Evidence that Phospholipase D Mediates ADP Ribosylation Factor–Dependent Formation of Golgi Coated Vesicles

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Abstract. Formation of coatomer-coated vesicles from Golgi-enriched membranes requires the activation of a small GTP-binding protein, ADP ribosylation factor (ARF). ARF is also an efficacious activator of phospholipase D (PLD), an activity that is relatively abundant on Golgi-enriched membranes. It has been proposed that ARF, which is recruited onto membranes from cytosolic pools, acts directly to promote coatomer binding and is in a 3:1 stoichiometry with coatomer on coated vesicles. We present evidence that cytosolic ARF is not necessary for initiating coat assembly on Golgi membranes from cell lines with high constitutive PLD activity. Conditions are also described under which ARF is at most a minor component relative to coatomer in coated vesicles from all cell lines tested, including Chinese hamster ovary cells. Formation of coated vesicles was sensitive to ethanol at concentrations that inhibit the production of phosphatidic acid (PA) by PLD. When PA was produced in Golgi membranes by an exogenous bacterial PLD, rather than with ARF and endogenous PLD, coatomer bound to Golgi membranes. Purified coatomer also bound selectively to artificial lipid vesicles that contained PA and phosphatidylinositol (4,5)-bisphosphate (PIP2). We propose that activation of PLD and the subsequent production of PA are key early events for the formation of coatomer-coated vesicles.

An early critical event for the movement of membrane and proteins between organelles is the demarcation of a specific membrane site for the formation of a transport-competent intermediate. Since, in all cases, the goal is the efficient and correct delivery of a given set of cargo from point A to point B, it is attractive to speculate that transport intermediates are formed from various organelles using similar mechanisms and general components. For trafficking from the ER to the Golgi complex, as well as within the Golgi stacks, such underlying mechanistic and component similarities have already been uncovered (Pryer et al., 1992; Rothman, 1994). A set of seven cytosolic coat proteins, termed coatomer, seem to mediate transport within the Golgi stacks. Translocation of coatomer onto membranes is directed by a small GTP-binding protein (smg), ADP ribosylation factor (ARF) (Serafini et al., 1991; Waters et al., 1991). Similarly, another set of at least four cytosolic proteins, termed COPII, mediate transport from the ER to the Golgi in the yeast Saccharomyces cerevisiae using a distinct smg, Sar1p, for membrane translocation (Barlowe et al., 1994). In both instances, activation of the smg by GTP in the presence either of cytosol or of purified coat proteins leads to the production from donor membranes of small (70–90 nm) coated vesicles that contain the appropriate cargo and are competent to fuse with the acceptor membrane. Although related in their function, coatomer and COPII have very different compositions and limited sequence homologies among their polypeptides. It is also unclear at present to what extent coatomer-coated vesicles are involved in ER to Golgi transport (Pepperkok et al., 1993; Peter et al., 1993; Balch et al., 1994), and/or in the retrograde pathway of recycling from the Golgi back to the ER (Cosson and Letourneur, 1994; Letourneur et al., 1994; Letourneur et al., 1995; reviewed by Pelham, 1995; and Salama and Schekman, 1995).

Despite the rapid progress in identifying the components involved in coated-vesicle production, the exact mechanisms that cause cytosolic proteins to become membrane bound and induce the necessary curvature to transform a planar membrane into a spherical bud remain unknown. For the coatomer paradigm, it is clear that the only cytosolic proteins required to produce coated vesicles from Golgi-enriched membranes are coatomer and ARF (Orci et al., 1993). The finding that coated vesicles produced with either GTP or with its hydrolysis-resistant analogs contain ARF in approximately a 3:1 molar ratio to...
coatomer has led to the suggestion that ARF itself is an abundant component of the coat (Serafini et al., 1991). In this view, cytosolic ARF is first activated by GTP and then binds to Golgi membranes, either to non-saturable sites or to an “ARF-receptor” (Helms et al., 1993). The ARF population bound to trypsin-sensitive, saturable sites then initiates stoichiometric coatomer binding to membranes (Palmer et al., 1993) and eventual formation of coated vesicles. The putative “ARF receptor,” which must be of critical importance and of high abundance in this view, remains unknown.

It has been reported recently that, in addition to its role in intracellular transport, ARF is an effective activator of phospholipase D (PLD) (Brown et al., 1993; Cockcroft et al., 1994). PLD activated with ARF hydrolyzes phosphatidylcholine (PC) to produce phosphatidic acid (PA) and choline. High levels of ARF-stimulatable PLD activity were obtained in Golgi-enriched membranes from a variety of cell lines and this activity sedimented in a microfuge under conditions that leave smaller membranes, such as endosomes, in the supernatant (Ktistakis et al., 1995). Stimulation by ARF was inhibited by Brefeldin A (BFA), a fungal metabolite that prevents binding of ARF to Golgi membranes and inhibits secretion. Moreover, Golgi from BFA-resistant cell lines that contain a tightly bound ARF species had very high basal PLD activity; this activity was not further stimulated by exogenous ARF and was resistant to BFA (Ktistakis et al., 1995). These results suggest that the population of ARF responsible for stimulating vesicle formation from Golgi also activates PLD.

In an effort to investigate the possibility that PLD plays a direct role in intracellular transport, we made the unexpected observation that, with Golgi having high basal PLD activity, exogenous ARF was neither required for production of coatomer-coated vesicles nor was itself an abundant component of the vesicles thus produced. These observations were extended to cells in which ARF is not stably associated with Golgi membranes. If Golgi-enriched membranes from Chinese hamster ovary (CHO) cells were preincubated with activated ARF and reisolated, they could support the production of coated vesicles if purified coatomer was added to them. These vesicles did not contain detectable ARF and their production was inhibited by ethanol. Since ethanol diverts the production of PA by PLD to the production of phosphatidylethanol, we hypothesized that production of PA may be directly involved in coatomer binding to membranes. In support of this, we found that pure coatomer bound to either Golgi membranes in which PA has been produced by added bacterial PLD, or to artificial lipid vesicles as a function of their content of PA and phosphatidylinositol (4,5)-biphosphate (PIP2). We propose that, following its activation by ARF, PLD hydrolyzes PC and the PA produced facilitates budding of coated vesicles.

