc., Westbury, NY) (30 μl total volume) at 4°C. Next, 10 μl of perforated cells (~2×10^5 cells) resuspended in 4× BSST were added, the tubes were gently mixed, and transferred to 37°C. The final concentration of BSST in the reaction was 2–3 μg/ml. After 30 min, the tubes were returned to 4°C and pelleted in a refrigerated Eppendorf centrifuge for 20 s at 14 k rpm. The supernatants were carefully aspirated and the pellets were subjected to internalization or sequestration assays described briefly below and in detail elsewhere (Smythe et al., 1992*a*).

Internalization Assay. The internalization of BSST was measured by its acquisition of resistance to the small (150 mol wt) membrane impermeant reducing agent, MesNa (β-mercaptoethane sulfonate, sodium salt; Sigma Chemical Co.), which occurs as a result of its inclusion into sealed coated vesicles. The cell pellets were resuspended in 50 μl of 10 mM MesNa. The tubes were agitated at 4°C. At 30 min 12.5 μl of 50 mM MesNa was added to each tube, and at 60 min this was supplemented with 16 μl of 50 mM MesNa. The MesNa solutions were prepared just before each addition in 100 mM NaCl, 1 mM EDTA, 50 mM Tris, 0.2% BSA, pH 8.6. After 90 min, the MesNa was oxidized by the addition of 25 μl of 500 mM iodoacetic acid (Sigma Chemical Co.). After a final 10 min agitation, the membranes were solubilized by adding 0.1 ml blocking buffer to each tube and vortexing briefly. For each tube, 0.1 ml was plated into a well on the ELISA plate which contained 0.1 ml blocking buffer. Total cell-associated BSST was determined from cells incubated at 37°C in the absence of cytosol or ATP, by plating 100 μl of a cell lysate which had been subjected to the same buffer additions without MesNa. The plates were incubated overnight at 4°C.

Sequestration Assay. The sequestration of BSST which occurs either as a result of its inclusion into sealed, coated vesicles or its inclusion into deeply invaginated coated pits was determined by its acquisition of inaccessibility to the large (68 kD) probe, avidin. A stock solution of avidin (Canadian Lysozyme Inc., Vancouver, Canada) was prepared weekly in water and the concentration determined by A280 ($E_{1\%}$ = 15.5). The cell pellets were resuspended in 0.1 ml of 50 μg/ml avidin which was diluted daily in KSHM + 0.2% BSA. The tubes were agitated at 4°C for at least 1 h. Biocytin (Sigma Chemical Co., 20 mg/ml in H_2O) was then added to a final concentration of 50 μg/ml, and agitation was continued for 10 min. The cells were lysed by the addition of 0.1 ml of blocking buffer. After a brief vortex, 0.1 ml from each tube was plated per well on the ELISA plates to which 0.1 ml blocking buffer had been added. Total cell-associated BSST was determined from cells incubated at 37°C in the absence of cytosol or ATP, by plating 100 μl of a cell lysate which had been subjected to the same buffer additions without avidin. The plates were incubated overnight at 4°C.

ELISA-based Detection of Internalized and/or Sequestered BSST. After the overnight incubation of either avidin- and MesNa-treated cell lysates, the plates underwent a wash cycle. Streptavidin-HRP (Boehringer-Mannheim Biochemicals) was diluted 1/5,000 in blocking buffer and 0.2 ml was added to each well. The plates were incubated for at least 60 min at room temperature. After another wash cycle, 0.2 ml of substrate solution (10 mg *o*-phenylenediamine, 10 μl H_2O_2 in 25 ml of 50 mM Na_2HPO_4, 27 mM citrate, pH 5) was added to each well and the incubation allowed to proceed until sufficient color was developed, typically 2–4 min. This reaction was terminated by the addition of 50 μl per well of 2 M H_2SO_4. The A490 was read on an ELISA plate reader (Bio-Rad Laboratories, Cambridge, MA) and corrected for the A655.

Results

Sensitive ELISA-based Assays for Coated Pit Assembly, Invagination, and Coated Vesicle Budding

We have recently developed stage-specific assays which enable measurement of three biochemically distinct events involved in receptor-mediated endocytosis in vitro (Schmid and Smythe, 1991; Smythe et al., 1992*a*,*b*). These events which sequentially lead to coated vesicle formation are: (*a*) de novo coated pit assembly; (*b*) coated pit invagination; and (*c*) coated vesicle budding. The assays, diagrammed in Fig. 1, are performed using "perforated" human A431 cells which are prepared by scraping them from their substratum so as to fenestrate the plasma membrane enabling removal of endogenous cytosol and allowing full access to the cytoplasmic surface of the remaining plasma membrane. Transferrin which has been biotinylated via a cleavable disulphide bond (BSST) binds to the transferrin receptor and is constitutively internalized via clathrin-coated pits. Perforated A431 cells are incubated at 37°C in the presence of cytosol, ATP, and BSST to allow receptor-mediated endocytosis to occur. Distinct stages involved in CCV-mediated endocytosis are measured by the acquired inaccessibility of BSST to small and large probes. Thus, the "internalization" of BSST into sealed vesicles occurs as a result of coated vesicle budding and is measured by its acquired resistance to cleavage by β-mercaptoethane sulfonate (MesNa), a small membrane impermeant reducing agent. The "sequestration" of BSST from exogenously added avidin, a high molecular weight probe, can occur as a result of its inclusion into both sealed coated vesicles and into deeply invaginated coated pits which remain plasma membrane associated. The extent of "internalization" and/or "sequestration" of BSST is quantitated by capturing the transferrin on microtitre wells coated with anti-transferrin antibodies. The number of biotin residues on BSST remaining unmasked by avidin or uncleaved by MesNa are quantitated using streptavidin-HRP (Fig. 1). This ELISA-based assay is a modification of our previously published procedure offering several advantages: it is nonradioactive, more sensitive, more readily applicable to other ligands, and

