Cell, Vol. 75, 1015–1025, December 3, 1993, Copyright © 1993 by Cell Press

Stepwise Assembly of Functionally Active Transport Vesicles

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Summary

Budding of COP-coated vesicles (the likely carriers of newly synthesized proteins from the endoplasmic reticulum through the Golgi stack) from Golgi cisternae requires ADP-ribosylation factor (ARF), coatomer proteins from the cytosol, GTP, and fatty acyl–coenzyme A (CoA). The assembly of coated buds on the membranes requires coatomer, ARF, and GTP. When palmitoyl-CoA is added, membrane fission occurs at the coated bud, releasing coated vesicles. We show that COP-coated vesicles can be generated stepwise in vitro and isolated in a functionally active form, demonstrating that the minimal set of cytosolic components required for their formation as well as principal steps in their assembly have been identified.

Introduction

COP-coated vesicles have been proposed to mediate the transport of newly synthesized proteins from the endoplasmic reticulum (ER) through the Golgi stack, en route to the cell surface (Orci et al., 1986; Orci et al., 1989). Originally termed non-clathrin or Golgi-derived coated vesicles, these structures were first implicated as carriers because they form de novo in a cell-free system that reconstitutes intercisternal transport in the Golgi stack (Fries and Rothman, 1980; Balch et al., 1984a, 1984b; Braell et al., 1984; Orci et al., 1986) and accumulate when transport is blocked with GTPγS (Melancon et al., 1987). The cytoplasmic coating surrounds but does not extend beyond buds, and Golgi cisternae appear free of coating before incubation with cytosolic proteins. These observations led us to propose (Orci et al., 1986) that vesicle budding is driven by the coordinated assembly of the coat from dispersed cytoplasmic subunits during the course of the cell-free transport reaction, a process that requires both cytosol and a source of energy, such as ATP (Fries and Rothman, 1980; Balch et al., 1984a).

Considerable evidence has since accumulated in support of this simple concept, obtained from studies using both living cells and cell-free systems. Following the purification of COP-coated vesicles (Malhotra et al., 1989; Serafini et al., 1991a), the coat was found to consist principally of two proteins: a heptameric complex, termed coatomer (Waters et al., 1991), consisting of one copy each of α-, β-, β′, γ-, δ-, ε-, and ζ-COPs (Duden et al., 1991; Serafini et al., 1991a; Stenbeck et al., 1993), and the small GTP-binding protein ADP-ribosylation factor (ARF) (Serafini et al., 1991b). Microinjection of antibody to β-COP in living animal cells blocks transport out of the ER (Pepperkok et al., 1993). The γ-COP subunit of coatomer was found to be a homolog of the yeast *SEC21* gene (Stenbeck et al., 1992), required for ER to Golgi transport in yeast cells (Novick et al., 1980; Kaiser and Schekman, 1990), and the yeast equivalent of coatomer was found to contain the *SEC21*-encoded protein as its γ subunit (Hosobuchi et al., 1992). Also, ARF genes are essential in yeast, and lesions in ARF disrupt ER to Golgi transport in vivo (Stearns et al., 1990). It is thus clear that coat proteins of COP-coated vesicles are needed for transport in both yeast and animal cells.

Studies in cell-free systems have shed light on the mechanism of binding of the coat proteins to Golgi membranes. As anticipated from the electron micrographs of Golgi membranes (Orci et al., 1986), coatomer (Donaldson et al., 1991; Donaldson et al., 1992b) and ARF (Walker et al., 1992) are virtually absent from isolated membranes, but bind under conditions that reconstitute transport. Coatomer binding requires the prior binding of ARF, a process that can be reconstituted with Golgi membranes, recombinant N-myristoylated ARF protein, and a guanine nucleoside triphosphate (Donaldson et al., 1992b; Palmer et al., 1993). Cytosolic ARF contains GDP (Kahn and Gilman, 1986; Weiss et al., 1989), and ARF binding is triggered when a Golgi membrane–bound enzyme triggers exchange of this nucleotide with GTP (Donaldson et al., 1992a; Helms and Rothman, 1992; Tsai et al., 1993). The fact that nucleotide exchange and binding of ARF to membranes are inhibited by brefeldin A, a compound that prevents secretion in cells (Fujiwara et al., 1988), additionally underscores that the COP-coated vesicle pathway is the principal physiological pathway for biosynthetic protein transport.

Hydrolysis of GTP bound to ARF following budding triggers uncoating and the initiation of membrane fusion (Tanigawa et al., in press). Tight binding of ARF (resisting extraction by liposomes) is a saturable process, operationally defining an ARF receptor in Golgi membranes (Helms et al., 1993). Coatomer binds in a saturable fashion to these sites (Donaldson et al., 1992b; Palmer et al., 1993) to form complexes with a stoichiometry compatible with the ratio of ARF to coatomer in purified COP-coated vesicles. The coatomer binds from cytosol en bloc, without subunit exchange (Kuge et al., unpublished data), and is required for the process of budding as shown by the results of immunodepletion of coatomer from cytosol (Orci et al., 1993a). Coatomer and ARF are both necessary to reconstitute cell-free vesicular transport between Golgi cisternae (Elazar et al., submitted).

The recent finding that coatomer and ARF are the only cytosolic proteins required to form COP-coated vesicles (Orci et al., 1993b) has opened the door to a mechanistic dissection of the budding process itself. Golgi cisternae remain extended and flat when ARF alone is bound, with buds and vesicles only appearing when coatomer is additionally bound (Orci et al., 1993b). While it is clearly established that coatomer binding is required for the transition from flat membrane to fully formed vesicle, the essence of the budding mechanism could not be uncovered for the lack of defined intermediates in the steps following coatomer binding.

Yet, such intermediates should in principle exist, as the pinching off (fission) of a vesicle must involve the fusion of the membrane bilayers at the base of the bud, initiated on the lumenal (cisternal) side. In addition to Golgi membranes and coatomer and ARF proteins, the budding reactions described by Orci et al. (1993b) contained ATP, GTP, and palmitoyl-coenzyme A (CoA). These nucleotides were added because ATP is required for budding when cytosol is employed (Orci et al., 1986), GTP is needed for ARF binding (Kahn, 1991; Walker et al., 1992; Helms et al., 1993; Randazzo et al., 1993), and long chain fatty acyl–CoA is required in some fashion for budding (Pfanner et al., 1989).

We now report that GTP and acyl-CoA are the only nucleotides that are absolutely required for vesicle budding in a refined system. In the absence of acyl-CoA, coated buds accumulate. When acyl-CoA is added, fission occurs, releasing functionally active transport vesicles, providing the crucial demonstration that COP-coated vesicles are bona fide intermediates in transport, and establishing that the minimum set of nucleotide and protein cytosolic components needed to bud a transport vesicle has indeed been identified.