Materials and Methods

Tissue Culture and Isolation of Golgi-enriched Membranes

The growth conditions for all cell lines used have been reported previously (Ktistakis et al., 1991; Ktistakis et al., 1995). Golgi-enriched membranes were isolated from the 0.8/1.0 M-interface of sucrose step gradients as reported previously (Ktistakis et al., 1995). Multivesicular vesicles, identifiable endosomes, and identifiable ER are absent from this membrane fraction and ½ of the membranes are identifiable Golgi stacks, which sediment in a microfuge and are enriched in mannosidase II activity, 20-fold relative to whole cell membranes. For convenience, we will employ either “Golgi-enriched membranes” or “Golgi” to refer to the membrane fraction used in our experiments.

Isolation of Cytosol, ARF, and Coatomer

Cytosol was isolated after homogenization of cells in buffer A (90 mM KCl, 50 mM Hepes, pH 7.2) and centrifugation of postnuclear supernatant (100,000 g in a rotor (model TLA 100.1, Beckman Instruments, Fullerton, CA) for 60 min at 4°C. The supernatant from this centrifugation was dialyzed overnight at 4°C with three changes of buffer A, frozen in liquid nitrogen, and stored at −70°C. Native ARF was isolated from pig brain cytosol essentially as described (Brown et al., 1993; Brown and Sternweis, 1995). The Sepahex G-75 ARF pool contained 25−50% ARF as judged by staining with Coomassie blue. Coatomer was isolated from bovine liver cytosol as described previously (Waters et al., 1992) through the MonoQ step which yields coatomer that is about 50% pure. This material was used for binding to artificial vesicles. For all experiments with Golgi membranes, we used material that was 95% pure by staining with Coomassie blue that had been purified by gel filtration on Superose 6 as a last step.

Formulation of Coated Vesicles

These assays were adapted from publications from the Rothman laboratory (Orci et al., 1993; Ostermann et al., 1993; and references therein). Coated vesicles were formed in two ways: (a) using cytosol as the source of coatomer and (b) using purified coatomer fractions.

Generation of Coated Vesicles with Cytosol. Cytosol was prepared as described above and adjusted to a final concentration of 4 mg/ml. Golgi-enriched membranes (200 μg/ml) were diluted fivefold in buffer B (25 mM KCl, 2.5 mM MgSO4, 1 mM DTT, 0.2 M sucrose, 25 mM Hepes, pH 7.2) on ice with gentle vortexing in a volume of 200 μl. Cytosol (50 μl containing 10 μM GTP-γS where indicated) was added on ice and the samples were rapidly transferred to 37°C for 15 min. At the end of this incubation, the samples were cooled on ice and centrifuged for 10 min in a refrigerated microfuge at 12,000 g. The pellets were resuspended gently in 200 μl of buffer B, and 3 M KCl was added to a final concentration of 250 mM. After 10 min on ice, the samples were centrifuged as above and the supernatant containing the coated vesicles was transferred to a new tube and centrifuged for 60 min at 4°C in a rotor (model TLA 100.1, Beckman Instruments) at 65,000 rpm. After centrifugation, the supernatant was removed and the pellet was resuspended in Laemmli sample buffer for SDS-PAGE and immunodetection. If Golgi membranes were omitted from this procedure, no ARF or the β-COP coatomer subunit were recovered in this last pellet (see lane 5 of Fig. 7). For pretreatment with ARF, membranes were first incubated with ARF (5 μg), GTP-γS (10 μM), and cholera toxin as indicated for 10 min at 37°C. Treated membranes were isolated by centrifugation and cytosol was added as described above. In this case, ARF pretreatment constitutes a separate step in the reaction and therefore the membranes are subjected to three stages of treatment and reisolation (see Fig. 2).

Generation of Coated Vesicles with Purified Coatomer and ARF. Golgi-enriched membranes were diluted fivefold in buffer B, adjusted to 250 mM KCl, and were kept on ice for 10 min. At the end of this incubation, the membranes were collected by centrifugation in a refrigerated microfuge as above and resuspended in 200 μl of buffer B. Coatomer (10 μg), ARF (5 μg or as indicated), and GTP-γS (10 μM) were added from concentrated stocks and the samples were incubated at 37°C for 15 min. At the end of this time, samples were cooled on ice and the membranes were collected by centrifugation at 4°C, as described above. The membrane pellets were resuspended in buffer B containing 0.2 mM ATP, 2 mM creatine phosphate, 8 IU/ml creatine phosphokinase (all from Boehringer Mannheim Corp., Indianapolis, IN), and 10 μM palmityl-CoA, and incubated at 37°C for 5 min. At the end of this incubation, the samples were cooled on ice and 3 M KCl was added to a final concentration of 250 mM. After 15 min on ice, the samples were centrifuged for 10 min at 11,000 g and the supernatant containing coated vesicles was centrifuged in a rotor (model TLA 100.1, Beckman Instruments) as above. When the vesicles were col-
lected from fractions of a sucrose gradient, this assay was performed with 10-fold more material and the resulting supernatant (0.5 ml) was layered on top of a continuous 20-50% sucrose gradient (sucrose dissolved in buffer B containing 0.1 mg/ml fat-free bovine serum albumin and 250 mM KCl) and centrifuged in an SW-60 rotor at 40,000 rpm for 16-18 h at 4°C. The gradients were collected from the top in 200-μl fractions and 50 μl of potassium chloride was added. Coatomer was added immediately at concentrations of 20 μg/200 μl and the samples were incubated at 37°C for 5 min. After 10 min, and the supernatant was removed and the pellets were resuspended and centrifuged for an additional 5 min without precooling or centrifugation.

**SDS-PAGE and Immunochemistry**

ARF and the β-COP subunit of coatomer could be detected simultaneously on 13% polyacrylamide gels as follows: After SDS-PAGE, the gels were cut in half and each half was assembled separately for electrotransfer using the method of Towbin et al. (1979). Electrotransfer was initiated using constant current (150 mA) for 2 h. At the end of this time, the membranes to which ARF had been transferred were removed, wrapped in plastic, and stored at 4°C. The remainder of the gels were washed and electrotransferred at 200 mA constant current overnight to transfer β-COP quantitatively to the membranes. The following day both immunobLOTS were processed simultaneously for chemiluminescence detection as previously described (Kistakis et al., 1995). β-COP was detected using monoclonal antibody M3A5 (gift from Dr. T. Kreiss) and ARF using monoclonal antibody ID9 (gift from Dr. R. Kahn).