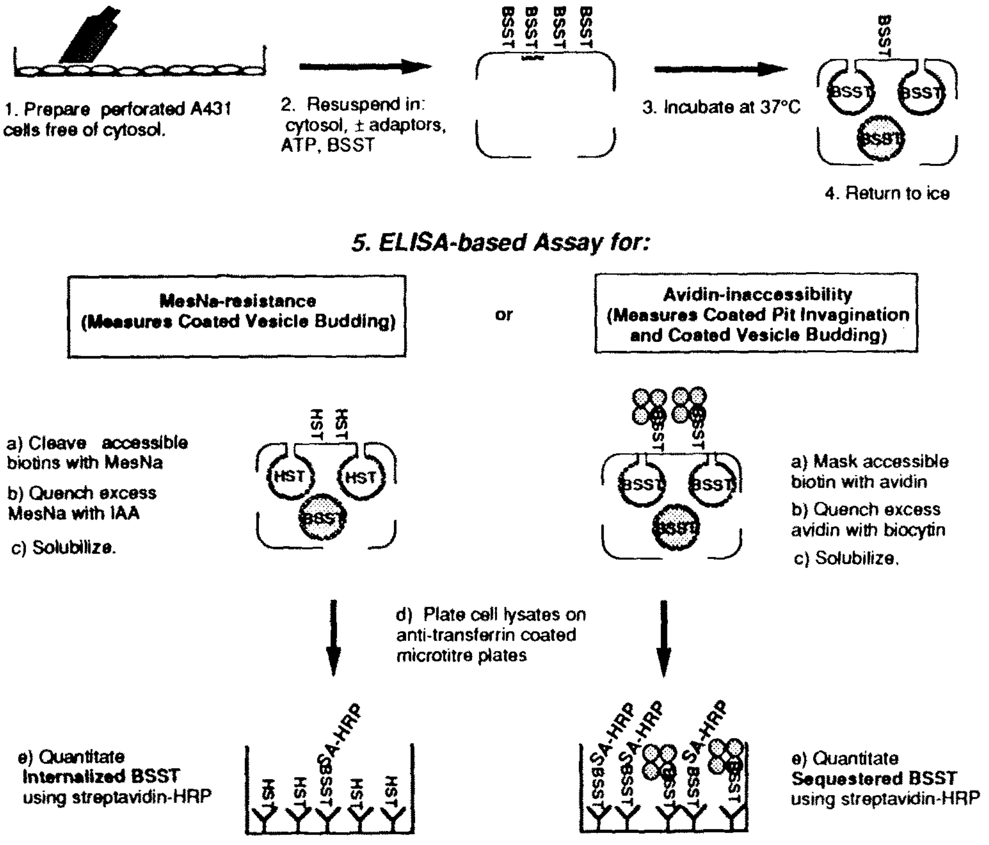


Figure 1. ELISA-based assay for receptor-mediated endocytosis into perforated A431 cells. Perforated A431 cells are prepared as described in Materials and Methods. Transferrin which is used as a ligand for receptor-mediated endocytosis is biotinylated via a cleavable disulphide bond and referred to as BSST. Assays were performed as described in Materials and Methods. Endocytic events are scored by either the inability of MesNa, a membrane impermeant reducing agent, to cleave accessible biotin residues or the inability of avidin to mask accessible biotin residues. BSST is captured on antibody-coated microtitre wells and remaining and/or unmasked biotin residues are quantitated using streptavidin-conjugated HRP (*SA-HRP*). As diagrammed, the inaccessibility to MesNa measures only internalization of BSST into sealed, coated vesicles. Inaccessibility of BSST to exogenously added avidin measures both internalization into sealed coated vesicles and its sequestration in deeply invaginated coated pits. The quantitative difference in signals obtained with the two assays provides a selective measurement of the sequestration of BSST into deeply invaginated coated pits. In this example, one of the five cell-associated BSST is internalized into a sealed, coated vesicle and becomes resistant to MesNa. Three of five BSST become sequestered from avidin. The difference in signals (two of five cell-associated BSST ligands) have been sequestered into deeply invaginated coated pits.

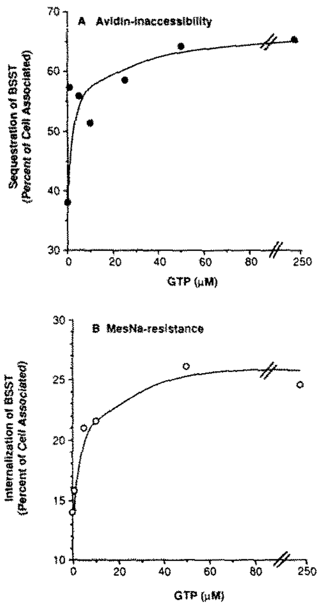


Figure 2. GTP stimulates both coated pit invagination and coated vesicle budding. Perforated A431 cells were incubated for 30 min at 37°C in 40 μl KSHM containing gel-filtered bovine brain cytosol (2.6 mg/ml), an ATP-regenerating system, 2 μg/ml BSST and increasing concentrations of GTP as indicated. Cells were returned to ice and processed for either avidin inaccessibility (*A*) or MesNa resistance (*B*) as described in Materials and Methods. The data are expressed as the percent of total cell-associated BSST which became inaccessible to either probe in an ATP and cytosol-dependent manner. Untreated cell lysates obtained following an incubation of cells in the presence of 2 μg/ml BSST but in the absence of ATP and cytosol were plated onto microtitre wells in serial dilutions to determine total cell-associated BSST and to ensure that the binding capacity of the wells for BSST was not exceeded.

backgrounds due to incomplete reduction of surface-bound BSST are reduced.

Extensive biochemical and morphological characterization of this assay system (Smythe et al., 1989; Schmid and Smythe, 1991; Smythe et al., 1992*b*) has shown that coated vesicles which form during the in vitro reaction are largely derived from pre-existing coated pits. As a result, MesNa-resistance selectively measures late events which correspond to coated vesicle budding and lead to the internalization of BSST. In contrast, the sequestration of BSST from avidin provides a measure of the sum of two biochemically distinct events, coated vesicle budding and coated pit invagination. Of the avidin signal, typically ∼60% derives from coated pit invagination while ∼40% derives from coated vesicle budding. For example, in the experiment illustrated in Fig. 2 at maximum efficiency, 65% of the total cell associated BSST became inaccessible to avidin (Fig. 2 *a*). In the same experiment, 25% of BSST became resistant to MesNa (Fig. 2 *b*). The extent of coated pit invagination is reflected by the quantitative difference between the avidin and MesNa signals. Thus, using this example, 40% of cell associated BSST became sequestered in deeply invaginated coated pits.