Results

Assembly of Coated Buds

Coated buds and coated vesicles form when Golgi membranes are incubated with the coat components coatomer and N-myristoylated ARF in the presence of the nonhydrolyzable GTP analog, GTPγS, as well as ATP and palmitoyl-CoA (Orci et al., 1993b). The use of GTPγS to accumulate vesicles has prevented us from assessing whether these vesicles are functional because hydrolysis of GTP by ARF is required for uncoating prior to fusion and consumption of the vesicles (Tanigawa et al., in press). We were limited to the use of GTPγS in earlier experiments using crude cytosol because the rapid hydrolysis of GTP (as transport proceeds) prevented sufficient accumulation of the normally transient coated vesicle carriers to permit their isolation. As a first step towards the isolation of functionally active vesicles, we therefore had to establish conditions under which coated structures form with equal efficiency whether in the presence of GTP or GTPγS. One possible reason for the need to use GTPγS rather than GTP to accumulate coated vesicles in our earlier studies was the presence of GTPases in our rabbit liver Golgi preparations. We recently found that Golgi preparations from CHO cells have a lower endogenous GTPase activity (Helms et al., 1993). Another possible explanation might be cytosolic uncoating factors loosely bound to the Golgi membranes.

To minimize these potential problems in this study, we now employ Golgi membranes from CHO cells that have been previously washed with 0.25 mM KCl to remove any bound cytosolic factors, the same salt concentration used previously to extract COP-coated vesicles as the first step in their purification (Malhotra et al., 1989) and to utilize pure coatomer and ARF, rather than crude cytosol. We find that coatomer binding to membranes is now equally efficient in the presence of GTP or GTPγS (Figure 1a). Neither ATP nor palmitoyl-CoA is necessary for or affects the extent of coatomer binding. However, GTP is absolutely required (Figure 1a).

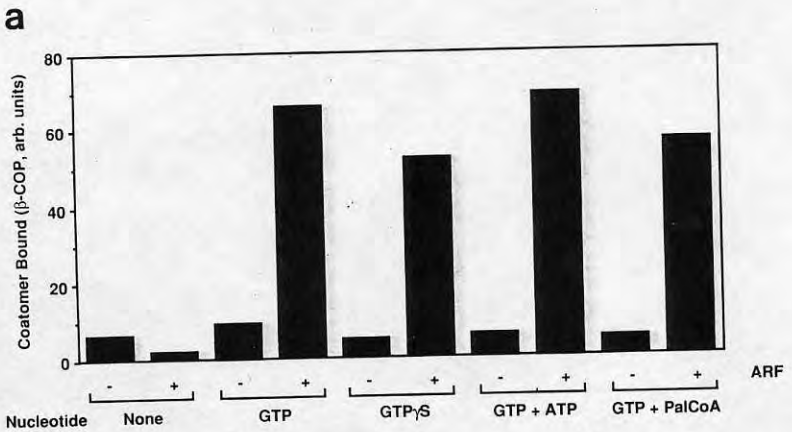
Electron microscopy revealed that the coated structures that are formed under these conditions (coatomer, ARF, Golgi, and GTP) are almost exclusively (95% ± 3% in serial sections) coated buds that are coated along their length, but whose bilayer remains continuous with the parent cisterna (Figure 1c). Coated buds required coatomer and ARF to form (Figure 1b); a total of 34 ± 3 coated buds were formed per square millimeter of Golgi in the presence of coatomer and ARF, as compared with 2.1 ± 0.8 in the absence of these proteins. Immunoelectron microscopy confirmed that the coated buds contained both coatomer and ARF proteins (data not shown).

Fission: Coated Buds Produce Coated Vesicles

Are these coated buds an intermediate stage in coated vesicle formation, or are these structures dead ends? To ascertain this, we carried out two stage reactions. In the first stage, we formed coated buds by incubating Golgi membranes with coatomer, ARF, and GTP. Any unbound coatomer, ARF, GTP, and any free coated vesicles were removed as the membranes were pelleted. Then, in the second stage, we added to the resuspended membranes both of the remaining nucleotides we have used in the past, palmitoyl-CoA and ATP, to investigate whether coated vesicles form, and if so, to determine which nucleotides would be needed in stage II.

To measure fully formed coated vesicles that had been produced in stage II, and to be able to test them for functional competence, we pelleted the membranes after stage II in a 0.25 M KCl-containing medium (coated buds will not be released), thus preparing the stage II supernatant in a manner corresponding to the first step in the purification of COP-coated vesicles (Malhotra et al., 1989). To allow us to follow cargo incorporated into these vesicles and test for transport competence, we used Golgi membranes from VSV-infected cells of the CHO mutant cell line 15B that had been pulse-labeled with [^{35}S]methionine for 15 min in vivo before homogenization, a time period that labels a transport-competent pool of VSV G protein in the Golgi (Fries and Rothman, 1981).

When the supernatant from a stage II reaction containing ATP and palmitoyl-CoA (but no other components) was centrifuged to equilibrium in a sucrose density gradient, the membranes containing VSV G protein peaked between 40% and 44% (w/w) sucrose (Figure 2a), corre-