**Electron Microscopy**

To prepare Golgi-enriched membranes for electron microscopy, 2 ml of membranes (at 200 μg/ml protein) were diluted fivefold in buffer B containing 250 mM KCl and resuspended by centrifugation. The membrane pellets were resuspended in 0.5 ml buffer B and ARF (40 μg), coatomer (80 μg), and GTP-γ-S (10 μM) were added from stocks as indicated in the text for various experiments. The samples were incubated at 37°C for 15 min, cooled on ice, and centrifuged in a refrigerated microfuge for 10 min at 11,000 g. After removal of the supernatant, the pellets were gently overlaid with 500 μl of ice-cold buffer B, gently vortexed for 3 s, and centrifuged again as above. The pellets were gently overlaid with 3% glutaraldehyde in PBS, incubated for 30 min on ice, resuspended by centrifugation, and incubated with 1% OsO₄ in phosphate buffer (0.1 M KPO₄, pH 7.2) for 1 h. After extensive washing with phosphate buffer, the samples were serially dehydrated in ethanol and then embedded in Epon 812 according to the manufacturer’s instructions. The polymerized samples were cut into sections 90-nm thickness, collected on grids coated with Formvar, and stained with 3% uranyl acetate followed by lead citrate. The samples were examined in an electron microscope (model 1200EX; JEOL, U.S.A., Peabody, MA) operated at 80 kV and photographs were recorded on Kodak film at a magnification of 20,000.

**Assays for PLD Activity**

**Exogenous Assay.** Hydrolysis of exogenous 3H-labeled PC by Golgi PLD was measured as the release of 3H-labeled choline as described (Brown et al., 1995), but with the concentration of free calcium being 20 μM. In addition, the production of 14C-labeled PA from Golgi PLD was measured essentially as described (Brown et al., 1995) using 10 μl of Golgi (200 μg of protein per ml) as a source of PLD activity and 10 μM ATP. Detection was done by thin-layer chromatography using a developing solvent of chloroform/methanol/acetic acid/acetone/water (10:2:2:4 by volume), and a radiographic imaging detector (model 4000; AMBIS Sys., Inc., San Diego, CA). When using PLD from Golgi, we note that assays measuring PA production from 3H-labeled arachidonyl PC had only 10% of the product formation as compared to assays measuring choline release from [3H]palmitoyl PC. Such difference in activity was not observed using partially purified PLD from brain.

**Determination of Chromofuscus PLD Activity on Labeled Golgi Membranes.** CHO cells were labeled overnight with 3H-palmitate (25 μCi/ml in DME containing 1% fetal bovine serum). Golgi was isolated from these cells as described above and the amount of PA production with bacterial PLD was determined under conditions identical to those used for coat binding (30 min at 37°C, see below) but with omission of coatomer. The resulting lipids were extracted and analyzed for PA content by thin layer chromatography as described above.

**Treatment of Golgi Membranes with Chromofuscus PLD**

Golgi-enriched membranes (40 μg protein) were diluted in coat-binding assay buffer B containing either CaCl₂ (0.1 to 0.4 mM) or EGTA (1 mM) as indicated. PLD from Streptomyces chromofuscus (Calbiochem-Novabiochem Corp., La Jolla, CA) was added to give an activity of 0.2 U/ml and the samples were incubated at 37°C for 5 min. Coatomer (20 μg/200 μl) was added for an additional 25 min at 37°C. Final sample volume was 200 μl. At the end of the incubation, the samples were cooled on ice, mixed 1:2 with 2.2 M sucrose, and placed in the bottom of centrifuge tubes (model TLS55; Beckman Instruments, Fullerton, CA). A step gradient was formed over the samples by adding in succession three 300 μl layers of 1.2 M, 1.0 M, and 0.8 M sucrose in buffer B. Samples were sedimented in an ultracentrifuge (model TL-100; Beckman Instruments) for 2 h at 30,000 rpm in a rotor (model TLS55; Beckman Instruments). At the end of the centrifugation, gradients were fractionated from the top into 15 fractions and the β-cop content of each fraction was determined by SDS-PAGE, immunoblots, and enhanced chemiluminescence. The optical density of bands on film was determined using the volume measurement of a laser scanning densitometer (model 300A; Molecular Dynamics, Sunnyvale, CA). To correct for variations in film exposure time for different experiments, the total optical density of all β-cop bands for each sample was normalized to the total from the sample having the highest total optical density.

**Coatomer Binding to Artificial Lipid Vesicles**

**Preparation of Vesicles.** Lipid vesicles for PLD assays and for part of the coatomer-binding experiments were made by ultrasonic irradiation as described previously (Brown et al., 1993). They contained a base composition of 600 μM phosphatidylethanolamine, 60 μM PI(4,5)P₂, 60 μM dipalmitoyl PC, and L-a-[choline-methyl-H]palmitoyl PC to give about 2,000 cpm per sample. Where indicated, the appropriate proportion of PA was substituted for PC and PI(4,5)P₂ was substituted for PI(4,5)P₂. Vesicles made by extrusion were first dispersed by ultrasonic irradiation as above. Following three cycles of rapid freezing and thawing (liquid N₂ to 37°C), the samples were extruded under N₂ pressure sequentially through polycarbonate filters with diameters of 500 and 200 nm, respectively. The extruded vesicles were collected, and the process repeated four times.

**Binding Reactions.** 160 μl of lipid vesicles at 720 nmol/ml were mixed at room temperature with 20 mg coatomer in buffer B containing 100 mg/ml fat-free BSA. (The molar ratio of coatomer to vesicle, assuming a molecular mass for coatomer of 1 million and that each vesicle has 100,000 lipid molecules, is approximately 20:1.) The samples were then transferred to 37°C and binding was allowed for 30 min. At the end of this time the samples were returned to room temperature for 10 min before sedimentation.