We have recently shown that adaptor proteins are limiting under our assay conditions so that even at high cytosol concentrations, the principal event resulting in the sequestration of BSST is the invagination of preformed coated pits (Smythe et al., 1992*b*). To directly measure de novo coated pit assembly, assays are performed in the presence of limiting concentrations of cytosol supplemented with purified adaptor proteins. When perforated A431 cells are incubated under these conditions adaptors specifically stimulate coated pit assembly which increases the extent of sequestration of BSST into deeply invaginated pits (Smythe et al., 1992*b*). Thus, adaptor-stimulated sequestration of BSST provides a measurement of the de novo assembly of functionally active coated pits (Smythe et al., 1992*b*). Using these three stage-specific assays to measure the biochemically distinct events of coated pit assembly, invagination and coated vesicle budding we have sought evidence for the role of GTP-binding proteins in clathrin-coated vesicle formation.

GTP Stimulates Coated Pit Invagination and Coated Vesicle Budding

Receptor-mediated endocytosis of BSST into perforated A431 cells requires both an ATP-regenerating system and added cytosol (Schmid and Smythe, 1991; Smythe et al., 1992*a*). Gel-filtration of the cytosolic fraction resulted in a reduction of its ability to support BSST internalization in vitro. This treatment removed >97% of the total perchloric acid extractable nucleotide pool (data not shown). Addition of GTP to an assay mixture containing perforated A431 cells, BSST, an ATP-regenerating system and gel-filtered cytosol resulted in an almost twofold stimulation of both the sequestration and internalization of BSST (Fig. 2, *a* and *b*). The GTP-dependent stimulation of sequestration was quantitatively greater than its effect on internalization indicating that both coated pit invagination and coated vesicle budding were affected. In both cases maximal stimulation was achieved at ∼50 μM added GTP. Stimulation was specific to GTP since neither UTP nor CTP had any effect (data not shown) and since assays were routinely performed in the presence of 800 μM ATP.

Nonhydrolyzable Analogues of GTP Inhibit Endocytosis of BSST

Given that GTP stimulated receptor-mediated endocytosis in vitro, the involvement of GTP-binding proteins was further investigated by examining the effects of nonhydrolyzable analogues of GTP on coated vesicle formation. The data in Fig. 3 *A* shows that coated vesicle budding, leading to the internalization of BSST was markedly inhibited by GTPγS. Half-maximal inhibition required <5 μM GTPγS and could be fully protected by 1 mM GTP (data not shown). In contrast, the extent of inhibition of BSST sequestration from avidin (<50%) could be largely accounted for by the selective inhibition of coated vesicle budding. Thus, coated pit invagination, itself, appeared to be relatively resistant to inhibition by GTPγS. Further, since GTP (Fig. 2) but not GTPγS stimulated invagination and coated vesicle budding, these results suggest that GTP hydrolysis was required for each of these events.

We next examined the effect of GDPβS on coated pit invagination and coated vesicle formation. In addition to serving as a potential competitive inhibitor for GTP binding, this guanine–nucleotide analogue cannot be phosphorylated and should therefore lock regulatory G-proteins in their GDP-bound state. In contrast to their differential sensitivity to GTPγS, the data in Fig. 3 *B* shows that GDPβS equally inhibits both coated vesicle budding and coated pit invagination (>70% inhibition, half-maximal at <25 μM).

Evidence for the Involvement of Heterotrimeric G Proteins in Clathrin-coated Vesicle Formation

To further characterize the GTP-binding proteins involved in endocytosis, AlF_4- and mastoparan, more selective inhibitors of heterotrimeric G proteins, were examined. The first

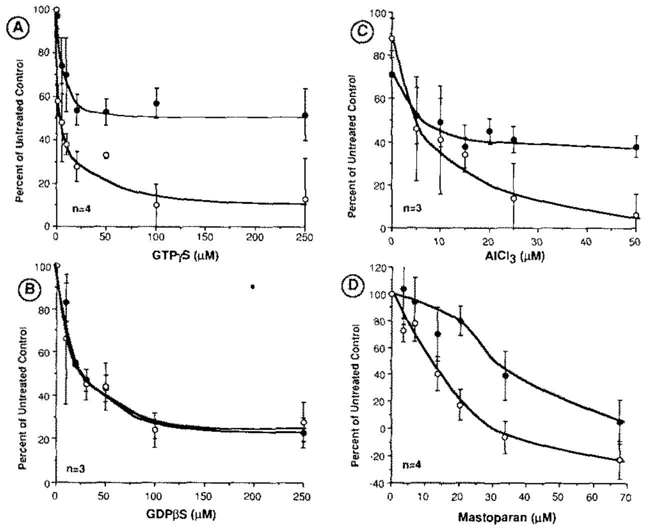


Figure 3. Differential effects of GTP-binding protein antagonists and agonists on coated pit invagination and coated vesicle budding. (*A* and *B*) Perforated A431 cells were incubated as described in Fig. 2 except in the presence of increasing concentrations of GTPγS (*A*) or GDPβs (*B*) as indicated. (*C*) Incubations were as above except that the buffer contained 5 mM NaF and increasing concentrations of $AlCl_3$ as indicated. (*D*) Assays were as described in Fig. 2 except that the volume was doubled. Cytosol, ATP, and BSST concentrations were maintained at standard levels, but the cells were at twofold lower concentration. Mastoparan was added at the indicated concentrations. After 30 min incubations at 37°C, cells were returned to ice and assayed for either avidin inaccessibility (●) or MesNa resistance (○). Signals obtained are expressed as the percentage of the ATP and cytosol-dependent signal obtained for untreated control cells. Points from three or four independent experiments (as indicated) ±SD are shown.