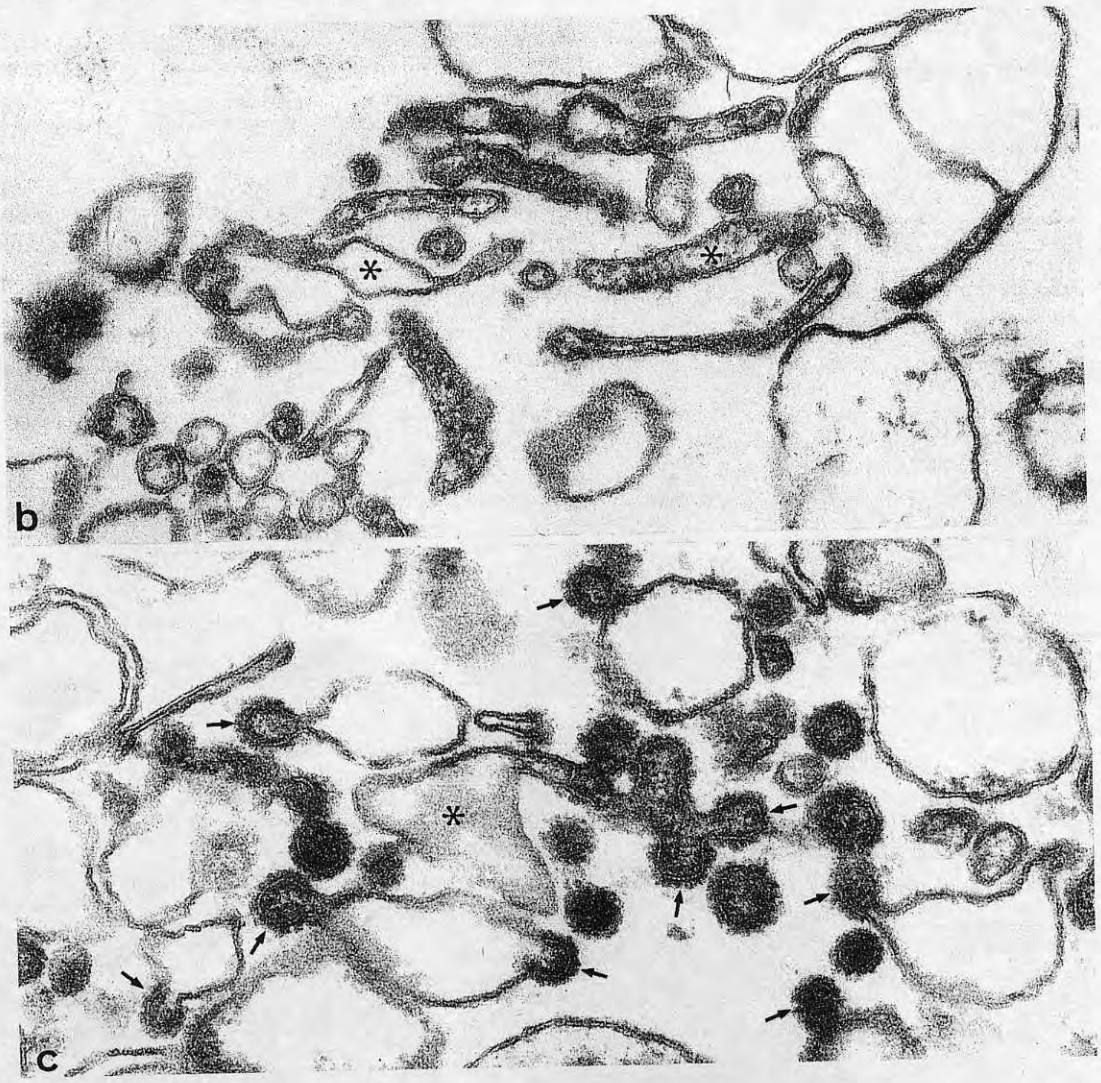


Figure 1. Coated Buds Accumulate on Golgi Membranes In the Presence of GTP or GTPγS In a Reaction Requiring Only the Coat Components Coatomer and ARF

(a) Binding of β-COP to Golgi membranes. Golgi membranes were prewashed with 0.25 M KCl to remove any cytosolic contaminations and were incubated with coatomer in the presence or absence of N-myristoylated ARF and the indicated nucleotides. ATP connotes the addition of ATP, creatine phosphate, and creatine kinase. Membranes were reisolated by flotation, and the amount of bound coatomer (as β-COP) was determined by Western blotting.

(b and c) Electron microscopy of Golgi membranes after incubation in the absence (b) or presence (c) of coatomer and ARF (95,000 ×). Note in (c) the abundant coated buds (arrows). The asterisk shows a cisternal element.

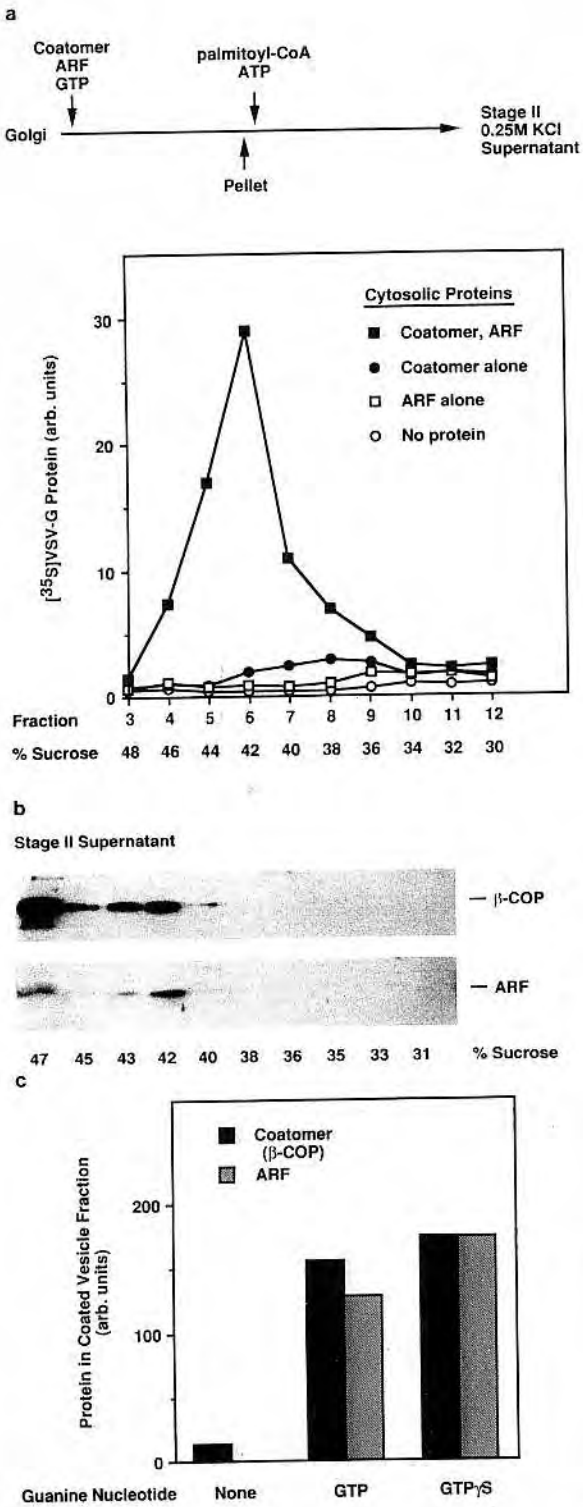


Figure 2. Coated Vesicles Pinch Off from Coated Buds

(a) VSV [^{35}S]G protein–containing membranes released from coated Golgi cisternae fractionate as coated vesicles. Both coatomer and ARF were added in stage I (closed squares), or only coatomer (closed circles), only ARF (open squares), or neither (open circles) was added. Palmitoyl-CoA and ATP were added in stage II in every case. Salt was added to 0.25 M KCl final concentration, Golgi was pelleted, and the supernatant (containing the vesicles) was loaded on a sucrose gradient. Labeled proteins were visualized by fluorography, and the amount of VSV G protein in each fraction was quantified with a phosphorimager.

(b) Coatomer and ARF were present in the vesicle fractions. Coatomer (as β-COP) and ARF present in the gradient fractions were detected by Western blotting.

(c) Vesicles made with GTP and GTPγS contain similar amounts of coat components. The amount of coatomer (i.e., β-COP) and ARF present in gradient fractions of 40%–44% sucrose, after budding in stage I either with no added guanine nucleotide, 1 mM MgGTP, or 20 μM GTPγS, was determined by Western blotting.

sponding to the density of COP-coated vesicles formed with cytosol and GTPγS (Malhotra et al., 1989; Serafini et al., 1991a). To confirm that fission in the second stage required the preassembly of coated structures in the first stage of incubation, two stage assays were performed in which coatomer, ARF, or both were selectively omitted from stage I and not added in stage II. Only trace amounts of vesicles were produced under these conditions (Figure 2a), when the stage I incubation was done without GTP (Figure 3b; see Figure 5a) on ice (rather than 37°C) or when the binding of ARF to membranes was prevented by the addition of brefeldin A (data not shown).

We conducted a number of studies to confirm that the major product of the stage II incubation (i.e., the peak containing VSV G protein at 40%–44% [w/w] sucrose) is in fact COP-coated vesicles. Western blotting analysis of gradient fractions (see Figure 2b) showed that coatomer (β-COP subunit) and ARF peak together with VSV G in the gradient at the expected density for coated vesicles. Coatomer and ARF were also found in the bottom fractions of the gradient, where the sample had been loaded, representing soluble material that had been extracted from the Golgi membranes during the preparation of the stage II supernatant. Whether GTP or GTPγS was used in stage I, no significant differences were found in the amount of vesicles formed (as judged by the amount of VSV G present, Figure 5a) or in the amount of coatomer or ARF proteins present in the coated vesicle–containing fractions (see Figure 2c). Previously, ARF had only been shown to be a component of coated vesicles formed in the presence of GTPγS (Serafini et al., 1991b). The fact that the ratio of ARF to coatomer is the same with GTP and GTPγS (see Figure 2c) implies that ARF is a stable component of the coat and that the hydrolysis of GTP bound to ARF, which is a requirement for uncoating (Tanigawa et al., in press), does not occur spontaneously; in other words, hydrolysis of ARFs bound to GTP must require other cytosolic factors, docking of the vesicle to its target membrane, or both.