**Detection of Binding.** If binding was to be detected by differential centrifugation, the samples were centrifuged at 15°C in a rotor (model TLA 100.1; Beckman Instruments) for 1 h at the indicated speeds. At the end of the centrifugation, the supernatant was removed and the pellets were re-suspended in sample buffer for SDS-PAGE and scintillation counting. For analysis by sedimentation in sucrose gradients, the samples were carefully layered on top of sucrose step gradients made in polyallomer tubes for the rotor (model TLS55; Beckman Instruments). Sucrose solutions in buffer B were layered from the bottom in the following order: 400 μl 0.6 M, 600 μl 0.4 M, and 400 μl 0.2 M. For analysis by floatation, the vesicles were mixed 1:1 with 1.2 M sucrose (to give a final sucrose concentration of 0.6 M) and were overlayed in polyallomer tubes (model TLS55; Beckman Instruments) with 200 μl 0.4 M, 200 μl 0.2 M, and 600 μl buffer B. Both sedimentation and flotation samples were centrifuged for 1 h at 30,000 rpm and the gradients were collected from the top into 200-μl aliquots for immunoblots.

**Results**

**Golgi Membranes with High Basal PLD Activity Do Not Require Addition of ARF for the Production of Coatomer-coated Vesicles.** Vesicles Thus Formed Do Not Contain Detectable ARF

The activity of PLD in Golgi-enriched membranes can be divided into two general categories, depending on the...
membrane source (Ktistakis et al., 1995; Fig. 1). In Golgi membranes from PtK1 and MDCK cell lines that are naturally resistant to BFA, PLD activity is very high and is stimulated very little by exogenous ARF and guanine nucleotides. In contrast, the basal PLD activity in all other Golgi membrane sources examined by us is low but can be stimulated as much as 10-fold by ARF and guanine nucleotides. For example, PLD activity measured in Golgi membranes from PtK1 or MDCK cells was rapid without addition of any soluble components (Fig. 1); this activity was stimulated by less than 50% with exogenous ARF and GTP-γS (data not shown). In contrast, Golgi membranes from CHO or MA104 cells had low activity but addition of ARF and guanine nucleotides increased the rate of PC hydrolysis to levels similar to the other cell types. A possible explanation for these differences in PLD activity is that Golgi membranes from PtK1 (Ktistakis et al., 1995) and MDCK cells (see below) contain a species of ARF that remains tightly bound to the membranes, even when they are incubated at 37°C in the absence of exogenous activators of ARF.

If PLD is involved in intracellular transport from the Golgi complex, the requirements for events known to occur during this stage of transport might differ for Golgi membranes with high versus low PLD activity. Rothman and colleagues (1994) have described an in vitro assay that measures the formation of coated vesicles from CHO Golgi-enriched membranes (Fig. 2). Formation of coated vesicles in this assay depends on the presence of membranes and addition of three other soluble components, ARF, coatomer, and GTP; ARF and coatomer can be provided either from cytosol or as pure components (Orci et al., 1993). We used either cytosol or purified components to compare the requirements for formation of coated vesicles from Golgi-enriched membranes isolated from five cell lines (CHO, PtK1, MA104, CV1, and MDCK) (Fig. 3). Use of CHO Golgi-enriched membranes and cytosol (Fig. 3 A) confirmed previous observations that coated vesicle formation required the presence of GTP-γS and that the vesicles formed contained ARF (Serafini et al., 1991). Similarly, if purified components were used, then both ARF and coatomer were required and the vesicles also contained ARF. However, when PtK1 Golgi-enriched membranes were used with cytosol (Fig. 3 A), GTP-γS was not required to form vesicles. Further, addition of purified coatomer was sufficient to induce formation of coated vesicles in PtK1 Golgi-enriched membranes; ARF and nucleotides were not required. The coated vesicles formed from PtK1 Golgi-enriched membranes in the absence of cytosolic ARF or GTP-γS did not contain detectable ARF, and their formation was as efficient as when ARF and nucleotides were used (the last four lanes in Fig. 3 A contain the same amount of β-COP, measured by densitometry). Examination of Golgi-enriched membranes from other cell types demonstrated that ARF and GTP-γS were required for the formation of coated vesicles from MA104 and CV1 membranes, but not from MDCK membranes (Fig. 3 B), where the efficiency of vesicle formation with coatomer alone was 75% that of the incubation that contained additional ARF and GTP-γS (compare the last two lanes of

**Figure 1.** PLD activity as a function of incubation at 37°C in Golgi-enriched membranes from various cell lines. Golgi-enriched membranes from the indicated cell lines were isolated using sucrose step gradients as described in Materials and Methods. Aliquots (0.5 μg of protein) of each preparation were used to determine the rate of hydrolysis of PC as described in Materials and Methods. Where indicated, ARF (0.8 μM) and GTP-γS (1 μM) were added from stock solutions at the start of the reaction.

**Figure 2.** Methods for the production of coatomer-coated vesicles. The assays used for producing coated vesicles from Golgi-enriched membranes are presented schematically. The requirements differ depending on whether the membranes were washed with KCl in the first step. For KCl-washed membranes, ARF and coatomer can be added together to bind to membranes in one step (2 b) or in sequential steps (2 a) with removal of free ARF before addition of coatomer.
Figure 4. Quantification of ARF and β-COP in cytosol from different cell lines. Purified fractions of ARF and β-COP do not contain the other protein. Each can be detected with the same sensitivity, either from pure fractions or from cytosols. 100 μg of cytosol were loaded in each of the last four lanes. Detection methods are described in Materials and Methods.

It has been reported that coated vesicles have a buoyant density of 1.18 g/ml and band at 41% sucrose in isopycnic gradients (Serafini and Rothman, 1992). Fig. 5 shows sucrose gradient profiles of β-COP following incubation of CHO or PtK1 Golgi-enriched membranes with the indicated components. Coated vesicles from CHO Golgi membranes were recovered only when both coatomer and ARF/GTP-γS were present (Fig. 5 A). In contrast, coated vesicles were formed equally well from PtK1 membranes with or without ARF/GTP-γS and their buoyant densities under these conditions were identical (Fig. 5 B). The absence of membranes (Fig. 5 A) or the absence of ARF and coatomer with PtK1 membranes (Fig. 5 B) did not result in detectable formation of coated vesicles.