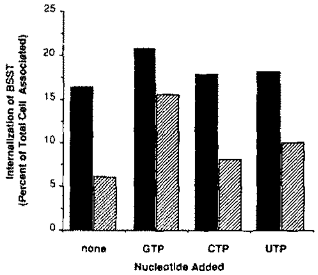


Figure 4. Mastoparan inhibition is prevented by addition of GTP. Perforated A431 cells were incubated in 80 μl of KSHM containing gel-filtered cytosol (2.6 mg/ml), an ATP-regenerating system (800 μM ATP), 2 μg/ml BSST with (▨) or without (■) 10 μM mastoparan and with the following nucleotides, as indicated: GTP (50 μM), CTP (500 μM), and UTP (500 μM). Internalization of BSST was determined by its inaccessibility to MesNa. The data are presented as the percent of cell associated BSST internalized in an ATP and cytosol-dependent manner.

of these was AlF_4^-. Complete assay mixtures containing perforated A431 cells, cytosol and an ATP-regenerating system were incubated at 37°C in the presence of 5 mM NaF and increasing concentrations of $AlCl_3$. These conditions result in the formation of AlF_4^-, which interacts with GDP in the nucleotide binding site of heterotrimer G proteins mimicking the γ-phosphate and converting the G protein to its active "GTP"-bound form (Chabre, 1990). Recent results have shown that while both $G_{\alpha i}$ and $G_{\alpha o}$ subunits of heterotrimeric G proteins are activated by AlF_4^-, ARF, and several members of the low molecular weight *ras*-related GTP-binding proteins are unaffected (Kahn, 1991). The data in Fig. 3 *C* shows that both assays were partially inhibited by 5 mM NaF alone. Addition of $AlCl_3$ further inhibited the internalization of BSST by 80–85%. In contrast, the sequestration of BSST was only inhibited an additional 30% in the presence of $AlCl_3$. For both reactions, half-maximal inhibition occurred at ~10 μM $AlCl_3$, $AlCl_3$ alone (up to 50 μM) had no effect and identical results were obtained using either gel-filtered or nongel-filtered cytosol (data not shown). Since ~40% of BSST sequestration results from coated vesicle budding, as was the case with GTPγS inhibition, these results suggested that coated pit invagination was resistant to AlF_4^-.

Mastoparan Specifically Inhibits Coated Vesicle Budding

Mastoparan is a cationic amphiphilic, α-helical peptide with the well-characterized property of interacting with the α-subunits of heterotrimeric G proteins to activate them by mimicking their interaction with G protein–coupled receptors. (Higashijima et al., 1990; Mousli et al., 1990; Weingarten et al., 1990). To test the effect of mastoparan on receptor-mediated endocytosis, perforated A431 cells were incubated in a complete assay mixture containing gel-filtered cytosol, an ATP-regenerating system, BSST, and increasing concentrations of mastoparan. The data in Fig. 3 *D* shows that although mastoparan was a potent and effective inhibitor of coated vesicle budding, coated pit invagination appeared more resistant. Internalization of BSST into sealed coated vesicles was completely inhibited in the presence of 20–30 μM mastoparan (note the expanded scale used in Fig. 3 *D*). Half-maximal inhibition occurred at <10 μM, concentrations consistent with its specific interaction with G_α-subunits (Higashijima et al., 1990; Mousli et al., 1990). In contrast, the sequestration of BSST was significantly less sensitive to mastoparan (Fig. 3 *D*). At 20 μM mastoparan, internalization was inhibited by ~90% while sequestration is only reduced by ~20%. The biphasic nature of the curve seen for inhibition of sequestration may reflect nonspecific effects of mastoparan at higher concentrations. These data were quantitatively consistent with those obtained using both GTPγS and AlF_4^- and further supported the model that heterotrimeric G proteins participate in coated vesicle budding, but not in coated pit invagination.

Since mastoparan is an amphiphilic α-helical peptide, its interaction with membranes may result in nonspecific inhibition of vesicular transport events. Specific inhibition by mastoparan should be related to its activity in increasing guanine-nucleotide exchange on G_α-subunits. Mastoparan stimulates the dissociation of bound guanine nucleotides from G_α-subunits, but does not directly affect GTP hydrolysis (Higashijima et al., 1990). We therefore tested the effect of GTP on mastoparan inhibition. The data in Fig. 4 shows that in the presence of 10 μM mastoparan (Fig. 4, *stippled bars*) and in the absence of added nucleotides coated vesicle budding was inhibited by ~65%. Mastoparan inhibition was blocked in the presence of 50 μM GTP. This protection was specific to GTP since UTP and CTP (both at 500 μM) and ATP (present at 800 μM) were much less effective.

The active inhibitory species of mastoparan is believed to be the α-helical conformation induced by binding to membranes (Higashijima et al., 1990). The effect of mastoparan on isolated G_α-subunits decreases with increasing concentrations of liposomes in the reaction mixture, presumably as

Table I. Effect of Mastoparan and Its Analogues on Coated Vesicle Budding and Coated Pit Invagination

		Coated vesicle budding		Coated pit invagination	
		(Percent of control)		(Percent of control)	
Peptide	μM	−GTP	+GTP	−GTP	+GTP
None	–	100	132	100	134
Mastoparan	12.5	65	96	96	114
INLKALAALAKKIL	25	22	40	70	111
Mast 7	12.5	66	99	81	128
INLKALAALAKALL	25	25	66	59	70
Mast 11	50	40	56	86	104
INLKALAALKKKLL	100	21	41	71	89
Mast 17	50	69	111	98	115
INLKAKAALAKKLL	100	62	78	81	106
1CS4	50	89	97	98	133
GLAQKLLEALQKALLA	100	54	68	85	97
Arf 26	25	55	119	86	121
GNIFANLFKGLFGKKE	50	46	85	88	108

Assays were performed in 80 μl of KSHM containing perforated A431 cells, 2 μg/ml BSST, 2.6 mg/ml gel-filtered cytosol, an ATP-regenerating system, the indicated concentrations of peptides with or without 50 μM GTP. Data are expressed as the percent of ATP and cytosol dependent internalization or sequestration obtained in a control incubation in the absence of either peptide or GTP.

