

The Production of Post-Golgi Vesicles Requires a Protein Kinase C-like Molecule, but Not Its Phosphorylating Activity

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Abstract. We have recently described a system that recreates in vitro the generation of post-Golgi vesicles from purified Golgi fractions obtained from virus-infected MDCK cells in which the vesicular stomatitis virus-G envelope glycoprotein had been allowed to accumulate in vivo in the TGN. Vesicle formation, monitored by the release of the viral glycoprotein, was shown to require the activation of a GTP-binding ADP ribosylation factor (ARF) protein that promotes the assembly of a vesicle coat in the TGN, and to be regulated by a Golgi-associated protein kinase C (PKC)-like activity. We have now been able to dissect the process of post-Golgi vesicle generation into two sequential stages, one of coat assembly and bud formation, and another of vesicle scission, neither of which requires an ATP supply. The first stage can occur at 20°C, and includes the GTP-dependent activation of the ARF protein, which can be effected by the nonhydrolyzable nucleotide analogue GTP γ S, whereas the second stage is nucleotide independent and can only occur at a higher temperature of incubation. Cytosolic proteins are required for the vesicle scission step and

they cannot be replaced by palmitoyl CoA, which is known to promote, by itself, scission of the coat-coated vesicles that mediate intra-Golgi transport. We have found that PKC inhibitors prevented vesicle generation, even when this was sustained by GTP γ S and ATP levels reduced far below the K_m of PKC. The inhibitors suppressed vesicle scission without preventing coat assembly, yet to exert their effect, they had to be added before coat assembly took place. This indicates that a target of the putative PKC is activated during the bud assembly stage of vesicle formation, but only acts during the phase of vesicle release. The behavior of the PKC target during vesicle formation resembles that of phospholipase D (PLD), a Golgi-associated enzyme that has been shown to be activated by PKC, even in the absence of the latter's phosphorylating activity. We therefore propose that during coat assembly, PKC activates a PLD that, during the incubation at 37°C, promotes vesicle scission by remodeling the phospholipid bilayer and severing connections between the vesicles and the donor membrane.

EXTENSIVE studies on the formation of clathrin-coated vesicles at the plasma membrane (for review see Robinson, 1994) and the TGN (Robinson and Kreis, 1992; Traub et al., 1993; Wong and Brodsky, 1992), and of the COPI- and COPII-coated vesicles that affect ER to Golgi and intra-Golgi transport (Barlowe et al., 1994; Bednarek et al., 1995; Ostermann et al., 1993; Salama and Schekman, 1995), have shown that the formation of a transport vesicle is initiated by the assembly of a protein coat on the donor membrane from cytosolic components. The coat is thought to serve as a mechanochemical device that imparts to the membrane the high degree of curvature necessary to form a budding vesicle (Pryer et al., 1992). The formation of a bud is followed by a second, not

yet fully biochemically characterized step of vesicle scission. This involves the fusion of regions within the ectoplasmic surfaces of the deformed phospholipid bilayer that have been brought in close proximity to each other at the base or neck of the forming vesicle.

Although a source of ATP appeared to be necessary to generate COPI- or COPII-coated vesicles when crude cytosolic fractions were used to support vesicle formation, it later became apparent that ATP only served to generate the GTP required to activate the respective low molecular weight GTP-binding proteins ADP ribosylation factor (ARF)¹ (Ostermann et al., 1993) and Sar (Aridor et al., 1995; Barlowe et al., 1994) that trigger coat assembly for each type of vesicle. Moreover, it is now also clear that the nonhydrolyzable GTP analogue GTP γ S can fully support

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1. *Abbreviations used in this paper:* ARF, ADP ribosylation factor; BFA, brefeldin A; PA, phosphatidic acid; PKC, protein kinase C; PLD, phospholipase D; VSV, vesicular stomatitis virus.

the formation of COPI- and COPII-coated vesicles. In these cases, GTP hydrolysis is only required for uncoating of the vesicles (Oka and Nakano, 1994; Tanigawa et al., 1993), a prerequisite for their fusion with the corresponding acceptor membranes. The scission of COPI-coated vesicles assembled *in vitro* on Golgi membranes from purified components, but not that of the COPII-coated vesicles formed on yeast ER membranes (Barlowe et al., 1994), requires the addition of palmitoyl CoA (Ostermann et al., 1993) whose synthesis may be promoted by ATP in a crude system. On the other hand, the hydrolysis of GTP by dynamin may be necessary to sever the neck of clathrin-coated endocytic vesicles from the plasma membrane (Carter et al., 1993).

There is still little information available about the process by which the vesicular carriers that transport proteins from the Golgi apparatus to the plasma membrane are generated. The *trans* face, or exit side, of the Golgi apparatus is characterized by the presence of an extensive network of tubulosaccular elements (see Rambourg and Clermont, 1990), now known as the TGN (Griffiths and Simons, 1986), from which the vesicular carriers that transport proteins to various post-Golgi destinations, including incipient lysosomes, secretory granules, and the plasma membrane, are formed. In certain types of polarized epithelial cells, such as MDCK cells, two classes of vesicles destined to the plasma membrane that are targeted to the different cell surface domains apparently emerge from the TGN (e.g., Rindler et al., 1985; Wandinger-Ness et al., 1990). These vesicles have been recovered in an uncoated state from semiintact MDCK cells incubated *in vitro*, and shown to have quite distinct protein compositions (e.g., Fiedler et al., 1995; Huber et al., 1993). Nevertheless, the process of formation of the vesicles, including the nature and mechanism of assembly of their coats, as well as the factors necessary for scission of the budding vesicles from the donor membrane, have not yet been elucidated.

Several types of regulatory proteins appear to control vesicular transport. In addition to the GTP-binding proteins whose activation promotes coat assembly, and of heterotrimeric G proteins that may regulate their activation (see Bomsel and Mostov, 1992; Donaldson and Klausner, 1994), these include protein (e.g., Davidson et al., 1992) and lipid (e.g., Brown et al., 1995; Stack and Emr, 1994) kinases, as well as macromolecules that may modify the composition of the lipid bilayer, such as the phosphatidylinositol transfer protein (Ohashi et al., 1995) and phospholipase D (PLD; Ktistakis et al., 1995).

Protein kinase C (PKC) has been found to participate in various intracellular transport steps, including regulated (Buccione et al., 1994) and constitutive exocytosis (De Matteis et al., 1993), as well as receptor traffic through the endosomal system (Cardone et al., 1994). Calphostin C, a PKC inhibitor that acts on the regulatory domain of the enzyme, has been shown to inhibit protein transport between the ER and the Golgi apparatus, and from this organelle to the cell surface (Fabbri et al., 1994). Several forms of PKC have been found to be associated with Golgi membranes (Lehel et al., 1995; Saito et al., 1989), and PKC modulators have been shown to affect the assembly of COPI coats on Golgi cisternae (De Matteis et al., 1993).

To study the molecular interactions that underlie the

generation of vesicles in the TGN, we have developed an *in vitro* system that reproduces this process and is suitable for experimental manipulation (Simon et al., 1996). The system uses purified Golgi fractions obtained from virus-infected MDCK cells in which a terminally glycosylated and labeled viral envelope glycoprotein had been allowed to accumulate in the TGN during a prolonged chase of the cells at 20°C. By following the release of labeled glycoprotein during an incubation with cytosolic proteins and an ATP-generating system, we demonstrated that the formation of post-Golgi vesicles involves the activation of an ARF-like, GTP-binding protein that serves to promote the assembly of a vesicle coat in the TGN, and that a PKC-like activity regulates vesicle production (Simon et al., 1996). Using an *in vitro* system derived from PC12 cells, other authors have also implicated a PKC in the release of post-Golgi vesicles containing a β -amyloid precursor, and in that case, the addition of a partially purified brain PKC was able to promote post-Golgi vesicle generation (Xu et al., 1995).

We now show that vesicle generation can be sustained by GTP γ S alone, even when ATP is depleted from the system. Therefore, the phosphorylating activity of the putative PKC is not required for vesicle generation. Nevertheless, in the absence of ATP, PKC modulators still suppressed or stimulated vesicle production. By dissecting the vesicle generation process into sequential stages of coat assembly/bud formation and vesicle scission, we were able to show that PKC inhibitors suppress vesicle scission without preventing coat assembly, yet, to exert their effect, they must be present before coat assembly takes place. These observations raise the possibility that PLD, a PKC target, which can be activated by PKC in the absence of the latter's phosphorylating activity (Conricode et al., 1992; Singer et al., 1996), plays an essential role in remodeling the phospholipid bilayer that is required for vesicle scission.

Materials and Methods

Reagents

GTP γ S was obtained as a lithium salt solution (catalogue No. 1 110 349; Boehringer Mannheim Biochemicals, Indianapolis, IN). According to the manufacturer, it contained undetectable levels of GTP (i.e., <0.1%). The tetralithium salt of guanylyl-imidodiphosphate (GMP-PNP), also from Boehringer Mannheim (catalogue No. 106 402), was also reported to contain <0.2% GTP as a contaminant. Apyrase, hexokinase, and 2-deoxyglucose were from Sigma Immunochemicals (St. Louis, MO). The sources of all other reagents not specifically mentioned have been indicated previously (Simon et al., 1996).