To confirm that the VSV G protein present in the 40%–44% (w/w) sucrose gradient fractions actually resides in coated vesicles, we immunoisolated COP-coated vesicles from these fractions with magnetic beads using a monoclonal antibody (CM1A10 [Palmer et al., 1993]) that binds native coatomer, both in solution and in coats (Figure 3a). Coatomer antibody–dependent binding of up to 50% of the added COP-coated vesicles (as determined by the amount of β-COP bound to the beads) and of the VSV

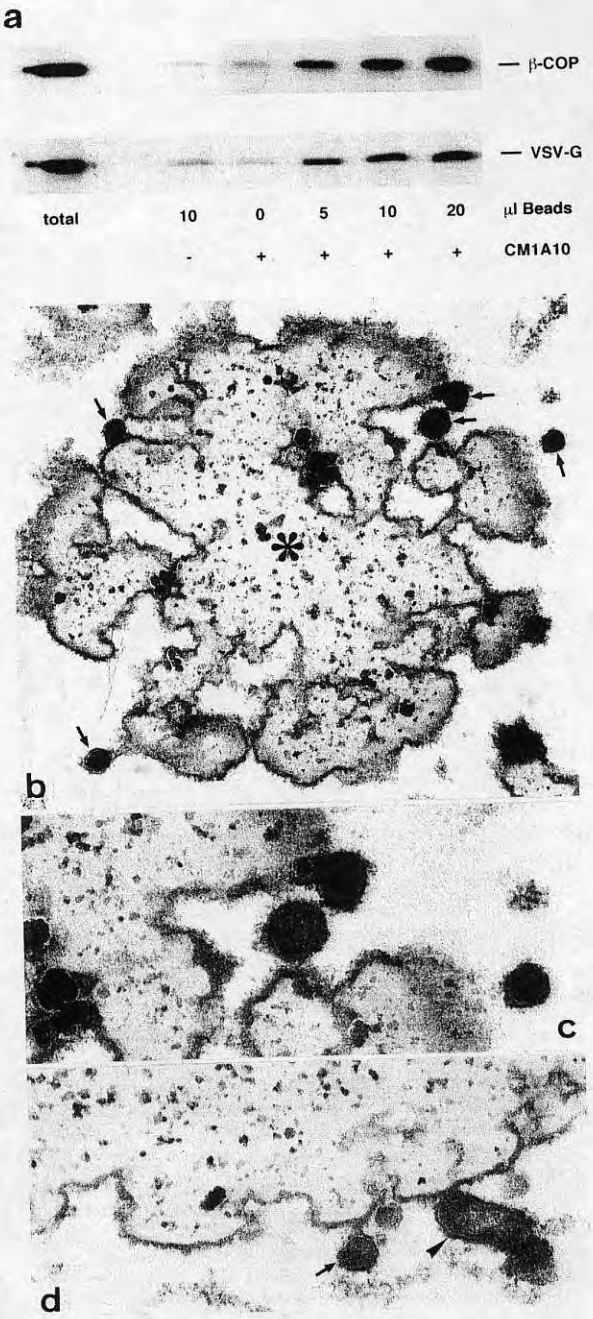


Figure 3. Isolation of Coated Vesicles by Binding to Immunobeads

(a) β-COP and VSV G protein present in the coated vesicle fractions are immunoprecipitated by a coatomer antibody. Vesicles were made in a two-stage incubation and isolated on the sucrose gradient. The peak fractions of the VSV G and β-COP distribution were pooled. Aliquots were incubated with CM1A10 antibody (Palmer et al., 1993) when indicated and increasing amounts of magnetic beads. VSV G protein was detected by fluorography, and β-COP was detected by Western blotting.

(b, c, and d) Electron microscopy of immunobeads. (b) Overview of a thin-sectioned immunobead (asterisk) with five membrane vesicular profiles (arrows) showing different degrees of coating. Note the irregular contour of the bead that may also contain small dark round spots not to be confused with coated vesicles. (c) Detail of a segment of the bead shown in (b) with three coated vesicles. The surface connection of the vesicle to the right is not apparent on this tangential plane of the section. (d) Periphery of a magnetic bead with a vesicular-coated (arrow) and a cisternal-coated (arrowhead) profile. Magnification is as follows: (b) 50,826×; (c) 101,910×; (d) 81,528×.

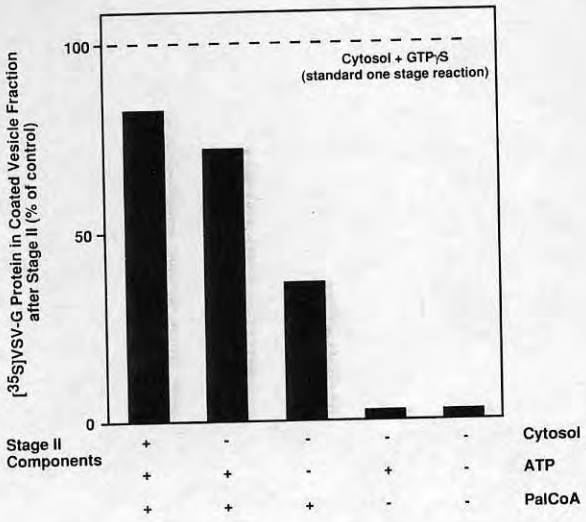


Figure 4. Fission of Coated Buds Does Not Require Addition of Cytosolic Protein Factors

Vesicles were made in a two-stage incubation with coatomer, ARF, and GTPγS in stage I and the indicated additions in stage II, or (for purposes of comparison) with cytosol and GTPγS in a one-stage incubation (dashed line), and were purified on the sucrose gradient. The amount of VSV G present in the COP-coated vesicle peak (fractions 5–8) of the gradient was determined with a phosphorimager and expressed as a percentage of the quantity of vesicles formed with cytosol and GTPγS, a standard reference. Various components were added or omitted from stage II as indicated. ATP connotes the addition of ATP, creatine phosphate, and creatine kinase.

G protein present in the vesicle containing fractions was observed. The beads were examined by electron microscopy. The structures observed consisted mainly (89% ± 3%) of fully formed COP-coated vesicles (as confirmed by serial sectioning; mean ± SEM, 10 sectioned profiles of magnetic beads were evaluated in 5 different fields). The remaining ~11% were COP-coated buds attached to a sealed cisternal fragment whose length was typically one to three times the diameter of the coated vesicle portion.