a result of partitioning of mastoparan between the solution and the competing lipid vesicles (Higashijima et al., 1990). The effectiveness of mastoparan in our system was similarly dependent on the concentration of cellular membranes present (data not shown). To further explore the specificity of mastoparan inhibition, other cationic amphiphilic, α-helical peptides and analogues of mastoparan were tested for their ability to inhibit both coated pit invagination and coated vesicle budding. The results are summarized in Table I. An active analogue of mastoparan, mast 7, inhibited coated vesicle budding at levels comparable to wild-type mastoparan. As with mastoparan, coated pit invagination was less affected. In contrast, mast 11 and mast 17, mastoparan analogues with mutations which disrupt their ability to adopt an α-helical conformation at the membrane surface (Higashijima et al., 1990) were four- to tenfold less potent inhibitors of BSST internalization. 1CS4, an unrelated peptide which is nonetheless cationic and α-helical in structure was also found to be at least 10-fold less effective than mastoparan. Peptide analogues corresponding to the amino-terminus of ARF are potent inhibitors of vesicular transport along the exocytic pathway (Kahn et al., 1992; Balch et al., 1992). We therefore tested an amino terminal 16-mer peptide analogue of ARF for its ability to inhibit CCV-mediated endocytosis. The data in Table I shows that arf26 inhibited coated vesicle formation with an EC$_{50}$ of $\sim$30 μM. As with mastoparan, coated pit invagination was resistant to arf26. Since ARF is presently not recognized to be a major coat protein of CCVs it was possible that this observed inhibition by the arf26 peptide was a reflection of its mastoparan-like structural properties. The arf26 is also a cationic, amphiphilic peptide capable of assuming an α-helical conformation at a membrane surface (Kahn et al., 1992). This possibility was supported by the finding that as with mastoparan, the presence of GTP reduces the observed inhibition by arf26 (Table I).

Involvement of GTP-binding Proteins in Coated Pit Assembly

Our results have so far suggested that distinct GTP-binding proteins participate in coated pit invagination and coated vesicle budding and that heterotrimeric G proteins may be selectively involved in coated vesicle budding. Work by others has demonstrated a role for heterotrimeric G proteins in regulating the assembly of β-COP, ARF, and γ-adaptins onto Golgi membranes (Ktiskakis et al., 1992; Robinson and Kries, 1992; Wong and Brodsky, 1992). Therefore, to directly measure whether GTP-binding proteins might also be involved in coated pit assembly at the cell surface, we examined the effects of these GTP-binding protein antagonists on adaptor-stimulated sequestration of BSST. Perforated A431 cells were incubated in a complete assay mixture in the presence of limiting amounts of gel-filtered cytosol, with or without purified adaptors and in the presence of various GTP-binding protein antagonists. The results shown in Fig. 5 indicate that the effects of GTP and its antagonists on BSST sequestration measured at low cytosol levels ($\sim$0.7 mg/ml, Fig. 5, *stippled bars*) were in agreement with those obtained in the presence of high cytosol ($\sim$2.6 mg/ml, cf. Fig. 3). The effect of the various GTP analogues and of mastoparan on adaptor-stimulated sequestration of BSST can be seen by comparing the stippled bars (−adaptors) with the solid bars (+adaptors) in each case. Adaptor-stimulated sequestration of BSST was inhibited by GTPγS and mastoparan (Fig. 5). As with inhibition of internalization, half-maximal inhibition of coated pit assembly required <5 μM GTPγS and <10 μM mastoparan (data not shown). Adaptor-dependent sequestration appeared to be unaffected by either GTP or GDPβS, although both these reagents altered the overall sequestration presumably by affecting invagination.

Discussion

Novel cell-free assays which enable measurement of three biochemically distinct stages of coated vesicle formation have been employed to demonstrate that multiple GTP-binding proteins are required for receptor-mediated endocytosis. These results along with a model are shown in Fig. 6 which summarizes the effect of various antagonists and

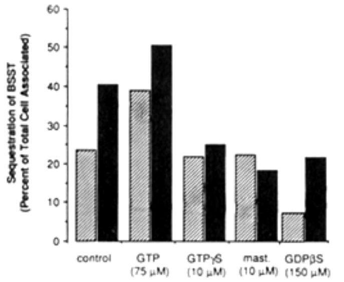
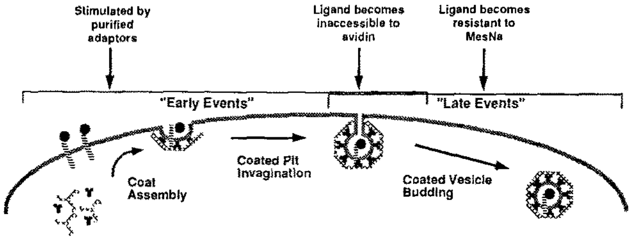


Figure 5. GTP-binding proteins participate in adaptor stimulated early events in coated pit assembly. Perforated A431 cells were incubated in 40 μl KSHM (or 80 μl for mastoparan experiment) containing 0.7 mg/ml gel-filtered cytosol, 2 μg/ml BSST, an ATP-regenerating system, the indicated concentration of guanine nucleotide or mastoparan with (■) or without (▨) 0.25 mg/ml bovine brain adaptors. Sequestration of BSST was determined by its inaccessibility to avidin. The data are presented as the percent of cell associated BSST sequestered in an ATP and cytosol-dependent manner.

agonists of GTP-binding proteins on coated pit assembly, coated pit invagination, and coated vesicle budding.

We have demonstrated elsewhere that adaptor-stimulated sequestration of BSST measures a very early event in coated vesicle formation, that it is supported by plasma membrane specific adaptors (referred to as AP2) but not by Golgi-specific adaptors (referred to as AP1) and that it requires cytosolic clathrin (Smythe et al., 1992*b*). These properties suggest that adaptor-stimulated sequestration of BSST measures the assembly of functionally active coated pits. This reaction was strongly inhibited by GTPγS and by mastoparan (Fig. 6), suggesting the involvement of heterotrimeric G protein(s) in clathrin-coated pit assembly. This result is intriguing given recent evidence for the involvement of trimeric G proteins in regulating the assembly of the coat constituents of COP-CVs. GTPγS and AlF_4^- promote association of ARF and β-COP onto Golgi membranes (Donaldson et al., 1991) and are antagonistic to the actions of brefeldin A, a fungal metabolite which inhibits transport along the exocytic pathway. BFA disrupts the membrane association of both ARF and β-COP (Orci et al., 1991; reviewed by Klausner et al., 1992). However, this effect of BFA is blocked by AlF_4^-, GTPγS and by activation of a pertussis toxin-sensitive G protein (Ktiskakis et al., 1992). Two recent reports have extended these observations and demonstrated that BFA also causes the rapid dissociation of γ and β′-adaptins (the ~100-kD subunits of AP1 adaptors) from Golgi-associated clathrin-coated pits. As for COP-CV coat constituents, AlF_4^- and GTPγS block the BFA effect and appear to enhance the binding of the AP1 adaptors to the Golgi membrane (Robinson and Kries, 1992; Wong and Brodsky, 1992). Interestingly, in these studies, the membrane association of α and β adaptins (the ~100-kD subunits of plasma membrane-specific AP2 adaptors) were unaffected. This finding was consistent with observations that BFA appears not to inhibit receptor-mediated endocytosis (Hunziger et al., 1991; Wood et al., 1991; Damke et al., 1991). Here we report that GTPγS and mastoparan strongly inhibit adaptor-stimulated sequestration of BSST, suggesting that these reagents interfere with de novo coated pit assembly at the plasma membrane. This result indicates an important mechanistic difference between COP-CV or Golgi CCV formation and plasma membrane CCV formation.