One-step *In Vitro* Vesicle Production Assay

Liver cytosolic protein fractions and Golgi fractions containing radiolabeled vesicular stomatitis virus (VSV)-G protein accumulated *in vivo* in the TGN were prepared as described previously (Simon et al., 1996). In Golgi fractions, the vast majority of the labeled VSV-G molecules were endo H resistant and contained sialylated oligosaccharides, as indicated by their sensitivity to neuraminidase digestion (Simon et al., 1996).

Standard incubation mixtures (200 μ l) for vesicle generation contained 20 μ l of a 10-fold concentrated assay buffer consisting of 600 mM K-aspartate, 30 mM MgCl₂, 5 mM EDTA in 20 mM Hepes-KOH, pH 7.3; 100 μ l of liver cytosolic proteins at a concentration of 20 mg/ml in 1 mM DTT, 1 mM PMSF, 20 mM Hepes-KOH, pH 7.3; 50 μ l of a Golgi membrane fraction suspended in 0.8 M sucrose, 1 mM DTT, 20 mM Hepes-KOH, pH 7.3;

20 μ l of either 10 mM ATP or 10 mM GTP γ S, both in 20 mM Hepes-KOH, pH 7.3, or this buffer alone; and 10 μ l of 20 mM Hepes-KOH, pH 7.3. To assess the effect of various reagents in vesicle generation, 5- μ l portions of the 20 mM Hepes buffer were replaced with 5 μ l of either one of the following: 100% DMSO, 400 μ M 12-O-tetradecanoylphorbol-13-acetate (TPA) in 100% DMSO, 40 μ M calphostin C in 100% DMSO, 4 mM *N,N*-dimethylsphingosine in 100% DMSO, 40% MeOH, 4 mM brefeldin A (BFA) in 40% MeOH, PBS, a monoclonal anti-PKC antibody in PBS (clone 1.9; Boehringer Mannheim), and 5 μ l of either 4 μ g/ml hexokinase and 400 mM 2-deoxyglucose, or 4 μ g/ml apyrase in 20 mM Hepes-KOH, pH 7.3. The mixtures were incubated for times ranging from 30 to 60 min at either 4°C, 20°C, or 37°C, as indicated in the figures, then chilled on ice for 10 min and loaded on continuous sucrose gradients (10 ml; ranging from 0.4 to 0.8 M sucrose) prepared over a 1-ml 2.0 M sucrose cushion (all sucrose solutions in 20 mM Hepes-KOH, pH 7.3). After the gradients were centrifuged at 4°C for 1 h at 125,000 *g* in an SW41 rotor (Beckman Instruments, Inc., Palo Alto, CA), the radioactivities in 500- μ l gradient fractions, as well as in the loading zone (S) and in the resuspended pellet (P), were measured by liquid scintillation counting. The radioactivity distributions are expressed as percentage of the total recovered in each gradient. Vesicle release was calculated as the percentage of the total radioactivity that was contained in the peak that sedimented in the top half of the gradient. In some experiments, depending on the Golgi preparation, the two to three first fractions of the gradients contained traces of radioactivity. This corresponded to TCA-soluble material, identified as ³⁵S-Met by TLC analysis.

Two-step In Vitro Vesicle Production Assay

The first step was performed as described above, but the mixtures (400 μ l) were always incubated for 60 min at 20°C instead of at 37°C. After this incubation, the samples were loaded on discontinuous sucrose gradients consisting of 2.5 ml of 0.4 M sucrose layered over a 1-ml 2.0 M sucrose cushion, both in 1 mM DTT, 20 mM Hepes-KOH, pH 7.3. The gradients were centrifuged at 4°C for 10 min at 45,000 rpm in an SW60 rotor. The Golgi membranes that accumulated at the interface between the two sucrose layers were collected in a small volume (\sim 50 μ l). The sucrose concentration in this sample (usually 1.2 M sucrose) was measured by refractometry and adjusted to 0.8 M in 100 μ l by the successive addition of appropriate volumes of two solutions, one containing 1 mM DTT, 20 mM Hepes-KOH, pH 7.3, and the other containing 0.8 M sucrose in the same buffer. In the second step of this assay, aliquots of the recovered membranes (50 μ l) were immediately reincubated for 60 min at 37°C in standard assay mixtures (200 μ l) that were similar to those for the one step assay, but contained or lacked the liver cytosolic protein fraction (10 mg/ml) and/or GTP γ S (1 mM). Vesicle release was assessed as in the one-step assay.

Density Shift Assay for Golgi Membrane Coating

Incubation mixtures (200 μ l) with or without 1 mM GTP γ S were first maintained at 20°C for 60 min, and then kept on ice for 10 min and loaded on discontinuous sucrose gradients (10.5 ml each) consisting of 3.5 ml layers of 2.0, 1.3, and 0.4 M sucrose in 20 mM Hepes-KOH, pH 7.3. The gradients were centrifuged at 4°C for 1 h at 125,000 *g* in an SW41 rotor, and the distribution of the VSV-G radioactivity was measured as described in the vesicle production assay.

Purification of Released Coated Vesicles

Vesicles were purified from a 12-ml reaction mixture that, after incubation, was divided into 1-ml aliquots. These were fractionated by velocity centrifugation on 10-ml continuous sucrose gradients similar to those used for analytical studies but prepared in assay buffer, as were all the sucrose solutions used in subsequent steps. Fractions 5–10 (500 μ l each), corresponding to the peak of radioactivity in coated vesicles, were pooled (32.4 ml) from the 12 gradients, and the sucrose concentration (0.56 M, measured by refractometry) was adjusted to 0.3 M by dilution to 60 ml with assay buffer. Aliquots (10 ml each) were then loaded over 1.5-ml cushions of 2.0 M sucrose prepared in SW41 rotor tubes, which were centrifuged at 150,000 *g* for 2 h at 4°C. The fractions banding between the two sucrose layers were collected and combined (9 ml total), and the final sucrose concentration was adjusted to 2.0 M by adding a 3.0 M sucrose buffer stock solution. Three 4-ml aliquots were then transferred to SW41 centrifuge tubes, and were overlaid with three layers of 1.6 M (3.5 ml), 1.2 M (3.5 ml), and 0.6 M (0.5 ml) sucrose. After centrifugation at 150,000 *g*

for 20 h at 4°C, the purified coated vesicles, banding at the 1.2/1.6 M sucrose interfaces, were collected to give a total volume of 1.5 ml and used for EM.

Sialyltransferase Assays

These were carried out on pooled fractions that were recovered from sucrose density gradients containing vesicles or residual Golgi membranes (fractions 5–10 and 17–22, respectively). The sucrose concentrations in the pooled fractions were diluted to 0.4 and 0.8 M, respectively, and the vesicles or membranes were recovered by centrifugation (150,000 *g* at 4°C in a TLS 100.3 rotor). The pellets were suspended in 30 μ l of 20 mM Hepes-KOH, pH 7.3, 1 mM DTT. Enzyme assays were carried out in triplicate in 10- μ l aliquots, as described previously (Simon et al., 1996).

ATP Measurements

These were carried out with Adenosine 5'-triphosphate (ATP) Bioluminescent Assay kit (Sigma) using a luminometer (Monolight 2010; Analytical Luminescence Laboratory, San Diego, CA). Measurements were made either in individual components of the assay mixture at the concentrations at which they were used, or in assembled mixtures.

EM

Purified vesicles were fixed in suspension by adding glutaraldehyde in 0.1 M cacodylate buffer, pH 7.5, to dilute the sucrose concentration to 0.3 M. The fixed vesicles were sedimented by centrifugation at 150,000 *g* for 2 h at 4°C in a TLS-55 swinging bucket rotor (Beckman Instruments), and the pellets were processed for routine thin-section EM, including tannic acid staining, as described previously (Simon et al., 1996). Golgi fractions incubated for vesicle generation were first recovered by centrifugation (10 min at 20,000 *g* in a TLS-55 swinging bucket rotor), fixed as pellets, and processed as described (Simon et al., 1996). Negative staining with 1% uranyl acetate was carried out in 1- μ l aliquots of unfixed released vesicles in the peak fractions from the standard sedimentation velocity gradients that were used to separate vesicles from Golgi remnants (Simon et al., 1996), or of purified coated vesicles.