The cisternal regions attached to the coated buds would be expected to contribute Golgi resident proteins, such as the enzyme mannosidase II, in substantial amounts to the isolated coated vesicle fraction. This is because the cisternal outpocketings of coated buds will disproportionately contribute membrane mass to the fraction as compared with coated vesicles. Resident proteins may also be present in the coated regions; it is not yet known whether retention in the Golgi is accomplished in part by retrieval (following transport) from later compartments or even whether the retention mechanism is effectively reconstituted in our system. Indeed, we find that up to 5% of the Golgi mannosidase II activity is recovered in the stage II

supernatant (data not shown). Electron microscopic analysis will be required to determine whether all of the mannosidase II is present in cisternal regions; preliminary data are consistent with this.

Fatty Acyl-CoA Alone Is Sufficient for Fission

The previous results show that it is not absolutely required to add protein components for coated buds to pinch off. To measure the relative efficiency of this reaction, we compared the amount of vesicles formed in stage II with the amount of vesicles formed with cytosol and GTPγS in a one-stage incubation (dashed line in Figure 4). When Stage II was performed with cytosol, ATP, and palmitoyl-CoA, the relative efficiency was about 85% (Figure 4). Omitting cytosol from stage II had almost no effect, showing that additional soluble proteins are not required at this stage. Additionally omitting palmitoyl-CoA completely prevented budding, showing that palmitoyl-CoA is absolutely required. Omitting ATP reduced budding by about 50%, implying that while not absolutely required and inactive by itself, ATP can stimulate the capacity of palmitoyl-CoA to sustain budding.

The absolute efficiency of budding was 5%–10%, measured as the fraction of the VSV G protein present in the starting Golgi fraction that was recovered in the stage II supernatant. This compares with 10% of input VSV G in coated vesicles with cytosol and GTPγS in a standard one-stage reaction and is a reasonable figure for a single round of a transient carrier. The number of coated vesicles formed could not be increased by incubating Golgi membranes in a one-stage reaction with coatomer, ARF, and GTP together with palmitoyl-CoA and ATP or by addition of GTP in stage II (data not shown). A limiting factor may be the proportion of ARF that is N-myristoylated, which varies between ARF preparations. The time course for budding (stage II) is linear for 3 min and maximal by 5 min, which was the time employed here (data not shown).

In previous studies (Pfanner et al., 1989), the requirement for fatty acyl–CoA could only be demonstrated following the extraction of endogenous pools with low concentrations of ethanol or detergent. We now observe a strict requirement for acyl-CoA even without these maneuvers and think it is likely that the endogenous pool of acyl-CoA is depleted during the first stage of incubation, releasing vesicles that we do not measure after stage II into the stage I supernatant. Acylation apparently must occur after the coated bud has assembled because the small amount of vesicles formed with coatomer, ARF, and GTP alone could not be increased by previous incubation with palmitoyl-CoA (data not shown). We do not think ATP stimulates palmitoyl-CoA-dependent budding indirectly, via the synthesis of acyl-CoA from fatty acid and CoASH, as is observed in reactions containing crude cytosol (Glick and Rothman, 1987) because when radiolabeled palmitoyl-CoA is added to stage II (in the absence of cytosol), about 90% is still intact after the incubation. Addition of hexokinase and glucose to stage II prior to palmitoyl-CoA does not reduce the yield of vesicles whose budding is driven by palmitoyl-CoA alone (data not shown). The identification of the acceptor molecule for the acyl-CoA and the membrane protein factors involved in fission will be required for further insights into the role of ATP at this stage.

Coated Vesicles Are Functional Intermediates In Transport

Although much correlative evidence implies that COP-coated vesicles are the carriers between Golgi cisternae, this has never been directly demonstrated. We therefore added de novo synthesized COP-coated vesicles to Golgi membranes to test for competence in fusion and, in so doing, to confirm that the coated vesicles assembled from the minimum set of cytosolic and nucleotide components are complete. Due to the absence of the N-acetyl–glucosamin (GlcNAc) transferase I of the mutant CHO cell line 15B (whose Golgi were used to assemble COP-coated vesicles), the asparagine-linked oligosaccharides attached to VSV G protein synthesized in these cells can be cleaved off by endoglycosidase H (endo H). This increases the mobility of VSV G during SDS gel electrophoresis. However, when VSV G protein is delivered by membrane fusion to the medial cisternae of Golgi from wild-type CHO cells, the oligosaccharide chains receive GlcNAc and become resistant to digestion by endo H, allowing a simple test for fusion competence (Fries and Rothman, 1980).

COP-coated vesicles pinched off with palmitoyl-CoA and ATP in stage II from coated buds that had been made (in stage I) with coatomer, and ARF and GTP were incubated with Golgi membranes from wild-type CHO cells (i.e., the standard acceptor membrane fraction [Balch et al., 1984a]) in the presence of cytosol, ATP, and an ATP regenerating system under the same conditions as those used in a standard transport assay (Balch et al., 1984a). Between 25%–35% of the VSV G protein contained in the COP-coated vesicles was converted to the endo H–resistant form (Figure 5a), depending on the experiment. As only about one-third of the Golgi cisternae contain GlcNAc transferase I (Dunphy and Rothman, 1985; Nilsson et al., 1993), the actual number of vesicles that fuse (including fusion at other locations) could well be higher. If vesicles bud and fuse in a sequential, compartment-specific way, as is the case in vivo (Rothman et al., 1984), then the COP-coated vesicles should be a mixture of subtypes of which only a fraction (from the cis cisternae) is programmed to fuse with the GlcNAc transferase–containing medial cisternae.

As controls, we carried out stage I reactions with GTPγS replacing GTP or with the myristoylated ARF mutant Q71L replacing ARF. This mutant ARF protein can bind but not hydrolyze GTP (Tanigawa et al., in press) (Figure 5a). These controls produce COP coats that cannot be removed and thus stop transport by accumulating COP-coated vesicles (Melancon et al., 1987; Tanigawa et al., in press). Both controls resulted in fusion-incompetent vesicles; only vesicles with GTP and wild-type ARF were fusion competent. The demonstration that uncoating is prerequisite for membrane fusion confirms that the VSV G protein converted to endo H–resistance resides in COP-coated vesicles, a conclusion that also follows from the high efficiency of fusion (see Figure 2a). This is an im-

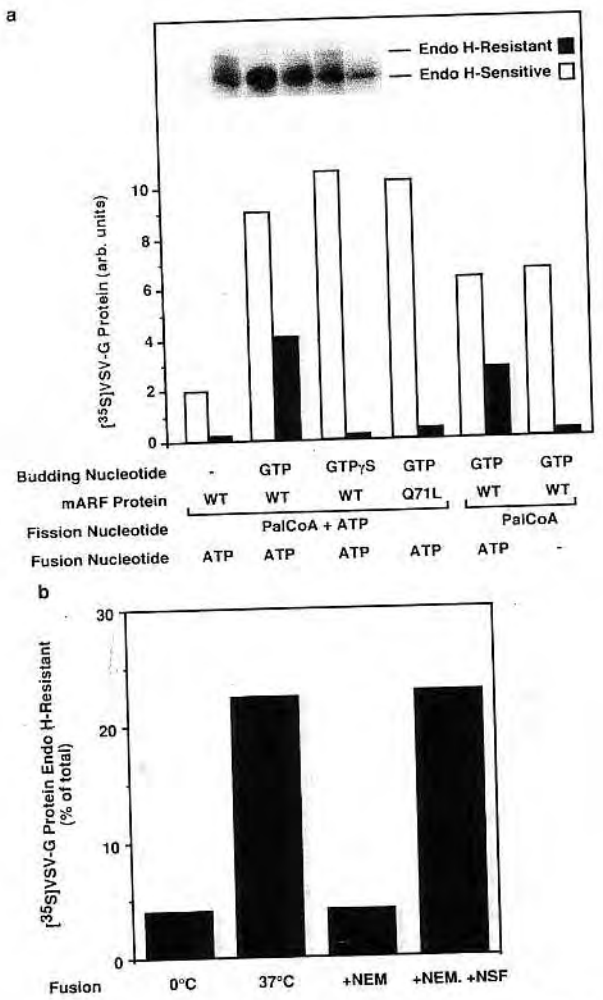


Figure 5. Vesicles Are Intermediates In the Transport of Protein between Golgi Cisternae