Membrane fractions were examined by electron microscopy to investigate the morphology of the vesicles produced during these experiments. For optimal examination of the structure of coated membranes, samples were not subjected to pre-embedding staining, which enhances membrane contrast but obscures fine detail. Previous work has demonstrated that isolation of treated Golgi membranes without the final wash with KCl (see the protocol in Fig. 2) allows visualization of numerous coated vesicles and buds (Ostermann et al., 1993). Fig. 6 shows such an experiment with CHO or PtK1 Golgi membranes and various combinations of ARF/GTP-γS and coatomer. Incubation of CHO Golgi with coatomer and ARF/GTP-γS resulted in the formation of numerous clustered, dark-stained, spheri-
particles (Fig. 6, d-f) that were indistinguishable from spherical vesicles of identical diameter (Fig. 6 b). PtK1 cal vesicles with a diameter of 90 nm (Fig. 6 a). Omission was also present at 10 μM. The supernatant from the last centrifugation step (see Fig. 2) was used. When ARF was used, GTP-γS gradients. Incubations contained ARF and coatomer as indicated.

Figure 5. Fractionation of coatomer-coated vesicles on sucrose gradients. Incubations contained ARF and coatomer as indicated and scheme 2 b in Fig. 2 was used. When ARF was used, GTP-γS was also present at 10 μM. The supernatant from the last centrifugation step (see Fig. 2) was loaded on 20–50% linear sucrose gradients. Following centrifugation, the gradients were fractionated from the top into 200 μl fractions. Aliquots (50 μl) of each fraction were analyzed for β-COP by SDS-PAGE and immunoblotting (see Materials and Methods). The protein blots were quantified by laser scanning densitometry. Intensity units were the same for A and B.

cal vesicles with a diameter of 90 nm (Fig. 6 a). Omission of ARF/GTP-γS in this system resulted in the absence of such vesicles (data not shown). PtK1 Golgi incubated with coatomer, ARF, and GTP-γS produced a similar profile of spherical vesicles of identical diameter (Fig. 6 b). PtK1 Golgi incubated with no additives showed no vesicular profiles (Fig. 6 c). PtK1 Golgi incubated with coatomer alone produced numerous profiles of clustered spherical particles (Fig. 6, d–f) that were indistinguishable from those produced when Golgi membranes were incubated with coatomer and ARF/GTP-γS (Fig. 6 b). In addition, incubations with coatomer alone produced many profiles, such as those shown in Fig. 6, e and f, where two vesicular structures were commonly observed emerging from larger membranes, instead of masses of clustered 90-nm particles. This may suggest that less of the Golgi membrane is involved in vesicle formation when excess activated ARF is absent from the reaction.

Coatomer-coated Vesicles That Do Not Contain Detectable Amounts of ARF Can Also Be Formed from CHO Golgi

Our observations on vesicle production from PtK1 Golgi membranes suggested that relatively small amounts of active endogenous ARF on the membranes were sufficient to facilitate binding of coatomer and vesicle production. Since it has already been reported that binding of ARF to Golgi membranes and the subsequent induction of coatomer binding can be temporally separated (Donaldson et al., 1992; Palmer et al., 1993), it was of interest to examine whether vesicles produced from such a two-step incubation would contain ARF. When ARF and coatomer were added simultaneously (see Fig. 2, pathway 2 b), the coated vesicles formed contained both components in approximately stoichiometric amounts (Fig. 7, lanes 1–4; see Fig. 4 for comparison of coatomer and ARF detection). We also confirmed previous observations that, with purified components, GTP works as well as hydrolysis-resistant analogs (Fig. 7, compare lanes 2 and 3). When membranes first treated with ARF and GTP-γS were reisolated and incubated with coatomer in the absence of additional ARF and GTP-γS, coated vesicles were produced that lacked detectable ARF (Fig. 7, lanes 8 and 9). Adding ARF during the second incubation did not alter the efficiency of vesicle production (compare β-COP detection in lanes 6 and 7 with lanes 8 and 9) but resulted in large amounts of ARF associated with the coated vesicle fraction (lanes 6 and 7). The two concentrations of ARF shown resulted in the production of very similar amounts of vesicles. A dependency of vesicle formation on ARF was observed at lower concentrations of ARF during pretreatment and subsequent incubation with coatomer alone (data not shown). We conclude from this data that the role of ARF in coatomer vesicle production is not stoichiometric. Rather, with respect to coatomer association with membranes, it seems likely that ARF plays a catalytic role.

The Process of Coat Formation Is Inhibited by Ethanol

A potential role of ARF that would account for its efficacy during preincubations is stimulation of PLD. This hypothesis, which would be consistent with the data presented above, cannot be tested directly at present because the enzyme is not purified, antibodies are unavailable, and specific inhibitors are not known. However, evidence supporting the idea comes from an indirect approach that takes advantage of the fact that in the presence of primary alcohols, PLD produces phosphatidylalcohols at the expense of PA (Heller, 1978). Thus, if the production of PA by PLD is critical for subsequent events, the presence of alcohols during PLD activation should be inhibitory. Ethanol has been shown to block in vitro transport at concentrations as low as 2% (Pfanner et al., 1989) and to delay transport in vivo (Stutchfield and Cockcroft, 1993). Here we show that inclusion of increasing amounts of ethanol during preincubation of CHO Golgi with ARF and GTP-γS
decreased the production of coated vesicles as a function of the ethanol concentration (Fig. 8 A). When cytosol was used as a source of coatamer, 3% ethanol inhibited 80% of vesicle production (Fig. 8 A, compare first and last lanes of the left panel). Similar inhibition was seen when pure coatamer was used (Fig. 8 A, right panel). Although palmitoyl CoA has been reported to reverse the inhibition of an in vitro Golgi transport assay by ethanol (Pfanner et al., 1989), the palmitoyl CoA that was included in the second step reaction to facilitate “budding” of coated vesicles in vitro (see Fig. 2) did not reverse the inhibition of vesicle formation. The inhibitory effect of ethanol was dependent on its near simultaneous addition with ARF and GTP-γS. If ethanol was not added within the first 5 min of the incubation of Golgi membranes with ARF and GTP-γS, there was little inhibition of vesicle formation (Fig. 8 B). In this experiment, ethanol at 3% was also present during the second incubation with coatamer; thus, any direct effects of the ethanol on coat binding would be identical for all samples. To further exclude the possibility that ethanol might extract from membranes a component that is required for ARF and/or coatamer binding, CHO Golgi were incubated in the presence or absence of 3% ethanol for 20 min at 37°C, reisolated, incubated with ARF/GTP-γS and coatamer, and pelleted through a sucrose cushion (Fig. 8 C). Pretreatment with ethanol had no effect on subsequent coatamer binding and had a slight stimulatory effect on binding of ARF.