Results

GTP γ S Promotes the Formation of Coated Post-Golgi Vesicles in the Absence of an ATP Supply

Previous studies have shown that the GTP-mediated activation of an ARF protein is involved in the generation of post-Golgi vesicles (Barr and Huttner, 1996; Chen and Shields, 1996; Simon et al., 1996). It is therefore possible that the requirement for an ATP supply reported by us as well as others (Salamero et al., 1990; Simon et al., 1996; Tooze and Huttner, 1990) reflects, at least in part, the need to generate the GTP required for coat assembly. To test this possibility, we determined whether GTP γ S could support the production of post-Golgi vesicles by itself, in the absence of an ATP supply. It was found (Fig. 1 A) that this was indeed the case. 1 mM GTP γ S promoted the release of vesicles containing labeled VSV-G protein to the same extent as an equivalent concentration of ATP, and when vesicle generation was promoted by GTP γ S alone, the vesicles sedimented more rapidly in sucrose density gradients than when it was promoted by ATP (Fig. 1 A). In addition, as has been observed previously (Simon et al., 1996), when ATP and GTP γ S were both present during the incubation, only vesicles (60–80 nm in diameter) that had a protein coat were found in the peak fractions containing the released labeled VSV-G protein (Figs. 1 C and 2). To exclude the possibility that endogenous or contaminat-

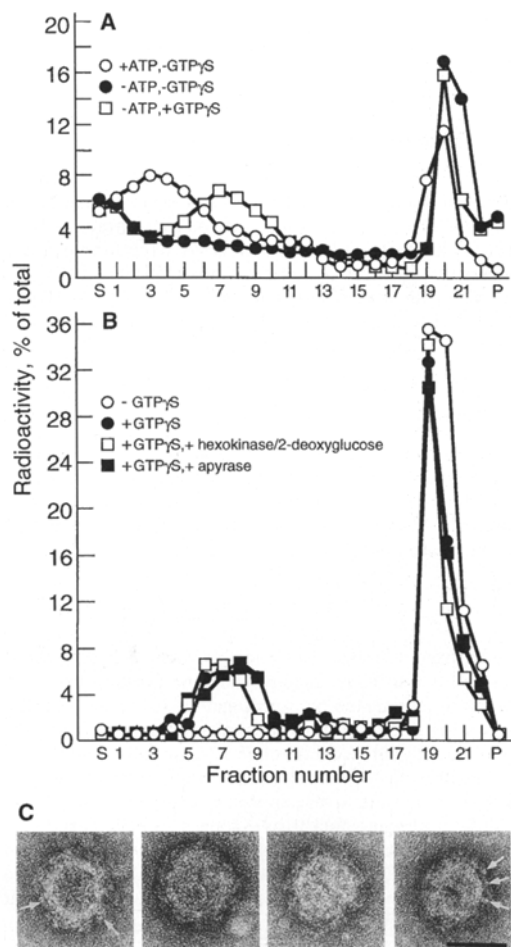


Figure 1. GTP γ S can fulfill the nucleotide requirement for the formation of post-Golgi vesicles from Golgi fractions containing labeled VSV-G protein accumulated in the TGN. (A) ATP and GTP γ S are equally effective in promoting vesicle release. Golgi fractions were incubated for 60 min at 37°C in the standard vesicle-generating assay, with either 1 mM ATP or 1 mM GTP γ S, or without either nucleotide, as indicated. The mixtures were then chilled on ice and analyzed by sucrose density gradient centrifugation. The vesicles released in the presence of GTP γ S (peak at fraction 7) sediment more rapidly than those generated in the presence of ATP (peak at fraction 3). The residual Golgi membranes are collected above the sucrose cushion (fractions 19–22). P, pellet; S, sample loading zone. (B) GTP γ S supports the formation of post-Golgi vesicles, even in the presence of an ATP-depleting system. Golgi fractions containing the labeled VSV-G protein were incubated at 37°C for vesicle release under the standard conditions, either with no added nucleotides (○—○), or with 1 mM GTP γ S and no other additions (●—●), or supplemented with an ATP-depleting system consisting of either hexokinase and 2-deoxyglucose (□—□), or with apyrase (■—■). Reaction mixtures were analyzed as described in A. (C) Coated vesicles found in the peak fraction of the gradient profile illustrated in (B), corresponding to a sample that was incubated with GTP γ S and apyrase to consume ATP. For negative staining, an aliquot (1 μ l) of gradient fraction 7 was applied to the grids without previous concentration. Only non-clathrin-coated vesicles (60–80 nm in diameter) were found, of which four examples are shown. The coat of the vesicles (~10 nm in thickness) appears to be composed of regularly spaced subunits that can be recognized along the circumferential profile of the vesicles (small arrows). Bar, 50 nm.

ing ATP or GTP molecules, provided by either the Golgi membranes, the cytosolic protein fraction, or the GTP γ S reagent, were being used to produce the vesicles, reactions promoted by GTP γ S were carried out in the presence of two commonly used ATP-depleting systems, hexokinase and 2-deoxyglucose, or apyrase. Neither of these diminished vesicle generation (Fig. 1 B), although, as shown below (Table I), they decreased the levels of ATP in the reaction mixture to the nanomolar range. Pretreatment of the individual components with the ATP-depleting systems before their mixing also did not reduce vesicle release (not shown).

For further assurance that contaminating GTP in the GTP γ S preparation—or slow hydrolysis of GTP γ S—did not contribute to vesicle production, we determined the dose response of this process for GTP γ S, and whether it could be sustained by GMP-PNP, which is believed to be more resistant to hydrolysis than GTP γ S. We found that a 10-fold lower concentration of GTP γ S (0.1 mM) also gave maximal vesicle release, and that even at a 100-fold lower concentration (0.01 mM), vesicle release was only reduced by 50% (not shown). In addition, 1 mM GMP-PNP supported vesicle production to the same extent as GTP γ S (not shown).

In our original studies on the cell-free formation of post-Golgi vesicles sustained by an ATP-generating system, we demonstrated that the release of vesicles does not result from a random fragmentation of the TGN, since their sialyltransferase content was very low (Simon et al., 1996). This was also the case for the vesicles produced in the presence of GTP γ S in the absence of an ATP supply: whereas 30% of the labeled viral glycoprotein was recovered in the released vesicles, only 1.5% of the sialyltransferase was in that fraction.

Coated vesicles released in the presence of GTP γ S and apyrase were purified (see Materials and Methods) from pooled peak fractions of sedimentation velocity gradients, and were characterized by EM (Fig. 2). This showed a relatively uniform population of non-clathrin-coated vesicles that were similar in appearance to the COPI-coated ones that are involved in intra-Golgi transport. COPI-coated vesicles, however, have been reported not to be released when Golgi membranes are incubated with GTP γ S (Melancon et al., 1987).

Table I. Effectiveness of ATP-depleting Systems in Reducing the ATP Levels in Vesicle Generation Mixtures

Components	ATP
	nM \pm SD
Golgi (G)	1.1 \pm 0.5
LCP	18.9 \pm 13.8
GTP γ S	9.0 \pm 1.8
G + GTP γ S	8.7 \pm 1.5
LCP + GTP γ S	110.8 \pm 22.5
G + LCP	19.2 \pm 3.2
G + LCP + GTP γ S	124.2 \pm 34.8
G + LCP + HK/2-DOG + GTP γ S	37.9 \pm 16.2
G + LCP + apyrase + GTP γ S	5.5 \pm 1.9

ATP concentrations were measured, as described in Materials and Methods, in aliquots of the individual components of reaction mixtures, and in the indicated combinations of components. Each value represents the average of 12 determinations using three different Golgi (G), liver cytosolic protein (LCP), and GTP γ S preparations. Buffers, 2-deoxyglucose (2-DOG), hexokinase (HK), and apyrase gave background levels.