PROPERTY	*Principal Event Detected* (Assay)		
	Coated Pit Assembly (Adaptor-stimulated sequestration)	*Invagination* (Avidin-Inaccessibility)	*Coated Vesicle Budding* (MesNa-resistance)
Stimulated by GTP	no?	yes	yes
Inhibited by GDPβS	no?	yes	yes
Inhibited by GTPγS	yes	no	yes
Inhibited by AlF_4^-	nd	no	yes
Inhibited by mastoparan	yes	no	yes

Figure 6. A model for the participation of GTP-binding proteins in biochemically distinct events involved in receptor-mediated endocytosis. Stage-specific assays for receptor-mediated endocytosis of transferrin detect three biochemically distinct events in vitro. These are coated pit assembly, coated pit invagination, and coated vesicle budding. These events are differentially sensitive to antagonists of GTP-binding protein activity, suggesting the involvement of multiple GTP-binding proteins in the overall process of receptor-mediated endocytosis via clathrin coated pits.

The invagination of preformed coated pits appeared resistant to GTPγS, mastoparan and AlF_4^-, suggesting that this event was independent of trimeric G proteins. However other GTP-binding proteins were clearly implicated in coated pit invagination since the sequestration of BSST into deeply invaginated pits was stimulated by GTP and inhibited by GDPβS (Fig. 6). We did not examine whether GTPγS was able to inhibit GTP-stimulated invagination, since the assays for GTPγS inhibition shown here were performed using gel-filtered cytosol in the absence of added GTP. It remains to be demonstrated which GTP-binding protein(s) is involved in this event, however it is of interest to note that coated pit invagination is inhibited in mitotic cells both in vivo (Pypaert et al., 1987) and in vitro (Pypaert et al., 1991) and therefore this event appears to be regulatable. What, if any, role GTP-binding proteins play in the regulation of invagination requires further investigation.

The data also suggests that multiple GTP-binding proteins participate in the final stage of receptor-mediated endocytosis: coated vesicle budding (Fig. 6). As for invagination, coated vesicle budding was stimulated by GTP and inhibited by GDPβS. In addition, coated vesicle budding was inhibited by GTPγS, mastoparan and AlF_4^-, suggesting the involvement of heterotrimeric G protein(s). Inhibition of clathrin-coated vesicle budding by GTPγS again contrasts with results obtained for COP-coated vesicle formation. Addition of GTPγS to in vitro intraGolgi transport assays causes accumulation of COP-coated vesicles (Maholtra et al., 1989). Similarly, vesicle budding from the ER in digitonin permeabilized mammalian cells appears to occur in the presence of GTPγS (Schwaninger et al., 1992). In contrast, GTPγS appears to inhibit vesicle release from the ER in a yeast cell-free assay system (Rexach and Schekman, 1991). These results suggest additional mechanistic differences between CCV formation at the plasma membrane and COP-CV formation along the exocytic pathway.

The observed inhibition of coated vesicle budding by GTPγS differs from results obtained by Lin et al. (1991) using an indirect assay for coated vesicle formation based on the measurement of the loss of clathrin from isolated plasma membrane fragments. In this system 1 mM $GTP\gamma S$ failed to inhibit clathrin loss. Two other major differences in the biochemical requirements for coated vesicle budding observed in our system distinguish this process from that leading to clathrin loss as measured by Lin et al. (1991). First, ATP hydrolysis is absolutely required for coated vesicle budding in perforated A431 cells (Smythe et al., 1989; Smythe et al., 1992*b*) but not for clathrin loss from isolated plasma membranes (both $ATP\gamma S$ and ADP will fulfill the "ATP-requirements" for clathrin loss). Secondly, whereas 150–500 μM Ca^{2+} is required for clathrin loss from isolated membranes, coated vesicle budding in perforated A431 cells does not require Ca^{2+} (Smythe et al., 1989, 1992*b*; LaMaze, C., T. Redelmeier, and S. Schmid, manuscript in preparation). Given these differing biochemical properties, the clathrin loss detected by Lin et al. (1991) may not reflect coated vesicle formation.

The data demonstrates that multiple GTP-binding proteins participate in receptor-mediated endocytosis. Which GTP-binding proteins participate in which events and whether individual GTP-binding proteins might participate in more than one event remains to be determined. The observed inhibition of coated pit assembly and coated vesicle budding by AlF_4- and mastoparan suggests that heterotrimeric G proteins participate in at least two stages of receptor-mediated endocytosis. The specificity of these reagents for heterotrimeric G proteins is supported by our results that the concentrations required for inhibition were well within the range seen both for inhibition of other intracellular transport events (see for example Columbo et al., 1992) and for activation of isolated G_α-subunits in other reconstituted systems (see for example Higashijima et al., 1990; Kahn, 1991). Furthermore, inhibition by mastoparan was blocked in the presence of GTP. This result adds further support to the importance of GTP hydrolysis in these events.

Although these specificity controls strengthen a model for the participation of trimeric G proteins in endocytosis, the data falls short of directly demonstrating their involvement. Several attempts were made to examine the effects of isolated bovine brain $\beta\gamma$ subunits on endocytosis in vitro. In systems reconstituted with purified components, $\beta\gamma$ subunits inactivate G_α subunits when present in the 10–1,000 pM range. Addition of up to 250 nM bovine brain $\beta\gamma$ subunits had no effect on our in vitro endocytosis assay. Detergent effects prevented testing at higher concentrations. Isolated $\beta\gamma$ subunits have been shown to inhibit endosome fusion at ∼400 nM (Columbo et al., 1992) and β-COP association with Golgi membranes at 3 μM (Donaldson et al., 1991). The effect of purified transducin $\beta\gamma$ subunits (50–500 nM) were also tested in an effort to bypass the detergent requirements. Although the results obtained using these subunits were suggestive of stimulating internalization, they were poorly reproducible for as yet unexplained reasons. The possibility therefore remains that the effects of mastoparan and AlF_4- reflect the involvement of a G protein coupled signalling pathway which regulates endocytosis rather than the direct involvement of G proteins as constitutive participants in this process.

