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, AlF4-, 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, AlF4-, 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) 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 NH2-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., 1992b). In contrast, COPs are present in the cytosol as a large multimeric precursor termed a "coatamer" 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 non-selective, 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
implicated a role for dynamin, a microtubule-stimulated GTPase (Schietner 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., 1992a,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 this evidence suggests 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). Nucleosides 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 H2O; Sigma Chemical Co., St. Louis, MO) was stored at 4°C and was used within 2 wk. Immediately before use, the mastoparan solution was diluted 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., 1992a). Human diferric transferrin (Boehringer-Mannheim Biochemicals) was biotinylated via a cleavable disulfide 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., 1992a). 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 at -70°C. Bovine brain adaptors were prepared exactly as described by Smythe et al. (1992b). Internalization and sequestration assays were performed exactly as previously described (Schmid and Smythe, 1991; Smythe et al., 1992a). Duplicate samples were run for each experimental point.

Briefly, assay components including KSHM, an ATP-regenerating system containing an ATP-regenerating system (containing 1 mM ATP, creatine phosphate 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 × 10^6 cells) resuspended in 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 and reagent(s) used in the experiment. 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 1% BSA, 1 mM EDTA, 50 mM NaCl, 0.2% BSA, pH 7.6. 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. 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 1% BSA, 1 mM EDTA, 50 mM NaCl, 0.2% BSA, pH 7.6. 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.

Preparation of ELISA Plates

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-phenylenecliamiae, 10 μl H2O2 in 25 ml of 50 mM Na2HPO4, 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 H2SO4. 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., 1992a,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 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.

Extensive biochemical and morphological characterization of this assay system (Smythe et al., 1989; Schmid and Smythe, 1991; Smythe et al., 1992b) 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 a 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 next examined the effect of GDPβS on coated pit invagination and coated vesicle budding. 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 A shows that coated vesicle budding, leading to the internalization of BSST was markedly inhibited by GDPβS. Half-maximal inhibition required <5 μM GTPγS and could be fully protected by 1 mM GDP (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 budding. 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 A 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, AIFr- and mastoparan, more selective inhibitors of heterotrimeric G proteins, were examined. The first
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; Moussi 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. 3D 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. 3D). Half-maximal inhibition occurred at <10 μM, concentrations consistent with its specific interaction with Gα-subunits (Higashijima et al., 1990; Moussi et al., 1990). In contrast, the sequestration of BSST was significantly less sensitive to mastoparan (Fig. 3D). 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 AlF₄⁻ 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 Gα-subunits. Mastoparan stimulates the dissociation of bound guanine nucleotides from Gα-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 Gα-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

<table>
<thead>
<tr>
<th>Peptide</th>
<th>µM</th>
<th>Coated vesicle budding</th>
<th>Coated pit invagination</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>~</td>
<td>100 - GTP</td>
<td>100 - GTP</td>
</tr>
<tr>
<td>Mastoparan</td>
<td>12.5</td>
<td>65 - GTP</td>
<td>96 + GTP</td>
</tr>
<tr>
<td>Mast 7</td>
<td>12.5</td>
<td>66 - GTP</td>
<td>99 + GTP</td>
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<td>Mast 11</td>
<td>50</td>
<td>25 - GTP</td>
<td>66 + GTP</td>
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<tr>
<td>Mast 17</td>
<td>100</td>
<td>40 - GTP</td>
<td>56 + GTP</td>
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<tr>
<td>Mast 7</td>
<td>100</td>
<td>69 - GTP</td>
<td>111 + GTP</td>
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<td>Mast 17</td>
<td>100</td>
<td>62 - GTP</td>
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<td>Mast 17</td>
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<td>55 - GTP</td>
<td>119 + GTP</td>
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<tr>
<td>Mast 17</td>
<td>50</td>
<td>46 - GTP</td>
<td>85 + GTP</td>
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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. ICS4, 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 EC₅₀ of ~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 (~0.7 mg/ml, Fig. 5, stippled bars) were in agreement with those obtained in the presence of high cytosol (~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 GDPγS, 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
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 API) and that it requires cytosolic clathrin (Smythe et al., 1992b). 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 AlF4- 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 AlF4- and GTPγS and by activation of a pertussis toxin-sensitive G protein (Kiskakis 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 API adaptors) from Golgi-associated clathrin-coated pits. As for COP-CV coat constituents, AlF4- and GTPγS block the BFA effect and appear to enhance the binding of the API 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.

The invagination of preformed coated pits appeared resistant to GTPγS, mastoparan and AlF4-, 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 could 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 AlF4-, 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 intra-Golgi 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 Scheiman, 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

![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.](attachment:image.png)

![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: 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.](attachment:image.png)
the measurement of the loss of clathrin from isolated plasma membrane fragments. In this system 1 mM GTPγS 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., 1992b) but not for clathrin loss from isolated plasma membranes (both ATPγS and ADP will fulfill the "ATP-requirements" for clathrin loss). Secondly, whereas 150-500 μM Ca2+ is required for clathrin loss from isolated membranes, coated vesicle budding in perforated A431 cells does not require Ca2+ (Smythe et al., 1989, 1992b; LaMaze, C., T. Reddelmeier, 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 AlF4− 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 Go-subunits in other reconstituted systems (see for example Higashijima et al., 1990; Kahn, 1991). Furthermore, inhibition by mastoparan was blocked in the presence of GST. 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 βγ subunits on endocytosis in vitro. In systems reconstituted with purified components, βγ subunits inactivate Gα subunits when present in the 10-1,000 pM range. Addition of up to 250 nM bovine brain βγ subunits had no effect on our in vitro endocytosis assay. Detergent effects prevented testing at higher concentrations. Isolated βγ 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 βγ 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 AlF4− 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 knowl-