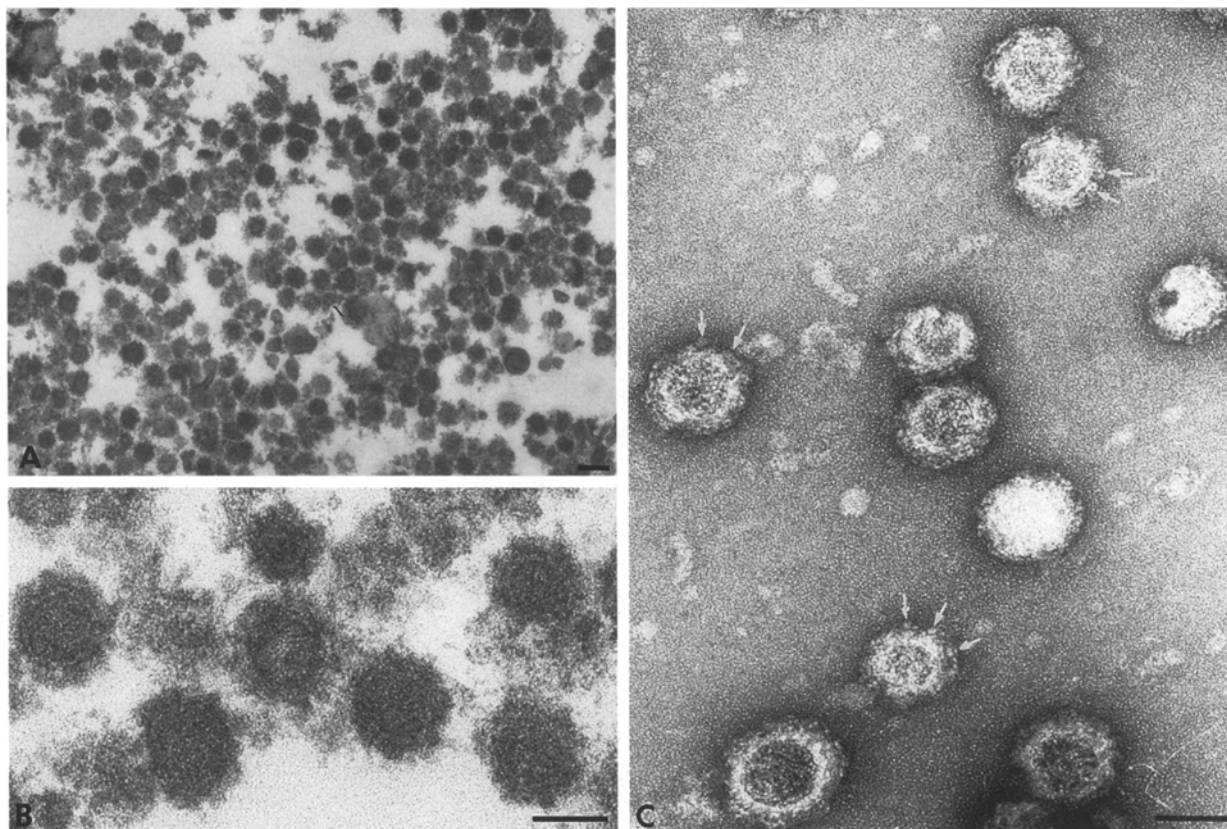


Figure 2. Purified coated vesicles released *in vitro*. The vesicles were purified from pooled peak fractions obtained from large-scale reaction mixtures (see Materials and Methods). *A* shows the purity of the vesicle preparation. In *B*, the presence of a coat is apparent in all the vesicles in the field. (*C*) The vesicle coat is also well visualized by negative staining, which reveals its subunit structure (*small arrows*). Bars: (*A*) 100 nm; (*B*) 50 nm; (*C*) 50 nm.

The Production of Coated Vesicles Occurs in Two Cytosolic Protein-dependent Phases: Membrane Coating/Bud Formation and Vesicle Scission

We have observed previously (Simon et al., 1996) that post-Golgi vesicle production did not take place when *in vitro* incubation in the presence of an ATP supply was carried out at 20°C, a temperature at which exit from the TGN does not occur *in vivo* (Matlin and Simons, 1983). We therefore determined whether coat assembly (i.e., the putative GTP-dependent step) proceeds normally, but vesicle release, which requires a membrane fusion event initiated at the luminal face of the donor Golgi membrane, is halted at that temperature. As shown in Figs. 3 and 4, this was indeed the case. At 20°C, vesicle production with GTP γ S as the sole added nucleotide was almost negligible (Fig. 3 *A*). The membranes preincubated in this fashion, however, released normal amounts of post-Golgi coated vesicles that contained terminally glycosylated, labeled VSV-G protein when, after their recovery by sedimentation, they were reincubated at 37°C with cytosolic proteins in the absence of GTP γ S or any other nucleotide (Fig. 4 *A*). Vesicle release during this second incubation did not take place if either cytosolic proteins or GTP γ S had been omitted during the 20°C preincubation (Fig. 4 *A*), and this cannot be attributed to an inactivation of the Golgi membranes, since in all cases, normal amounts of vesicles were generated when GTP γ S was present during the reincuba-

tion at 37°C (Fig. 4 *B*). This indicates that during the 20°C preincubation with cytosolic proteins, a priming step promoted by GTP γ S had taken place. Two observations showed that this corresponded to the assembly of a protein coat on Golgi membranes. First, after the incubation with GTP γ S, a substantial portion (~55%) of the Golgi fraction containing the labeled glycoprotein increased in isopycnic density, sedimenting through a 1.3-M sucrose layer to band above a 2.0-M sucrose cushion (Fig. 3 *B*). On the other hand, Golgi membranes incubated in the absence of the nucleotide analogue did not penetrate into the 1.3-M sucrose layer. Second, examination of the Golgi membranes in the electron microscope (Fig. 5) revealed that during the incubation at 20°C, an extensive formation of coated buds whose membranes remained connected to those of the cisterna, as well as the formation of apparently complete but undetached vesicles, had taken place. This occurred in both dilated terminal cisternae and tubules that may correspond to TGN elements (Fig. 5, *A* and *B*), as well as in other cisternae throughout the Golgi stack (Fig. 5, *B*, *C*, and *E*), where they have been described previously (Melancon et al., 1987; Weidman et al., 1993). The associated vesicles could frequently be seen connected to the donor membranes or to each other, sometimes by fine threads (Fig. 5, *F*, *G*, and *H*, *arrows*). Strikingly, it was frequently observed that whole tubular regions or tubular extensions of the Golgi cisternae became coated and began to vesiculate, generating entire chains of linked coated

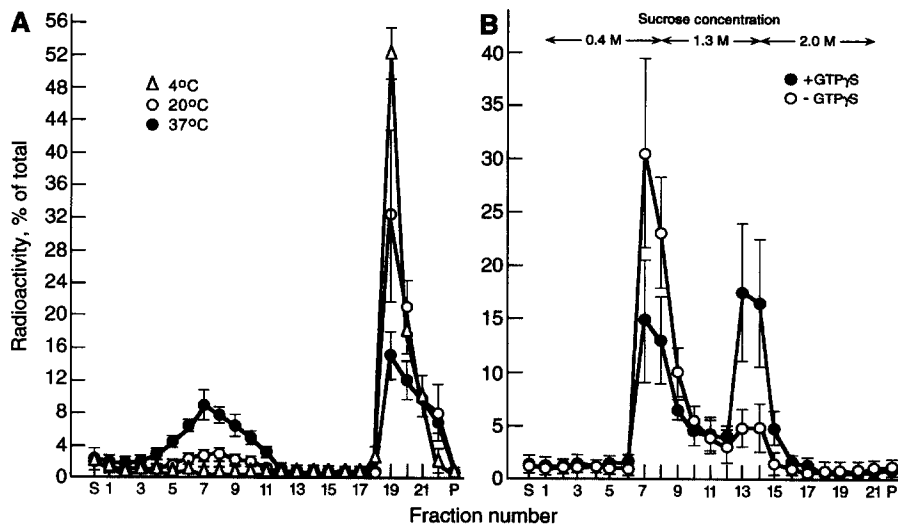


Figure 3. At 20°C, a temperature at which vesicle release does not take place, GTP γ S promotes the binding of cytosolic proteins to the Golgi membranes, as indicated by a density shift assay. (A) The *in vitro* release of post-Golgi vesicles promoted by GTP γ S is markedly reduced at 20°C, a temperature at which exit from the TGN is arrested *in vivo*. Golgi fractions were incubated for 60 min for vesicle release with GTP γ S (1 mM) at the temperatures indicated, and the reaction mixtures were analyzed by velocity gradient centrifugation, as described in Fig. 1. Each point represents the average (\pm SD) of three independent experiments. (B) After the incubation at 20°C, the Golgi membranes increase in isopycnic density. After a 60-min incubation at 20°C in the standard assay

with (-●-●-) or without (-○-○-) GTP γ S, the samples were analyzed by sedimentation on a discontinuous density gradient (10.5 ml) consisting of 2.0, 1.3, and 0.4 M sucrose layers, as described in Materials and Methods. The values displayed are the averages from four independent experiments (\pm SD).

vesicles (Fig. 5, D and G). The demonstration that BFA completely blocked vesicle formation when added before the incubation at 20°C, but had no effect when added after it, established that the activation of the ARF-like protein that promotes the assembly of a coat for TGN-derived vesicles takes place during the 20°C, GTP-dependent, priming step (Fig. 6).

These observations indicate that the second phase of vesicle release, which also requires the cytosolic fraction but proceeds in the absence of nucleotides and occurs only at a higher temperature of incubation, can be considered as a vesicle scission step. During this phase, the coated buds become vesicles and the connections between vesicles and between vesicles and Golgi membranes are severed. The fact that vesicle release did not take place if the cytosolic fraction used for the second phase was either heated to 100°C or treated with trypsin or *N*-ethylmaleimide (NEM) (not shown) made clear that the proteins present in the cytosolic fraction are required for this scission step. Palmitoyl CoA, a cofactor that is necessary for the release of intra-Golgi transport vesicles *in vitro* (Ostermann et al., 1993; Pfanner et al., 1989), could not replace the cytosolic protein fraction or enhance vesicle release when added to limiting or optimal concentrations of cytosolic proteins (not shown).