Both coated pit invagination and coated vesicle budding were stimulated by GTP, suggesting that it was an important limiting component in gel-filtered cytosol. To our knowledge, the GTP requirement observed in this system has not been reported for other in vitro membrane transport systems, despite the well-documented involvement of multiple GTP-binding proteins in COP-coated vesicle mediated transport events (Balch, 1990). Given the nucleoside diphosphotransferase activity present in the crude cytosolic fractions and the presence of high ATP levels, low concentrations of GTP are undoubtedly present in each of these assay systems. Therefore our ability to detect a GTP requirement may reflect the involvement of a GTP-binding protein with a higher turnover rate for GTP and/or a lower affinity for GTP than either small or large regulatory G proteins. One candidate for such a protein might be dynamin. Work is in progress to directly demonstrate a role for dynamin in clathrin-coated vesicle formation.

A third class of GTP-binding proteins implicated in vesicular trafficking events are the rab-related family of low molecular weight GTP-binding proteins. This class of proteins have to date been implicated in vesicle targeting and fusion events but not in vesicle formation (Balch, 1990; Goud and McCaffrey, 1991). Recent studies have demonstrated that coated vesicles carry at least one to two molecules of the small GTP-binding protein rab5 and that this protein regulates endocytic vesicle fusion (Bucci et al., 1992). Whether rab5 or another small-GTP binding protein participates in coated vesicle formation remains to be determined.

In summary, we have provided several lines of evidence for the participation of multiple GTP-binding proteins in clathrin-coated vesicle formation. These findings provide new insight into the processes involved in receptor-mediated endocytosis and suggest that COP-CV–mediated transport events and CCV-mediated transport events are in some ways mechanistically related and in others, mechanistically distinct. The identification of the GTP-binding proteins which participate in these processes now becomes essential. In contrast to more complex assay systems which require vesicle formation or both vesicle formation and consumption, the assays used here dissect vesicle formation into three biochemically distinct events, coat assembly, invagination and coated vesicle budding. Thus the stage-specific assays used here provide valuable tools for the detailed biochemical dissection of the involvement of identified GTP-binding proteins in the sequential events leading to clathrin-coated vesicle formation.

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References

Balch, W. E. 1990. Small GTP-binding proteins in vesicular transport. *Trends in Biochem. Sci.* 15:473–477.

Balch, W. E. 1992. From G minor to G major. *Curr. Biol.* 2:157–160.

Balch, W. E., R. A. Kahn, and R. Schwaninger. 1992. ADP-ribosylation factor (ARF) is required for vesicular trafficking between the endoplasmic reticulum (ER) and the cis Golgi compartment. *J. Biol. Chem.* 267:13053–13061.

Barr, F. A., A. Leyte, S. Moller, T. Pfeuffer, S. A. Tooze, and W. B. Huttner. 1991. Trimeric G-proteins of the trans-Golgi network are involved in the formation of constitutive secretory vesicles and immature secretory granules. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 293:1–5.

Barr, F. A., A. Leyte, and W. B. Huttner. 1992. Trimeric G proteins and vesicle formation. Trends Cell Biol. 2:91–94.

Bourne, H. R., D. A. Sanders, and F. McCormick. 1990. The GTPase superfamily: a conserved switch for diverse cell functions. *Nature (Lond.).* 348: 125–132.

Braulke, T., S. Tippmer, E. Neher, and K. von Figura. 1989. Regulation of the mannose 6-phosphate/IGF-II receptor expression at the cell surface by mannose 6-phosate, insulin like growth factors and epidermal growth factor. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:681–686.

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

Chabre, M. 1990. Aluminofluoride and beryllofluoride complexes: new phosphate analogs in enzymology. *Trends Biochem. Sci.* 15:6–10.

Chen, M. S., R. A. Obar, C. C. Schroeder, T. W. Austin, C. A. Poodry, S. C. Wadsworth, and R. B. Vallee. 1991. Multiple forms of dynamin are encoded by Shibire, a Drosophila gene involved in endocytosis. *Nature (Lond.).* 351:583–586.

Collins, C. 1991. Dynamin: a novel microtubule associated GTPase. *Trends Cell Biol.* 1:57–60.

Columbo, M. I., L. S. Mayorga, P. J. Casey, and P. D. Stahl. 1992. Evidence of a role for heterotrimeric GTP-binding proteins in endosome fusion. *Science (Wash. DC).* In press.

Damke, H., J. Klumperman, K. von Figura, and T. Braulke. 1991. Effects of Brefeldin A on the endocytic route. *J. Biol. Chem.* 266:24829–24833.

Donaldson, J. G., R. A. Kahn, J. Lippincott-Schwartz, and R. D. Klausner. 1991. Binding of ARF and β-COP to Golgi membranes: possible regulation by a trimeric G protein. *Science (Wash. DC).* 254:1197–1199.

Duden, R., G. Griffiths, R. Frank, P. Argos, and T. E. Kries. 1991. β-COP, a 110 kD protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to β-adaptin. *Cell.* 64:649–665.

Goud, B., and M. McCaffrey. 1991. Small GTP binding proteins and their role in transport. *Curr. Opin. Cell Biol.* 3:626–633.

Higashijima, T., J. Burnier, and E. M. Ross. 1990. Regulation of G_i and G_o by mastoparan, related amphiphilic peptides, and hydrophobic amines. *J. Biol. Chem.* 265:14176–14186.

Hunziker, W., P. Male, and I. Mellman. 1990. Differential microtubule requirements for transcytosis in MDCK cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3515–3525.

Hunziker, W., J. A. Whitney, and I. Mellman. 1991. Selective inhibition of transcytosis by Brefeldin A in MDCK cells. *Cell.* 67:617–627.