(a) Consumption of COP-coated vesicles requires uncoating and ATP. (b) Vesicle fusion requires NSF. Budding (stage I) and fission (stage II) reactions were performed in the presence of coatomer, ARF or ARF Q71L, the indicated guanine nucleotide in the (stage I) budding reaction, and palmitoyl-CoA, with or without ATP, at the times indicated in the (stage II) fission reaction. The stage II 0.25 M salt supernatant containing COP-coated vesicles was prepared as described before and either was diluted 4-fold into a (stage III) fusion reaction containing wild-type Golgi membranes or was first fractionated on a sucrose gradient to allow isolated coated vesicles to be tested for fusion competence in the absence or presence of NSF. The gradient fractions containing the peak of the VSV G protein distribution were pooled and added to a (stage III) fusion reaction containing either untreated acceptor membranes or acceptor membranes that had been treated with 1 mM N-ethylmaleimide for 10 min on ice, followed by 1.5 mM dithiothreitol for 5 min on ice (to quench endogenous NSF), as indicated. Recombinant NSF (0.1 μg/ml [Whiteheart et al., 1993]) was added when indicated. After a 1 hr incubation at 37°C, Golgi membranes were pelleted and incubated with endo H. The endo H–resistant and sensitive forms of the VSV G protein were visualized by fluorography ([a], insert) and quantified by a phosphorimager.

portant point, since direct (uncoupled) fusion can occur between Golgi membranes in the absence of coat proteins (Elazar et al., submitted) and because the cisternal regions attached to the coated buds present (~11% of coated membranes isolated) may also contribute VSV G protein to the processing reaction.

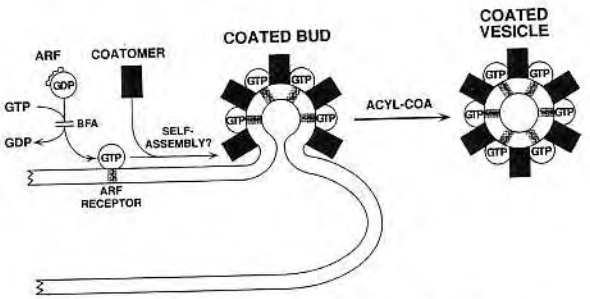


Figure 6. Steps In the Formation of COP-Coated Vesicles

Myristoylated ARF–GDP in the cytosol exchanges its bound nucleotide with GTP and binds to a receptor on the Golgi membrane (Kahn and Gilman, 1986; Weiss et al., 1989; Donaldson et al., 1992a; Helms and Rothman, 1992; Tsai et al., 1993). Coatomer binding at these sites results in the self-assembly of coated buds (Donaldson et al., 1991; Donaldson et al., 1992b; Orci et al., 1993a, 1993b; Palmer et al., 1993). Fission, releasing fusion-competent COP-coated vesicles, requires addition of acyl-CoA and is stimulated by ATP.

When vesicles were pinched off in stage II with palmitoyl-CoA and no ATP, fusion only occured when ATP was added subsequently (Figure 5a). This shows that ATP (or at least, a nucleoside triphosphate) is required for fusion and that vesicles produced with palmitoyl-CoA (and no ATP) are fusion competent.

We examined the sensitivity of fusion to N-ethylmaleimide to test whether this reaction utilizes N-ethylmaleimide-sensitive factor (NSF). When acceptor Golgi membranes were preincubated with 1 mM N-ethylmaleimide for 10 min on ice, the fusion reaction was completely inhibited, but could be reconstituted by the addition of recombinant NSF (Figure 5b). For this experiment, COP-coated vesicles were harvested from a sucrose gradient, allowing us also to confirm that density gradient–purified vesicles retain fusion competence. In that N-ethylmaleimide treatment of the acceptor membranes alone is sufficient to inhibit fusion of vesicles, it follows that the coated vesicles themselves do not contain functional molecules of NSF. For these experiments, we used cytosol whose endogenous NSF had been inactivated (by preparation in the absence of ATP [Clary et al., 1990]), so that the only potential sources of NSF were the COP-coated vesicles and the acceptor Golgi (which are known to contain bound NSF [Block et al., 1988]).

Discussion

The discovery of the coat components coatomer and ARF (Serafini et al., 1991a and b; Waters et al., 1991) and the finding that they are the only cytoplasmic proteins required for vesicle formation (Orci et al., 1993b) have enabled us to reveal intermediate steps in the process that forms a functionally competent transport vesicle (Figure 6). The accompanying technical advances have made it possible to accumulate COP-coated vesicles by omitting uncoating and fusion components (rather than by adding inhibitors), allowing fusion competence to be assessed.

The first step (Figure 6) in the assembly of a COP-coated vesicle from a Golgi cisterna is the formation of a site committed to budding, as marked by activated ARF protein bound to its membrane receptor. ARF protein is recruited from the cytosol, where it resides in its GDP-bound form (Kahn and Gilman, 1986; Weiss et al., 1989). Nucleotide exchange (catalyzed by a protein at the Golgi surface [Donaldson et al., 1992a; Helms and Rothman, 1992; Tsai et al., 1993]) produces ARF (GTP), which inserts into the local lipid bilayer via its myristic acid chain, whose exposure is triggered by GTP binding (Kahn, 1991; Helms et al., 1993). ARF then becomes more tightly bound when it associates with its receptor, creating high affinity binding sites for coatomer (Donaldson et al., 1992b; Palmer et al., 1993). At this stage, the bound ARFs are not clustered, and the cisternal membrane is still flat (Orci et al., 1993b).

When coatomer binds, coated buds form that need only be pinched off at their base (a process we term fission) to yield complete and fusion-competent coated vesicles. The only nucleotide required to form coated buds is GTP, which remains bound to ARF protein and unhydrolyzed; GTPγS can substitute for GTP and an ARF mutant that can bind but not hydrolyze GTP can substitute for wild-type ARF (Tanigawa et al., in press). Coatomer binding simultaneously results in the formation of buds and the clustering of ARF (presumably still bound to its receptor) with coatomer into buds; little bound coatomer remains outside of assembled coats (Orci et al., 1993a; Palmer et al., 1993). These facts, together with the observation that bound GTP remains unhydrolyzed at this stage, implies that ARF–coatomer complexes rapidly and spontaneously laterally self-assemble to form buds. Presumably, the shape of the coated vesicle is determined by a regular arrangement of coatomer and ARF in their polymerized form, as has long been thought to be the case for clathrin coats (Heuser, 1980; Pearse and Bretscher, 1981; Lin et al., 1991). The energy made available when GTP binds to ARF is presumably used to force this protein into a higher energy conformation in which fatty acid is more exposed to water; recycling of ARF with attendant uncoating will follow when GTP hydrolysis is triggered later in the pathway to initiate membrane fusion (Tanigawa et al., in press).