PKC Inhibitors Suppress and TPA Stimulates Vesicle Release Even When the System Is Depleted of ATP

We have shown previously (Simon et al., 1996) that post-Golgi vesicle production supported by ATP was markedly reduced by various specific PKC inhibitors and substantially enhanced by the phorbol ester TPA, a PKC activator. Because the experiments described above conclusively demonstrated that GTP γ S and cytosolic proteins are sufficient to fully support the formation and release of post-Golgi vesicles in the absence of an ATP supply, we re-investigated the role of PKC in this process, since the activity of this enzyme requires ATP. The effect of the various

PKC modulators on post-Golgi vesicle production was studied in two-step vesicle generation assays that were carried out in the presence of an ATP-depleting system (Fig. 7). Preincubation of the Golgi membranes and cytosolic proteins at 20°C with the PKC inhibitors calphostin C or *N,N*-dimethylsphingosine, together with hexokinase and 2-deoxyglucose, before the addition of GTP γ S, markedly suppressed (70–80%) vesicle generation (Fig. 7, C and E), whereas preincubation with TPA stimulated it (>60%) (Fig. 7 A). Moreover, TPA and *N,N*-dimethylsphingosine, which are known to compete for the diacylglycerol-binding site in the regulatory domain of PKC (Nishizuka, 1992), counteracted each other's effects when added together at the appropriate concentrations, since vesicle release promoted by subsequent addition of GTP γ S proceeded normally (Fig. 7 E). An Ab (clone 1.9) that recognizes all forms of PKC also substantially reduced vesicle release when it was added before GTP γ S (Fig. 7 G). None of the PKC modulators, however, had a significant effect on vesicle release when added to the system after the priming incubation at 20°C with GTP γ S had been completed (Fig. 7, B, D, F, and H). The PKC modulators were equally effective when apyrase, instead of hexokinase and 2-deoxyglucose, was added to reduce ATP levels (Fig. 8). Control mAbs to endolyn 78 (Croze et al., 1989) or to influenza hemagglutinin (Wandinger-Ness et al., 1990), which were used at the same concentration as the anti-PKC 1.9 mAb, did not affect vesicle formation (not shown).

The PKC Inhibitor Calphostin C Only Inhibits Vesicle Scission and Not Membrane Coating, but to Exert Its Effect, It Must be Added before Membrane Coating Takes Place

Pretreatment of the Golgi membranes with the irreversible PKC inhibitor calphostin C inactivated them with respect to their capacity to release post-Golgi vesicles during a subsequent incubation at 37°C with GTP γ S and cytosolic proteins (Fig. 9). An examination of the Golgi membranes

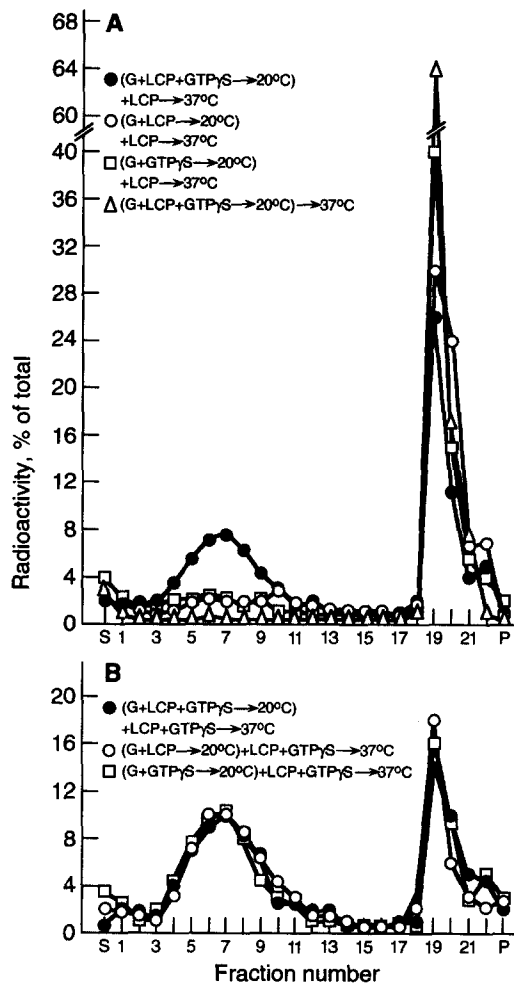


Figure 4. The *in vitro* formation of post-Golgi vesicles can be carried out in sequential coat assembly and vesicle release steps. (A) The coating of Golgi membranes can take place at 20°C, requires cytosolic proteins, and is triggered by GTP γ S, whereas the subsequent vesicle release reaction requires a higher temperature of incubation (37°C) and cytosolic proteins, but no added nucleotides. Aliquots of a labeled Golgi fraction were first incubated at 20°C for 60 min in assay buffer with 1 mM GTP γ S and liver cytosolic proteins (LCP, 10 mg/ml; ●—● and Δ—Δ), or with only one of the latter two components (○—○ and □—□), as indicated. At the end of this incubation, the Golgi membranes were recovered as described in Materials and Methods, and reincubated for 60 min at 37°C in mixtures containing the assay buffer without GTP γ S, and with (●—●, ○—○, and □—□) or without (Δ—Δ) liver cytosolic proteins. (B) All incubations were carried out as described in A, except that 1 mM GTP γ S was present during the second incubation.

by EM, however, revealed (Fig. 10) that calphostin C treatment did not prevent the formation of coated buds and undetached vesicles promoted by GTP γ S and cytosolic proteins at either 20°C or 37°C (Fig. 10 A). A quantitative analysis of the number of coated buds and undetached vesicles found in randomly selected areas of sections of Golgi stacks showed no measurable effect of calphostin C. Thus, the average number of coated buds and undetached vesicles per square millimeter in a total area of ~ 20 mm² was 25 ± 1.7 in the absence of calphostin C and 26.1 ± 2.0 in its presence, with a total of ~ 500 vesicles counted in

each case. As was the case with controls (Fig. 5), in the presence of calphostin C, entire coated tubules were frequently observed (Fig. 10 B) that apparently gave rise to rows of budding vesicles (Fig. 10 C).

The possibility that calphostin C only prevented the incorporation of the labeled VSV-G glycoprotein into the vesicles, but that these continued to be formed, was excluded in two ways. First, an examination by EM of samples taken from the region of the sucrose density gradient where the vesicles are normally found showed that very few vesicles were present. Moreover, when the vesicle generation assay was carried out with Golgi fractions from metabolically labeled, uninfected cells, calphostin C also prevented the release (80% inhibition) of labeled vesicles (not shown). The fact that in a density shift assay similar to that in Fig. 3, the GTP analogue caused a shift of $\sim 50\%$ of the labeled G protein molecules to the position of coated membranes, even in the presence of calphostin C, demonstrated that calphostin C and several other PKC inhibitors, such as the autoinhibitory peptide and the anti-PKC mAb (clone 1.9), did not diminish GTP γ S-induced membrane coating. Thus, although the inhibitors prevented vesicle generation only when added before membrane coating and bud formation had taken place, their effects were only manifested during the second phase of the process, i.e., when membrane scission and the ensuing release of the vesicles occur.

These experiments established that a PKC-like protein plays an essential role in post-Golgi vesicle generation, even when this process is promoted by GTP γ S as the sole added nucleotide and ATP is depleted from the system. Thus, the phosphorylating activity of the putative PKC would not appear to be necessary for its participation in vesicle generation. Direct ATP measurements using the luciferin-luciferase assay verified that the ATP levels in the reaction mixtures are not sufficient to sustain the phosphorylating activity of a PKC (Table I). This showed that the total concentration of ATP that could be contributed by the individual components was ~ 30 nM, which is >150 -fold lower than the K_m for ATP measured for a PKC (Kikkawa et al., 1982). When the various components of the vesicle generation reaction were mixed, the ATP levels increased to ~ 125 nM, but this increase was prevented by the ATP-depleting systems and, in particular, by apyrase, which reduced the ATP levels to ~ 5 nM. It is perhaps worth mentioning that the increase in ATP levels that takes place upon mixing the components must be caused by the production of ATP by cytosolic nucleoside diphosphokinases from GTP known to contaminate ($<0.1\%$) the GTP γ S preparation used. Thus, mixing GTP γ S with the cytosolic protein fraction led to nearly the same level of ATP production (110 nM) as in the complete system (Table I), whereas mixing the GTP γ S with the Golgi fraction or mixing the Golgi fraction with the cytosolic proteins did not lead to an increase in ATP levels (Table I).