Kahn, R. A. 1991. Fluoride is not an activator of the smaller (20–25 kDa) GTP-binding proteins. *J. Biol. Chem.* 266:15595–15597.

Kahn, R. A., P. Randazzo, T. Serafini, O. Weiss, C. Rulka, J. Clark, M. Amherdt, P. Roller, L. Orci, and J. E. Rothman. 1992. The amino terminus of ADP ribosylation factor (ARF) is a critical determinant of ARF activities and is a potent and specific inhibitor of protein transport. *J. Biol. Chem.* 267:13039–13046.

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

Klausner, R. D., J. G. Donaldson, and J. Lippincott-Schwartz. 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* 116:10171–1080.

Kosaka, T., and K. Ikeda. 1983. Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of Drosophila melanogaster, shibire. *J. Cell Biol.* 97:499–507.

Ktistakis, N. T., M. E. Linder, and M. G. Roth. 1992. Action of brefeldin A blocked by activation of a pertussis-toxin-sensitive G protein. *Nature (Lond.).* 356:344–346.

Lin, H. C., M. S. Moore, D. A. Sanan, and R. G. W. Anderson. 1991. Reconstitution of clathrin-coated pit budding from plasma membranes. *J. Cell Biol.* 114:881–891.

Mahaffey, D. T., J. S. Peeler, F. M. Brodsky, and R. G. W. Anderson. 1990. Clathrin-coated pits contain an integral membrane protein that binds the AP2 subunit with high affinity. *J. Biol. Chem.* 265:16514–16520.

Maholta, V., T. Serafini, L. Orci, J. C. Shepherd, and J. E. Rothman. 1989. Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell.* 58:329–336.

Morgan, E. H., and B. J. Iacopetta. 1987. Vinblastine but not other microtubule inhibitors block transferrin endocytosis and iron uptake by reticulocytes. *Clin. Exp. Pharmacol. Phys.* 14:119–126.

Mousli, M., J.-L. Bueb, C. Bronner, B. Roudot, and Y. Landry. 1990. G protein activation: a receptor-independent mode of action of cationic amphiphilic neuropeptides and venom peptides. *Trends Biochem. Sci.* 11: 358–364.

Orci, L., B. S. Glick, and J. E. Rothman. 1986. A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. *Cell.* 46:171–184.

Orci, L., M. Tagaya, M. Amherdt, K. Perrelet, J. G. Donaldson, J. Lippincott-Schwartz, R. D. Klausner, and J. E. Rothman. 1991. Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell.* 64:1183–1195.

Pearse, B. M. F., and R. A. Crowther. 1987. Structure and assembly of coated vesicles. *Ann. Rev. Biophys. Chem.* 16:49–68.

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

Pypaert, M., J. M. Lucocq, and G. Warren. 1987. Coated pits in interphase and mitotic A431 cells. *Eur. J. Cell Biol.* 45:23–29.

Pypaert, M., D. Mundy, W. Souter, J.-C. Labbé, and G. Warren. 1991. Mitotic cytosol inhibits invagination of coated pits in broken mitotic cells. *J. Cell Biol.* 114:1159–1166.

Rexach, M. F., and R. W. Schekman. 1991. Distinct biochemical requirements for budding, targeting and fusion of ER-derived transport vesicles. *J. Cell Biol.* 114:219–229.

Robinson, M. S., and T. J. Kreis. 1992. Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of brefeldin A and G protein activators. *Cell.* 69:129–138.

Rothman, J. E., and L. Orci. 1992. Molecular dissection of the secretory pathway. *Nature (Lond.).* 355:409–415.

Schmid, S. L. 1992. The mechanism of receptor-mediated endocytosis: more questions than answers. *BioEssays.* 14:581–588.

Schmid, S. L., and E. Smythe. 1991. Stage-specific assays for coated pit formation and coated vesicle budding in vitro. *J. Cell Biol.* 114:860–880.

Schwaninger, R., H. Plutner, G. M. Bokoch, and W. E. Balch. 1992. Multiple GTP-binding proteins regulate vesicular transport from the endoplasmic reticulum to Golgi membranes. *J. Cell Biol.* 119:1077–1096.

Serafini, T., L. Orci, M. Amherdt, M. Brunner, R. A. Kahn, and J. E. Rothman. 1991. ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. *Cell.* 67:239–253.

Shpetner, H. S., and R. B. Vallee. 1989. Identification of dynamin, a novel mechanochemical enzyme that mediates interactions between microtubules. *Cell.* 59:421–432.

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

Smythe, E., T. E. Redelmeier, and S. L. Schmid. 1992*a*. Receptor-mediated endocytosis in semi-intact cells. *In* Methods in Enzymology "Reconstitution of Intracellular Transport." J. E. Rothman, editor. Vol. 219. 223–234.

Smythe, E., L. L. Carter, and S. L. Schmid. 1992*b*. Cytosol- and clathrin-dependent stimulation of endocytosis *in vitro* by purified adaptors. *J. Cell Biol.* 119:1163–1172.

Stow, J. L., J. B. De Almeida, N. Narula, K. J. Holtzman, L. Ercolani, and S. A. Ausiello. (1991). A heterotrimeric G protein, $G_{\alpha i3}$, on Golgi membranes regulates the secretion of a heparan sulfate proteoglycan in LLC-PK epithelial cells. *J. Cell Biol.* 114:1113–1124.

van der Bliek, A. M., and E. M. Meyerowitz. 1991. Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. *Nature (Lond.).* 351:411–414.

Waters, M. G., T. Serafini, and J. E. Rothman. 1991. 'Coatomer': a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. *Nature (Lond.).* 349:248–251.

Weingarten, R., L. Ransnas, H. Mueller, L. A. Sklar, and G. M. Bokoch. 1990. Mastoparan interacts with the carboxyl terminus of the α subunit of G_i. *J. Biol. Chem.* 265:11044–11049.

Wong, D. H., and F. M. Brodsky. 1992. 100 kD proteins of Golgi- and *trans*-Golgi network-associated coated vesicles have related by distinct membrane binding properties. *J. Cell Biol.* 117:1171–1179.

Wood, S. A., J. E. Park, and W. J. Brown. 1991. Brefeldin A causes a microtubule-mediated fusion of the trans-Golgi network and early endosomes. *Cell.* 67:591–600.