Membrane fission requires the fusion of adjoining regions of the same lipid bilayer at the base of the bud, initiated from the lumenal (cisternal) side of the membrane. Cisternal fusion is thus topologically distinct from cytoplasmic fusion, which is initiated from the cytoplasmic side and employs two separate lipid bilayers as substrates. It is thus expected that different protein machineries will be required for cytoplasmic and cisternal fusion, even though the underlying biophysical mechanisms may be similar. Cytoplasmic fusion generally requires NSF, soluble NSF attachment protein (SNAP), and most likely SNAP receptor (SNARE) proteins (Malhotra et al., 1988; Wilson et al., 1989; Clary et al., 1990; Clary and Rothman, 1990; Söllner et al., 1993; Whiteheart et al., 1993); the cisternal fusion machinery is still unknown. The simplest interpretation of the requirement for fatty acyl–CoA in fission is that acylation of a fusion protein facing the lumen triggers fission when the coat brings the lumenal surfaces of the lipid bilayers into proximity at the base of the bud. Cytoplasmic fusion also requires fatty acyl–CoA (Pfanner et al., 1990), a fact that hints at fundamentally similar mechanisms. Studies of ethanol acyl transferase (Polokoff and Bell, 1978) imply that microsomal membranes can take up long chain fatty acyl–CoAs, making acylation on the lumenal side a possibility. Identification of the postulated acyl protein may help to uncover the cisternal fusion machinery.

While we have found that coated bud assembly on and fission from Golgi cisternae can occur in minimal fashion with only coatomer and ARF proteins from the cytosol, this in no way rules out the possibility that additional components may modulate the process or even be required for coated vesicle budding from other compartments. For example, while the assembly of COP-coated vesicles from Golgi cisternae does not require cargo (Orci et al., 1986) (hence cargo is not concentrated during budding) it appears that cargo is concentrated in COP-coated vesicles that bud from the ER (W. Balch, personal communication), implying that assembly of coats must be coupled to the loading of cargo in this case. Indeed, additional machinery (SEC12, SEC23, SEC24 [Schekman, 1992]), including the SAR1 protein, is required for vesicle budding from yeast ER (d'Enfert et al., 1991; Barlowe et al., 1993), and SAR1 is required for COP-coated vesicle budding from the ER in animal cells, but not for transport in the Golgi (O. Kuge et al., submitted). Also, the $G\alpha_{i3}$ chain may regulate COP-coated vesicle assembly (Ercolani et al., 1990; Stow et al., 1991; Donaldson et al., 1991); perhaps $G\alpha_{i3}$ (GTP) is part of the ARF receptor.

Coated vesicles bud without an absolute requirement for free ATP, minimally requiring only GTP to trigger ARF binding to the membrane and acyl-CoA for fission. The apparent requirement for ATP when COP-coated vesicle budding was first studied using crude cytosol as a source of coat proteins (Orci et al., 1986) is readily explained by the action of two metabolic enzymes present in crude extracts, nucleoside diphosphokinase and fatty acyl–CoA ligase, which can utilize ATP to maintain levels of GTP and acyl-CoAs. Numerous cell-free systems have been described that require ATP in crude extracts (Smythe et al., 1989; Lin et al., 1991; Rexach and Schekman, 1991; Schmid and Smythe, 1991), and a GTP requirement for budding has previously been described (Barlowe et al., 1993). While it is clear that ATP per se is needed at the least for NSF action in fusion (Block et al., 1988; Tagaya et al., 1993), it may well be that additional nucleotides are required, along with acyl-CoA, in fusion. In the specific case of the budding of clathrin-coated vesicles from the plasma membrane, the fission process has been shown to utilize ATP-derived energy in crude extracts (Smythe et al., 1989; Lin et al., 1991; Schmid and Smythe, 1991). Whether this step may utilize acyl-CoA, as for COP-coated vesicles, remains to be determined.

The simple view that vesicles having different coats bud from different organelles using similar underlying mechanisms seems increasingly tenable. This is suggested by certain similarities in the sequences of clathrin and COP-

coated vesicle proteins (Duden et al., 1991; Serafini et al., 1991a; Kuge et al., in press) and by the requirement for ARF in binding reactions involved in the assembly of both types of coats from Golgi membranes (Stamnes and Rothman, 1993).

It has been claimed that budding of fusion-competent COP-coated vesicles from Golgi membranes requires NSF and α-SNAP (Wattenberg et al., 1992). However, addition of α-SNAP does not stimulate vesicle budding in our assays (data not shown), nor is its addition required. Furthermore, α-SNAP is not found in purified COP-coated vesicles (W. Whiteheart, unpublished data; T. Mayer et al., unpublished data), nor can it be detected in appreciable amount on coated buds/vesicles by immunocytochemistry (L. Orci et al., unpublished data) even though α-SNAP is easily demonstrated in adjoining cisternal regions. Similarly, NSF is not required for vesicle budding, nor can functional NSF be provided to fusion reactions by COP-coated vesicles (Figure 5b). The earlier conclusions (Wattenberg et al., 1992) were based mainly on a kinetic analysis of the effects of a compound capable of only partial inhibition of transport that indeed blocks budding, but whose possible additional effects on steps in fusion could not be ruled out given the indirect methods of analysis used. Most likely, the assays used (Wattenberg et al., 1992), in which SNAP overcomes partial inhibition, actually measure fusion.

Experimental Procedures

Materials

The following reagents were isolated as described elsewhere: Golgi membranes from CHO cells (Balch et al., 1984a), bovine brain cytosol (Malhotra et al., 1989), recombinant NSF (Whiteheart et al., 1993), coatomer (Waters et al., 1992), and recombinant N-myristoylated wild-type (Helms and Rothman, 1992) and mutant Q71L ARF (Tanigawa et al., in press). [^{35}S]-Express label was obtained from New England Nuclear, and magnetic beads were obtained from Dynal.