Discussion

Post-Golgi Vesicle Production Requires GTP to Assemble a Vesicle Coat, but Does Not Require ATP at All

The fact that the nonhydrolyzable GTP analogue GTP γ S

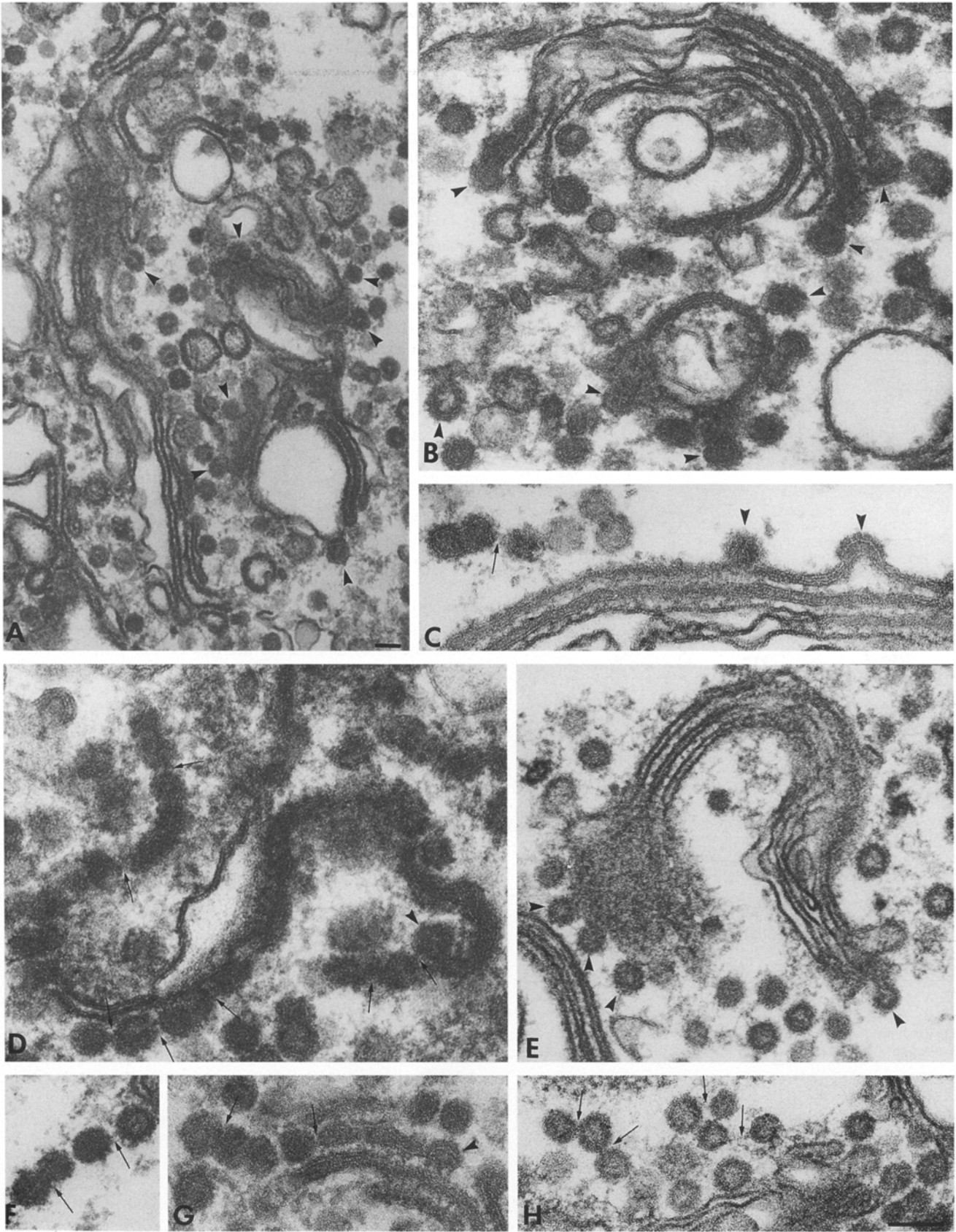


Figure 5. Formation of membrane buds and undetached vesicles from the TGN and other Golgi cisternae during a 20°C incubation with GTP γ S and cytosolic proteins. After *in vitro* incubation for 30 min at 20°C, the Golgi membranes were recovered by sedimentation and processed for thin-section EM. Forming coated buds (arrowheads) are abundant on dilated cisternae within the TGN region (A), but they also arise from the rims of individual cisternae within a stack (B and E), as well as from the central region of the outer cisternae within a stack (C). Frequently, entire tubular elements of the cisternae (D) become coated and give origin to rows of buds or vesicles that remain connected through their membranes or that are linked by fine threads (small arrows; C, F, G and H). B–H are at the same magnification. Bars, 100 nm.

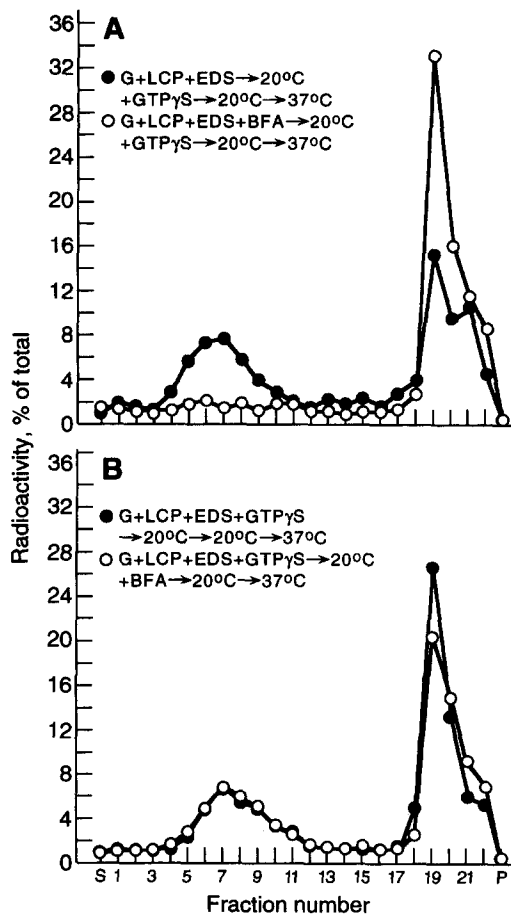


Figure 6. BFA inhibits vesicle production by blocking the GTP γ S-promoted membrane-coating step. (A) Golgi fractions were preincubated at 20°C for 30 min with liver cytosolic proteins (LCP, 10 mg/ml) and an energy-depleting system (EDS, 0.1 mg/ml hexokinase, 10 mM 2-deoxyglucose), with (○—○) or without 100 μ M BFA (●—●) before the addition of 1 mM GTP γ S to both samples and a second incubation for 30 min at 20°C, followed by 60 min at 37°C. (B) Incubations were as described in A, but GTP γ S was present instead of BFA in the first 20°C incubation, and BFA was added to the sample before the second 20°C incubation. Control samples not treated with BFA (A and B, ●—●) contained 1% methanol, the BFA solvent. The mixtures were analyzed for vesicle release as described in Fig. 1.

fully supported post-Golgi vesicle production in the presence of ATP-depleting systems clearly indicates that, as is the case for COPI- and COPII-coated vesicles (Barlowe et al., 1994; Ostermann et al., 1993), the activation of a GTP-binding protein, but not hydrolysis of the nucleotide, is required for the process. The capacity of GTP γ S to promote post-Golgi vesicle production with purified MDCK cell-derived Golgi fractions, demonstrated here, contrasts with the inhibitory effect of the nonhydrolyzable GTP analogue on post-Golgi vesicle production observed with permeabilized, pituitary-derived GH3 cells (Xu and Shields, 1993), or with PC12 cell-derived postnuclear supernatant or crude membrane fractions (Ohashi and Huttner, 1994; Tooze et al., 1990). In the latter case, the inhibitory effect of GTP γ S was attributed to the stimulation of an inhibitory heterotrimeric G protein (Barr et al., 1991) that, in

balance with a stimulatory G protein, would regulate vesicle production. Our results indicate that such an inhibitory G protein could not play a dominant role in the MDCK cell-free system.

The Formation of Post-Golgi Vesicles Can Be Dissected into Two Sequential Stages, Both of Which Require Cytosolic Proteins but Have Different Temperature and Nucleotide Requirements

We found that incubation of the Golgi fraction at 20°C with cytosolic proteins and GTP γ S does not lead to vesicle release, but primes the membranes so that they can release vesicles during a subsequent incubation at 37°C, even in the absence of nucleotide. Priming of the membranes can be inhibited by BFA and, therefore, includes the activation of an ARF-like protein. This stage corresponds to the assembly of a coat on the TGN membranes that increases their isopycnic density and leads to the extensive formation of coated buds and vesicles that remain attached to the membranes and/or to each other by membranous or thin, electron-dense, thread-like connections. Connections of this type have been previously observed by freeze-etch EM in Golgi fractions that were attached to glass beads and incubated in vitro with cytosol and an ATP supply to which GTP γ S was added (Weidman et al., 1993). In that work, it was suggested that these connections were responsible for the retention of newly formed vesicles on the donor membrane (Weidman et al., 1993).