In separate experiments, we determined the efficiency of transphosphatidylation by Golgi-derived PLD as a function of ethanol concentration. When PLD partially purified from pig brain is used, 3% ethanol results in production of 80% phosphatidylethanol and 20% PA (Brown et al., 1995). Similarly, when Golgi membranes were used as a source of PLD, 3% of ethanol resulted in production of 70% phosphatidylethanol and 30% PA.

**Production of PA in Golgi-enriched Membranes with Bacterial PLD Allows Coatamer to Bind without Addition or Stimulation of ARF**

If the primary function of ARF during transport is to stimulate PLD for subsequent coat binding and vesicle formation, it should be possible to eliminate the ARF requirement completely by providing an alternative ARF-independent source of PLD activity. Such a PLD activity from *Streptomyces chromofuscus* is commercially available, requires Ca^{2+} cations, and is inhibited by EGTA. When Golgi membranes were incubated with *S. chromofuscus* PLD in the presence of coatamer and reisolated, we found that activated enzyme increased coat binding to membranes by fourfold (Fig. 9). Under these conditions,
The PA made by PLD could be directly required for the PA and PIP2 in vitro coatomer binding increased as a function of the amount of fold higher than in the absence of PA. In all cases, approx-

ervesicles were made with increasing amounts of PA, which is required for PLD activity (Brown et al., 1993), and 3). In lanes

by measuring the amount of pure coatomer that bound to formation of coatomer-coated vesicles or could be used as labeled with palmitate (data not shown).

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Figure 7. Coatomer-coated vesicles that do not contain detect- able ARF can be formed from CHO Golgi-enriched membranes. Lanes 1–4 show the production of coated vesicles from salt- washed CHO Golgi by simultaneous addition of ARF and coatomer as indicated (scheme 2b in Fig. 2). In this case, both ARF and β-COP are easily observed with the vesicles (lanes 2 and 3). In lanes 5–9, the coated vesicles were formed after sequential steps (scheme 2a in Fig. 2); the first step was binding of ARF/GTP-γS in the absence of coatomer. During the second step of coatomer binding, some samples received additional ARF (lanes 6 and 7).

approximately 20% of endogenous PC was converted into PA, as determined by TLC analysis of Golgi membranes labeled with palmitate (data not shown).

Coatomer Binds to Artificial Lipid Vesicles Containing PA and PIP2 In Vitro

The PA made by PLD could be directly required for the formation of coatomer-coated vesicles or could be used as the precursor of a second product, for example diacylglycerol or lysophosphatidic acid. We tested for the potential involvement of PA in the recruitment of coat from cytosol by measuring the amount of pure coatomer that bound to artificial lipid vesicles containing different mole ratios of PA. In these experiments, we also tested whether PIP2, which is required for PLD activity (Brown et al., 1993), could also facilitate association with coatomer. When lipid vesicles were made with increasing amounts of PA, coatomer binding increased as a function of the amount of PA used (Fig. 10 A). This was true in the presence or absence of PIP2, although the latter increased the overall level of binding by approximately fivefold. In the presence of 2.5 mole% PA, binding of coatomer was five- to sevenfold higher than in the absence of PA. In all cases, approximately 18% of the lipid, as measured by incorporated 3H-PC, was recovered in the 30,000 g pellet that was analyzed for bound coatomer proteins. The differential binding of coatomer to PA-containing vesicles was neither dependent on nor improved by the presence of ARF and GTP-γS. Fig. 10 B shows a binding experiment using PIP2-containing vesicles in which the amount of incorporated PA was either 0 or 5%. These vesicles were incubated with coatomer either in the presence or absence of ARF/GTP-γS and were subjected to two sequential centrifugation steps, at 30 or 50K rpm. In the pellets from both speeds, the vesicles that contained PA bound four to sevenfold more coatomer than those that did not, independent of the presence of ARF/GTP-γS.

Attempts to fractionate lipid vesicles on sucrose gradients indicated that they were heterogeneous in size. Moreover, their preparation with ultrasonic irradiation should result in small vesicles with diameters 25–40 nm, smaller than reported for coated vesicles produced in vitro (90 nm). To more closely resemble coated vesicles in size, large unilamellar vesicles were made by extrusion of multilamellar vesicles through polycarbonate filters of 200-nm diameter. Using these vesicles, it was possible to separate bound from free coatomer by flotation and, to some extent, by sedimentation through sucrose (Fig. 11). Exogenous lipid vesicles that contained a background of phosphatidylethanolamine and PC (as described in Materials

Figure 8. Ethanol blocks production of coated vesicles. (A) Golgi-enriched membranes from CHO cells were used to pro-
duce coatomer-coated vesicles in the presence of increasing concentrations of ethanol. If cytosol was used (lanes 1–6), the mem-
branes were pretreated with ARF/GTP-γS plus the indicated amount of ethanol for 10 min. The membranes were reisolated and cytosol plus the indicated amount of ethanol was added for an additional 10 min. If pure components were used (lanes 7–9), membranes were first washed with KCl, reisolated, incubated with ARF/GTP-γS plus the indicated amount of ethanol for 10 min, and then coatomer was added without a centrifugation step. Ethanol at 3% blocked the formation of coated vesicles by approximately 80% in both assays, as indicated by the amount of β-COP detected in the vesicle fraction. (B) Vesicle formation was inhibited as a function of time in the presence of ethanol. Mem-
branes were washed with KCl and reisolated. ARF/GTP-γS was added to all samples and they were incubated at 37°C for a total of 20 min. At various intervals, ethanol was added (3% final concent-
tration) to the incubations for the times shown. At the end of 20 min, the membranes were reisolated and coatomer was added to all samples for 5 min in the presence of 3% ethanol. Coated vesicles were then isolated according to the scheme in Fig. 2 and detected by immunoblotting for β-COP. (C) Ethanol does not ex-
tract components necessary for ARF or coatomer binding. Mem-
branes were washed with KCl and incubated at 37°C for 10 min with or without 3% ethanol without additional components. The membranes were then isolated again by centrifugation, incubated with ARF/GTP-γS plus coatomer for 10 min at 37°C, and recov-
ered by centrifugation for 30 min at 11,000 g. β-COP and ARF on these membranes were detected by immunoblotting.
Treatment of CHO Golgi-enriched membranes with bacterial PLD allows coatomer to bind and coated vesicles to form without addition of ARF. Membranes were incubated in two stages at 37°C, first for 5 min with *S. chromofuscus* PLD as indicated, and then for an additional 25 min with coatomer in the continued presence of the bacterial enzyme. At the end of incubation, the entire sample was loaded into the bottom of a centrifuge tube and overlaid with a sucrose gradient. Membranes that had bound coatomer were separated from soluble proteins by flotation and β-cop content was determined by immunoblotting. Figure 9 shows β-cop profiles from such an experiment, whereas B shows the quantification of β-cop binding (the amount detected in 1 M sucrose) from three different experiments employing equal amounts of Golgi-enriched membranes. The values presented are the volume optical density of the samples measured by a laser scanning densitometer (see Materials and Methods). Standard deviations are indicated by error bars.