Coatomer Binding Assay

Golgi membranes (0.2 mg/ml) were prewashed with 0.25 M KCl by incubation for 2 min on ice in assay buffer (25 mM HEPES [pH 7.4], 2.5 mM $Mg(OAc)_2$, 25 mM KCl, 1 mg/ml SBTI [soybean trypsin inhibitor], 0.2 M sucrose) containing an additional 225 mM KCl, reisolated by centrifugation (10 min) in a refrigerated Eppendorf centrifuge, and resuspended in assay buffer. Incubations were then performed with these Golgi membranes (0.1 mg/ml final concentration), which also contained 40 μg/ml coatomer and the following additional components, as indicated in Figure 1a: 40 μg/ml ARF, 1 mM Mg/GTP (1 mM $Mg(OAc)_2$ and 1 mM GTP [pH 7.0], 20 μM GTPγS, 50 μM ATP and an ATP-regenerating system of 2 mM creatine phosphate and 8 IU/ml creatine kinase, and 10 μM palmitoyl-CoA. Samples (100 μl) were incubated for 10 min at 37°C. To determine the amount of bound β-COP, Golgi were reisolated by flotation. Sucrose was added to 55% final concentration (w/w) from a 70% solution in gradient buffer (25 mM HEPES [pH 7.4], 2.5 mM $Mg(OAc)_2$, 250 mM KCl, 0.1 mg/ml bovine serum albumin), and these samples were overlayed in 0.7 ml ultracentrifuge tubes with 300 μl 45% sucrose and 100 μl 30% sucrose (both in gradient buffer) and centrifuged in an SW 55 rotor for between 10 hr and 16 hr at 4°C. The top 200 μl of each gradient (containing the Golgi) were diluted 2-fold with water. Membranes were pelleted by 30 min centrifugation in a TL100.2 rotor at 100,000 rpm at 4°C. The membrane pellet was dissolved in SDS–polyacrylamide gel electrophoresis sample buffer, and proteins were separated on a 12% polyacrylamide gel and transferred to nitrocellulose. The amount of β-COP in each fraction was determined by quantitative Western blotting (Palmer et al., 1993).

Pulse-Labeling of VSV-Infected CHO Cells

CHO cells were harvested 3 hr after infection (Balch et al., 1984a), collected by centrifugation (for 5 min at 20°C at 1500 rpm), resuspended in methionine-free medium with 1 mCi/ml [^{35}S]-Express label, and incubated for 15 min at 37°C.

Two-Stage Budding Reaction

Golgi membranes from VSV-infected 15B CHO cells that had been pulse-labeled with [^{35}S]methionine or from wild-type CHO cells were isolated as described and prewashed with 250 mM KCl. These membranes (0.1 mg/ml) were then incubated (stage I) with coatomer (40 μg/ml) and ARF (40 μg/ml) in 0.5 ml assay buffer with either MgGTP (1 mM) or GTPγS (20 μM) for 10 min at 37°C. After incubation, membranes were reisolated by centrifugation (10 min in a refrigerated Eppendorf centrifuge), resuspended (for Stage II) by repeated micropipetting in 0.5 ml assay buffer containing 10 μM palmitoyl-CoA, 50 μM ATP plus 2 mM creatine phosphate, and 8 IU/ml creatine kinase, and incubated for 5 min at 37°C. KCl was then added to a final concentration of 250 mM from a 3 M stock solution, and Golgi membranes were pelleted as above.

Sucrose Gradient Purification of Vesicles

The 0.25 M salt supernatant (approximately 0.5 ml) was mixed with 1 ml of 70% (w/w) sucrose in gradient buffer and overlayed in 5 ml SW 55 ultracentrifuge tubes with 0.5 ml 45% (w/w) sucrose and 1 ml 40%, 35%, and 30% (w/w) sucrose in gradient buffer. The gradients were centrifuged for 14 hr at 50,000 rpm at 4°C and fractionated into 14 fractions of 350 μl. The indicated fractions (300 μl) were diluted 2-fold with water, and membranes were pelleted by ultracentrifugation (for 40 min at 4°C in a TL100.2 rotor at 100,000 rpm). The membrane pellet was dissolved in SDS–polyacrylamide gel electrophoresis sample buffer, and proteins were separated by electrophoresis.

Immunoisolation of Coated Vesicles with Magnetic Beads

Vesicles were made in a two-stage budding reaction and purified on the sucrose gradient. The peak fractions of the VSV G distribution were collected, diluted 2-fold in 25 mM HEPES (pH 7.4) and 2.5 mM $Mg(OAc)_2$ (final volume of 1.5 ml), and 200 μl aliquots were incubated with 0.4 μg/ml CM1A10 antibody (Palmer et al., 1993) for 1 hr on ice when indicated. Increasing amounts of magnetic beads cross-linked with anti-mouse immunoglobulin G (Dynabeads M280, 6×10^8 to 7×10^8 beads/ml) were added, and the samples were incubated for 1 hr at 4°C with gentle agitation. The beads were collected with a magnet, and the bound proteins were analyzed by electrophoresis. For electron microscopy, vesicles were formed in a 5 ml reaction with wild-type CHO Golgi membranes with coatomer, ARF, and GTPγS in the budding reaction (stage I) and palmitoyl-CoA and ATP in the pinching off reaction (Stage II). Vesicles from the 0.25 M salt supernatant of stage II were first concentrated by pelleting on a cushion of 0.5 ml of 70% (w/w) sucrose in gradient buffer (for 2 hr at 4°C in a SW 55 rotor at 55,000 rpm). Material obtained 1 ml from the bottom of the tube (containing the vesicles) was mixed with 0.5 ml of 70% (w/w) sucrose in gradient buffer and overlayed with the gradient described before. The gradient fraction that contained the highest amount of β-COP (as determined by Western blotting) was diluted 2-fold in buffer and incubated with CM1A10 antibody and M280 magnetic beads.

Vesicle Fusion Assay

The vesicle containing stage II supernatant or sucrose gradient fractions was diluted into a fusion reaction (Stage III) containing wild-type Golgi (0.1 mg/ml), bovine brain cytosol (1 mg/ml), ATP (50 μM), creatine phosphate (2 mM), creatine kinase (8 IU/ml), UDP–GlcNAc (0.5 mM), and palmitoyl-CoA (10 μM) in 25 mM HEPES (pH 7.4) and 2.5 mM $Mg(OAc)_2$. The final concentration of KCl was 60 mM. After 1 hr of incubation at 37°C, Golgi membranes were pelleted, dissolved in 50 mM sodium citrate (pH 5.5), 1 mM dithiothreitol, and 0.04% SDS gel, incubated for 3 min at 95°C, and diluted 2-fold in 50 mM sodium citrate (pH 5.5). Endo H (2.5 mU) was added, and the samples were incubated

for at least 5 hr at 37°C. Proteins were precipitated with trichloroacetic acid and separated by SDS gel electrophoresis.

Electron Microscopy

Golgi membranes were pelleted by a 30 min centrifugation, and magnetic beads were collected with a magnet. Samples were fixed in 1% glutaraldehyde in 50 mM potassium phosphate (pH 7.0) for 30 min on ice and processed as described (Orci et al., 1986; 1991).

Western Blotting

Proteins were separated by electrophoresis and transferred to nitrocellulose. Coat proteins were detected with antibodies directed against β-COP (M3A5 antibody [Duden et al., 1991]) and ARF (Palmer et al., 1993) as primary and peroxidase-coupled secondary antibodies. The bands were visualized by enhanced chemiluminescence (ECL, Amersham).

Acknowledgments

We wish to thank Drs. Gary Tanigawa and Bernd Helms for ARF protein, Dr. David Palmer for CM1A10 antibody, and Drs. Jim Shepherd, Thomas Söllner, Mark Stamnes, and Wally Whiteheart for critically reading the manuscript. This work was supported by grants from the National Institutes of Health (to J. E. R.), the Human Frontier Science Program (to J. E. R. and L. O.), the Swiss National Science Foundation (to L. O.), and the Mathers Charitable Foundation, as well as by a Fellowship from the Deutsche Forschungsgemeinschaft (to J. O.). We also acknowledge assistance from the Memorial Sloan Kettering Cancer Center microchemistry facility.

Received September 2, 1993; revised September 29, 1993.

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