Scission and release from the donor membrane of the buds and vesicles that accumulated at 20°C represents the second phase in vesicle production, which requires a higher incubation temperature and proceeds in the absence of added nucleotides, but is also totally dependent on the presence of cytosolic proteins. The production of COPI-coated vesicles has also been dissected into two separable phases, one of coated bud formation that was promoted by GTP and required only ARF and coatamer, and a second one of vesicle release that depended only on the addition of palmitoyl CoA (Ostermann et al., 1993). In our case, however, vesicles could not be released in phase II by the simple addition of palmitoyl CoA, but cytosolic proteins, including an NEM-sensitive factor distinct from the N-ethylmaleimide-sensitive fusion protein (NSF) (Simon et al., 1996) were required. A possible NEM-sensitive cytosolic component required for vesicle release could be the phosphatidylinositol transfer protein, which requires SH groups for its function (V.A. Bankaitis, personal communication) and has been shown to stimulate the production of post-Golgi vesicles in the PC12 cell-free system when cytosolic proteins are limiting (Ohashi et al., 1995). It has been suggested that the change in phospholipid composition brought about by this protein could result in changes in lipid orientation and membrane leaflet curvature that are necessary for vesicle fission (Ohashi et al., 1995).

The fact that Golgi membranes isolated from cells incubated for 2 h at 20°C were not already primed (i.e., fully coated) to release vesicles in a GTP γ S-independent fashion when incubated in vitro with cytosol at 37°C may have been caused either by insufficient levels of GTP in the cell during the cooling period to fully assemble a coat, or by

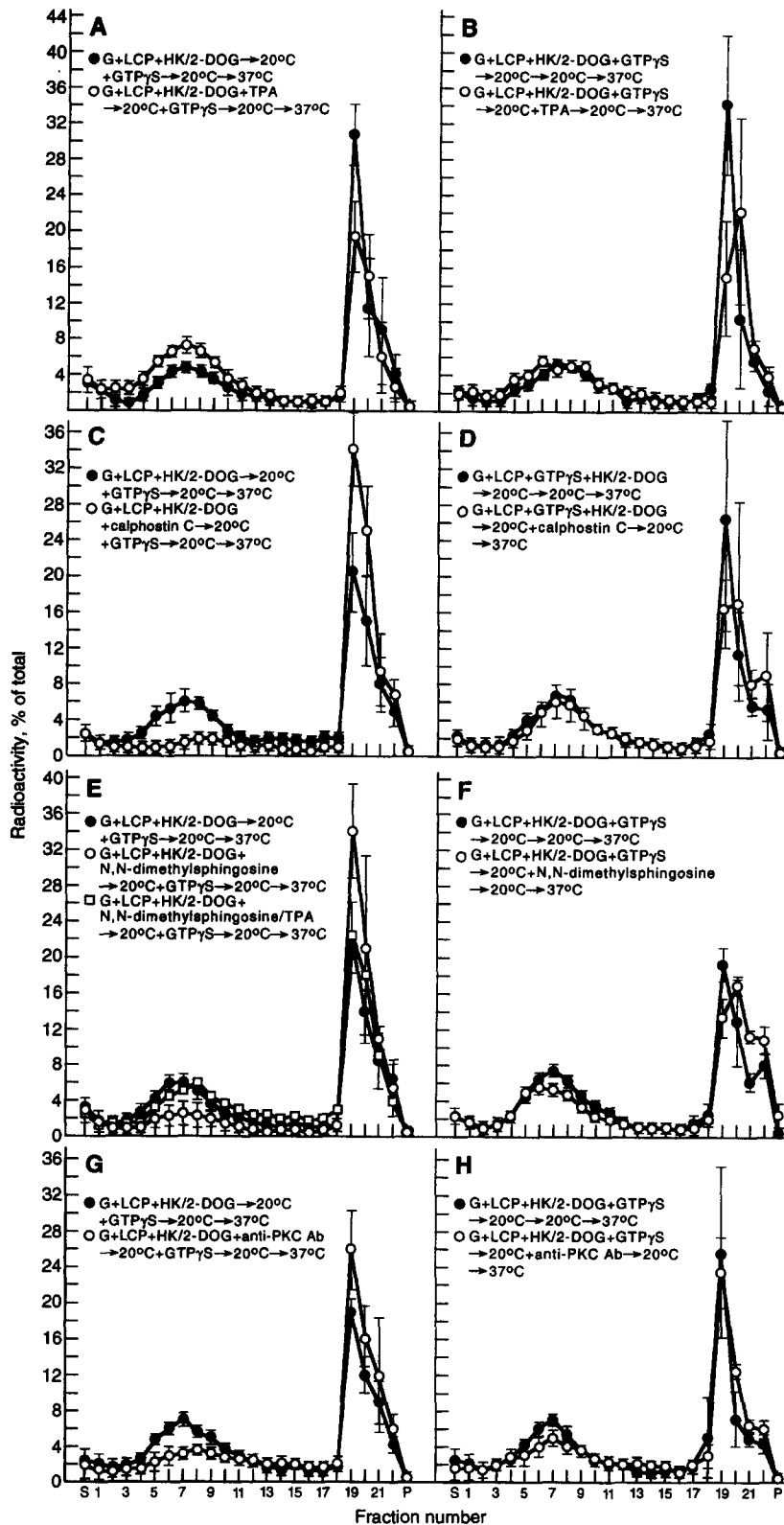


Figure 7. PKC modulators exert their effects on vesicle production during the membrane-coating step, and they do so even in the presence of an ATP-depleting system. (A, C, E, and G) Labeled Golgi membranes were preincubated (○—○) for 30 min at 20°C with liver cytosolic proteins and an energy-depleting system (*HK/2-DOG*, 0.1 mg/ml hexokinase and 10 mM 2-deoxyglucose), and either 10 μM TPA (A), 1 μM calphostin C (C), 100 μM *N,N*-dimethylsphingosine (E), or 0.15 mg/ml anti-PKC mAb (G). In controls for each panel, the PKC modulators were replaced by their solvents (●—●). In E, a sample (□—□) was also preincubated with both 10 μM TPA and 100 μM *N,N*-dimethylsphingosine. After the preincubations, 1 mM GTPγS was added, and incubation at 20°C continued for 30 min to complete the first phase. The reaction mixtures were then incubated at 37°C for 60 min to effect vesicle release. (B, D, F, and H) A preincubation (○—○) for 30 min at 20°C was carried out as described above, but 1 mM GTPγS was added instead of the PKC modulators, which were only added subsequently (B, TPA; D, calphostin C; F, *N,N*-dimethylsphingosine; H, anti-PKC antibody) followed by incubation for 30 min at 20°C and 60 min at 37°C. For each panel, a control (●—●) was carried out in which, instead of the PKC modulator, its solvent alone was added. The values displayed (except for E, open squares) are the averages (±SD) of three independent experiments.

disassembly of a preform coat during the lengthy preparation procedure that is necessary to obtain purified Golgi membranes.

EM showed that the formation of coated buds and vesicles that takes place during the priming step is not limited to the dilated terminal cisternae and to the adjacent tu-

bules that at one end of the isolated Golgi stack may represent the TGN. In fact, morphologically similar coated buds and attached vesicles were also present in cisternae throughout the Golgi stack. It seems likely that many of those buds and vesicles correspond to the well-characterized COPI-coated vesicles (see Rothman and Wieland,

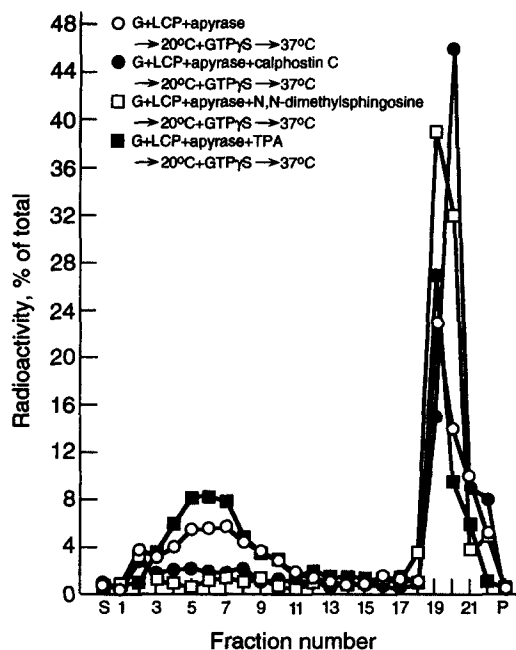


Figure 8. PKC modulators affect the GTP γ S-induced release of post-Golgi vesicles even in the presence of apyrase, which nearly completely depletes the system of ATP. Labeled Golgi fractions (G) were preincubated for 30 min at 20°C in assay mixtures containing liver cytosolic proteins (LCP), 0.1 mg/ml apyrase, and either 1 μ M calphostin C (●—●), 100 μ M *N,N*-dimethylsphingosine (□—□), or 10 μ M TPA (■—■). Control samples received 2.5% DMSO (○—○). After the preincubation, the samples were chilled on ice, supplemented with 1 mM GTP γ S, and reincubated at 37°C for 1 h. Vesicle release was determined as in Fig. 1.