Figure 9. Treatment of CHO Golgi-enriched membranes with bacterial PLD allows coatomer to bind and coated vesicles to form without addition of ARF. Membranes were incubated in two stages at 37°C, first for 5 min with *S. chromofuscus* PLD as indicated, and then for an additional 25 min with coatomer in the continued presence of the bacterial enzyme. At the end of incubation, the entire sample was loaded into the bottom of a centrifuge tube and overlaid with a sucrose gradient. Membranes that had bound coatomer were separated from soluble proteins by flotation and β-cop content was determined by immunoblotting. Figure 9 shows β-cop profiles from such an experiment, whereas B shows the quantification of β-cop binding (the amount detected in 1 M sucrose) from three different experiments employing equal amounts of Golgi-enriched membranes. The values presented are the volume optical density of the samples measured by a laser scanning densitometer (see Materials and Methods). Standard deviations are indicated by error bars.

PA and PIP2 facilitate coatomer binding to artificial lipid vesicles. (A) Lipid vesicles that contained mole ratios of 10% PC, 10% PIP, and 80% PE were made by ultrasonic irradiation. Various amounts of PA were substituted with respect to PIP2 versus PIP content and PC versus PA ratios, incubated with coatomer, loaded on the bottom of a sucrose step gradient, and centrifuged until the vesicles floated to equilibrium. Vesicles containing 2.5% PA and 10% PIP2 banded at the 0.2/0.4 M sucrose interface (Fig. 11 B, graph) whereas those substituted respectively with PC and PIP migrated to a slightly lower sucrose concentration (Fig. 11 A, graph). Coatomer bound more avidly to the vesicles that contained PA and PIP2 than to those that did not (compare fractions 4–6 in Fig. 11 A and B). The small amount of coatomer that bound to vesicles with no PA or PIP2 was shifted by one fraction relative to the peak of lipid (Fig. 11 A, graph). Figure 10 shows β-cop profiles from such an experiment, whereas B shows the quantification of β-cop binding (the amount detected in 1 M sucrose) from three different experiments employing equal amounts of Golgi-enriched membranes. The values presented are the volume optical density of the samples measured by a laser scanning densitometer (see Materials and Methods). Standard deviations are indicated by error bars.

Figure 10. PA and PIP2 facilitate coatomer binding to artificial lipid vesicles. (A) Lipid vesicles that contained mole ratios of 10% PC, 10% PIP, and 80% PE were made by ultrasonic irradiation. Various amounts of PA were substituted with respect to PIP2 versus PIP content and PC versus PA ratios, incubated with coatomer, loaded on the bottom of a sucrose step gradient, and centrifuged until the vesicles floated to equilibrium. Vesicles containing 2.5% PA and 10% PIP2 banded at the 0.2/0.4 M sucrose interface (Fig. 11 B, graph) whereas those substituted respectively with PC and PIP migrated to a slightly lower sucrose concentration (Fig. 11 A, graph). Coatomer bound more avidly to the vesicles that contained PA and PIP2 than to those that did not (compare fractions 4–6 in Fig. 11 A and B). The small amount of coatomer that bound to vesicles with no PA or PIP2 was shifted by one fraction relative to the peak of lipid (Fig. 11 A, graph). Figure 10 shows β-cop profiles from such an experiment, whereas B shows the quantification of β-cop binding (the amount detected in 1 M sucrose) from three different experiments employing equal amounts of Golgi-enriched membranes. The values presented are the volume optical density of the samples measured by a laser scanning densitometer (see Materials and Methods). Standard deviations are indicated by error bars.

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fractions 4 and 5). This could be due to a higher density for the vesicle population containing coatomer. When binding was assessed by sedimentation (Fig. 11, C and D), both types of vesicles migrated to the 0.2/0.4 M sucrose interface, consistent with their density during flotation. Again, substantial amounts of coatomer migrated with vesicles that contained 2.5% PA and 10% PIP₂, as opposed to the virtual absence of binding to the control vesicles. The trails of coatomer protein in the gradients suggest a dynamic association of coatomer with these vesicles and significant dissociation during the sedimentation (compare fractions 3–6 between C and D).

Discussion

Although the precise role played by lipids in the regulation of vesicular transport is unknown, their importance is clear. Thus, metabolism of PC (and its apparent relationship to acidic phospholipids such as phophatidylinositol) in the yeast Golgi is known to be critically important for efficient secretion (Bankaitis et al., 1990; Cleves et al., 1991; Skinner et al., 1995). PLD activity would directly affect metabolism of PC. The discovery of ARF as an efficacious activator of PLD (Brown et al., 1993; Cockcroft et al., 1994) suggests that this lipase, which is enriched in Golgi membranes (Ktistakis et al., 1995), could be a downstream mediator for the actions of ARF in intracellular transport. In this work, we provide evidence that PLD activity is relevant to the formation of coatomer-coated vesicles, a key event that follows activation of ARF. We have examined the requirements of vesicle formation from two types of Golgi that display distinct regulation of PLD activity in vitro (see Fig. 1). One type, exemplified by Golgi from CHO cells, has low PLD activity that is greatly enhanced when exogenous ARF and GTP-γS are provided. A second type includes Golgi from PtK1 and MDCK cells. PLD activity in these preparations is constitutively high and shows little response to exogenous ARF and GTP-γS. Formation of coatomer-coated vesicles from PtK1 and MDCK Golgi also does not require exogenous ARF and GTP-γS. One possibility is that exogenous ARF is not required for vesicle formation from these membranes because ARF is needed solely for PLD activation. Therefore, Golgi that contain activated PLD only need coatomer to form vesicles.

Figure 11. Binding of coatomer to artificial lipid vesicles analyzed by centrifugation in sucrose gradients. Lipid vesicles made by extrusion (see Materials and Methods) with the indicated amounts of phospholipids were incubated with 20 μg of coatomer in buffer B for 30 min at 37°C. Samples were then analyzed by either flotation (A and B) or sedimentation (C and D) through sucrose gradients; fractions were collected from the top. The graphs in each panel show the distribution ³H-PC as a marker for lipid mass, and the photographs show immunoblots for β-COP. (A and B) Analysis by flotation. Vesicles containing PA and PIP₂ bound five times more coatomer than vesicles with equivalent amounts of PC and PIP, respectively (fractions 4–6, compare C to D).
Vesicles formed from PtK1 and MDCK Golgi with addition of coatomer did not contain detectable ARF but appeared identical in morphology and sedimentation properties to their counterparts that were formed with, and contained, exogenous ARF. Vesicles that required but did not contain detectable ARF were also formed from CHO Golgi using a two-step incubation, first with ARF/GTP-γS and then with coatomer. It appears that ARF is not likely to be an abundant component of the coat and is therefore not required as the binding site on vesicles for each coatomer, as has been proposed (Rothman, 1994). This conclusion is supported by a recent observation that coatomer binding to Golgi membranes does not quantitatively parallel the binding of exogenous ARF to the same membranes (Finazzi et al., 1994). Our data also raise a question about the proposed possibility that hydrolysis of GTP on ARF serves as a switch for uncoating (Tanigawa et al., 1993; Rothman, 1994). It is still possible that small amounts of ARF assemble into each vesicle and trigger uncoating. Alternatively, it is possible that uncoating is uncoupled temporally from GTP hydrolysis as shown for COPII-coated vesicles. When COPII-coated vesicles are derived from yeast ER and purified cytosolic components, the smg Sarlp is required. However, these vesicles contain Sarlp only if hydrolysis-resistant analogs of GTP are used in the assay (Barlowe et al., 1994). If GTP is used, functional vesicles that contain a coat are formed and, while less stable, can nonetheless target and deliver their cargo correctly (Barlowe et al., 1994).

Formation of coatomer-coated vesicles was sensitive to ethanol at concentrations that stimulate a transphosphatidylolation reaction by either partially purified PLD (Brown et al., 1995) or PLD on Golgi membranes. Moreover, ethanol blocked vesicle formation when it was present together with ARF and GTP-γS; i.e., during PLD activation. The effect of ethanol was limited to events dependent on ARF because pretreatment of membranes with ethanol did not inhibit subsequent ARF or coatomer binding, and the presence of ethanol did not block coatomer binding after pretreatment with ARF and GTP-γS. Therefore, the block of coated vesicle formation in the presence of ethanol is consistent with an important role for PA (or a subsequent metabolite of PA).

A direct role for PA in the process of vesicle formation is suggested by the observation that pure coatomer associates better with artificial lipid vesicles containing PA than with control vesicles. Could PA facilitate coat binding directly? In terms of lipid dynamics, the most obvious change evoked by the conversion of PC to PA is a net increase in negative charge, which could facilitate binding of proteins through electrostatic interactions. If that were the case, binding of coatomer to vesicles, as in Figs. 10 and 11, would have to be to microdomains because the net increase in charge contributed by PA is small relative to that contributed by PIP and PIP₂. In addition, since the conversion of PC to phosphatidylethanol in the presence of ethanol is not expected to greatly alter the change in charge but only the size of the head group, the attenuation of vesicle formation by ethanol suggests a more complex role for PA in this pathway. One possibility is that PA may facilitate budding by altering the curvature of the membrane. Another possibility, not exclusive with the first, is suggested by the observation that PIP₂ markedly stimulates β-COP binding to artificial lipid vesicles. This suggests that a cooperative interaction between PIP₂ and PA in vivo may offer more optimal facilitation of coat assembly. Fig. 12 depicts a model for early events leading to vesicle assembly that introduces PLD as an important participant in the pathway. We position the activity of PLD distal to ARF activation (priming) and proximal to or concomitant with coatomer binding (vesiculation). Although we do not imply a direct role for PA and PIP₂ as coatomer “receptors,” we propose that these lipids are facilitators of coatomer association and perhaps contribute to the coat-induced curvature of the membrane.

The identity of a putative coatomer receptor introduced in Fig. 12 is unknown, but two recent observations may bear on this point. Coatomer has been shown to function during the retrieval of ER membrane proteins from the Golgi through binding to a di-lysine signal in the cytosolic domain of transmembrane proteins (Cosson et al., 1994; Letourneur et al., 1994). In addition, a family of small glycoproteins has been identified as components of either coatomer or COPII vesicles which function in some unknown way during secretion and contain the di-lysine motif in their extremely short cytosolic domains (Schimmoller et al., 1995; Stamnes et al., 1995). Production of negatively charged PA by PLD in the vicinity of such basic protein sequences might lead to the formation of microdomains stabilized by electrostatic interactions between lipid head groups and protein cytosolic domains which would act synergistically to form stable binding sites for coatomer. In this way, segregation of proteins bearing such positively charged cytoplasmic signals into transport-competent regions could serve as an effective sorting mechanism.

Figure 12. Involvement of PLD in formation of coated vesicles.

We propose that early events in the formation of coatomer-coated vesicles involve activation of PLD by ARF (priming). Subsequent production of PA in the vicinity of PIP can facilitate the binding of coatomer to its receptor and/or the transformation of the planar bilayer into a curved bud (vesiculation).
It is probable that similar mechanisms underlie the formation of coated vesicles for membrane transport throughout the cell. There are five known genes for ARF in human cells and two in yeast (Kahn et al., 1991). Most is known about ARF1, which associates selectively with the Golgi complex. Another isoform, ARF6, appears to be localized to the plasma membrane/endoosome membrane system and is also involved in some aspect of membrane transport (D’Souza-Schorey et al., 1995; Peters et al., 1995). As it is known that PLD is activated with all known mammalian ARF’s (Brown et al., 1995), one must consider the possibility that similar pathways of ARF activation of PLD isoforms may stimulate coated vesicle formation at many stages of intracellular transport.

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