1996, and references therein) that mediate intra-Golgi transport, whether in the anterograde or retrograde (Cosson and Letourneur, 1994) direction. We can conclude, however, that the terminally glycosylated, labeled VSV-G protein molecules, whose release we are studying, are incorporated into buds and attached vesicles that are formed in the TGN during phase I. This is demonstrated by the fact that during a subsequent incubation of the Golgi membranes in the absence of GTP γ S or any other added nucleotide that would be required for de novo coat assembly, the labeled VSV-G protein was released within vesicles that had acquired a coat, since they sedimented more rapidly in a sucrose gradient than the uncoated ones that had formed in the presence of the hydrolyzable nucleotide. Moreover, the fact that when BFA was added after phase I was completed it did not block release of the labeled VSV-G protein also indicates that the assembly of the coats for the TGN-derived vesicles carrying VSV-G protein takes place during phase I.

Thin-section EM failed to reveal easily recognizable morphological differences between the buds and vesicles produced from the TGN region and those found throughout the different Golgi cisternae. This would be expected if the coats of vesicles involved in intra-Golgi transport and those of post-Golgi vesicles have similar protein compositions and use at least partially overlapping sets of coat subunits. A precedent for this possibility is the reported

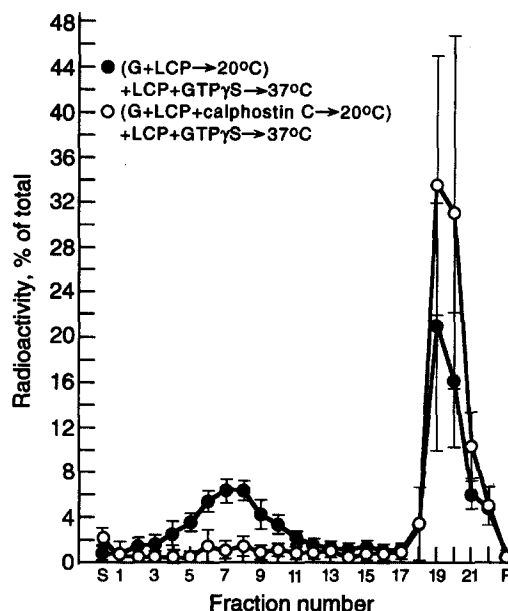


Figure 9. The target of the PKC inhibitor calphostin C is associated with Golgi membranes. Labeled Golgi fractions (G) were preincubated in the absence of GTP γ S for 60 min at 20°C with a liver cytosolic protein fraction (LCP) and 1 μ M calphostin C (○—○). The reaction mixtures were then chilled on ice, and the Golgi membranes were recovered by sedimentation through a sucrose step gradient. These membranes were then used in a standard vesicle release assay, i.e., incubated for 60 min at 37°C with (10 mg/ml) LCP and (1 mM) GTP γ S. Each point in the gradient profiles represents the average (\pm SD) from four independent experiments. Note that calphostin C acted on a membrane component, since its inhibitory effect could not be reversed by the addition of a fresh LCP fraction and GTP γ S.

existence of a variant of the COPI coat that lacks the δ and γ subunits and appears to play a role in transport from early to late endosomes (Whitney et al., 1995). On the other hand, COPI- and COPII-coated vesicles are morphologically very similar (Barlowe et al., 1994; Bednarek et al., 1995), and yet their coats have totally different protein compositions. A type of TGN-derived vesicle characterized by a “lace-like coat” morphologically distinguishable from that of COPI-coated vesicles found in the *cis* region of Golgi stacks has been recognized by tomographic analysis of high voltage electron microscope images (Ladinsky et al., 1994). With sections of intact cells, that study demonstrated the synchronous formation of multiple vesicles from a single TGN tubule, suggesting that a tubule may be consumed during vesicle formation, in accord with an earlier proposal (Rambourg et al., 1979). The electron micrographs we obtained with thin sections of isolated Golgi fractions, in which the process of vesicle formation was arrested before the scission step, either by using a low temperature of incubation (20°C) or by addition of the PKC inhibitor calphostin C, were also suggestive of the total consumption of TGN tubules by the synchronous generation of multiple buds and vesicles. As previously noted (Ladinsky et al., 1994), a process of this type is consistent with the notion that individual TGN tubules represent membrane domains into which proteins with different post-Golgi destinations have been already sorted.

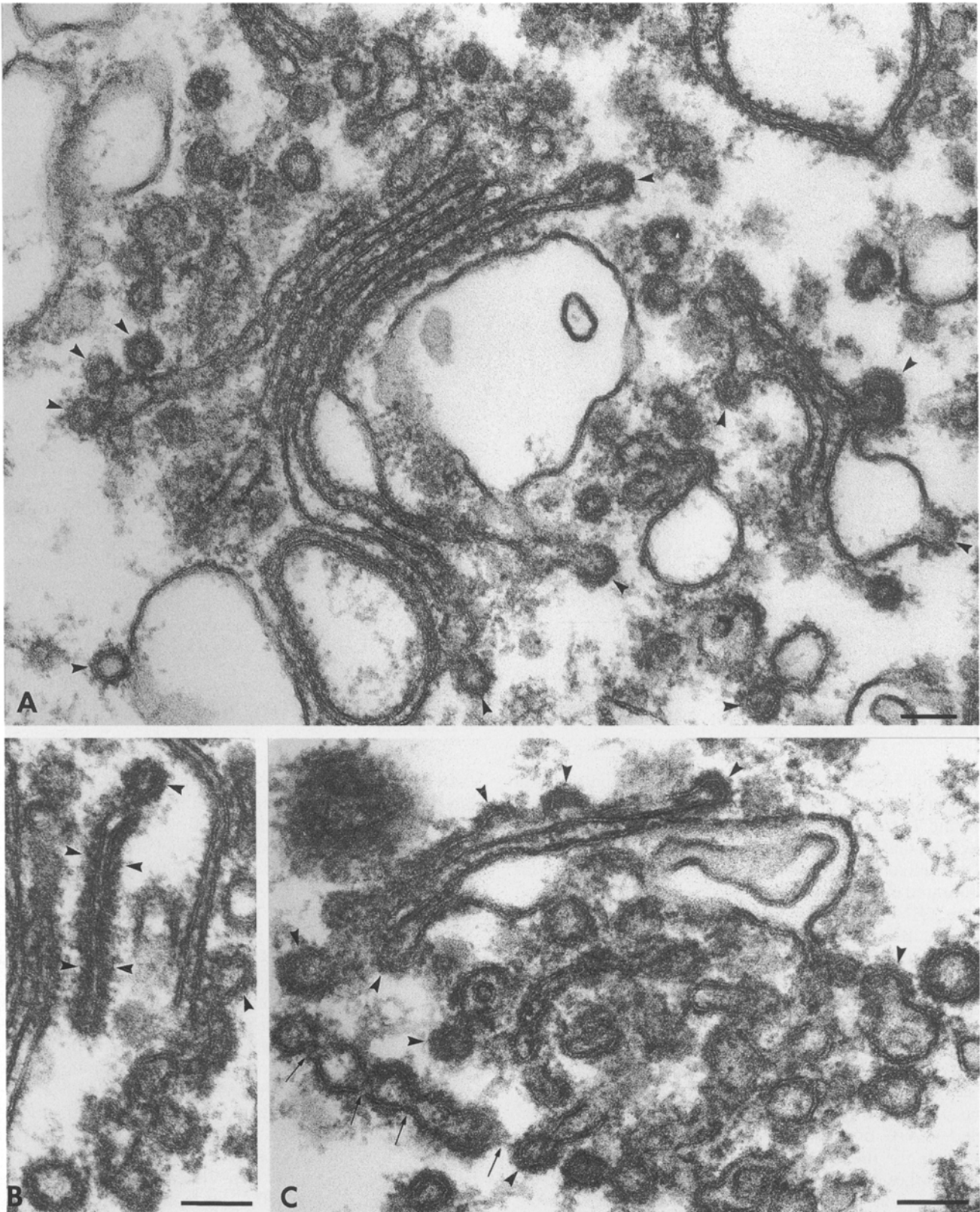


Figure 10. Calphostin C, which suppresses vesicle release, does not prevent the formation of coated buds on Golgi membranes that takes place during an incubation with GTP γ S and cytosolic proteins. A Golgi fraction was preincubated with calphostin C (1 μ M), apyrase, and liver cytosolic proteins for 30 min at 20°C. GTP γ S (1 mM) was then added and incubation continued for 60 min at 37°C, before recovery of the membranes and processing for EM. Bud formation was as abundant as observed after incubation at 20°C in the absence of the PKC inhibitors (see Fig. 5). Again, buds (arrowheads) originated from terminal cisterna, as well as from the rims and central regions of cisternae (A and C). Frequently, entire tubules became coated (B and C), (arrowheads) and gave rise to rows of budding vesicles (C) that remained connected to each other (arrows). Bars, 100 nm.

Although TGN-derived coated vesicles have not yet been purified, several proteins have been proposed as candidates for structural components of their coat. One of these is a complex of a 62-kD protein (p62) with several GTP-binding proteins (one of which is rab 6), which associates with the TGN integral membrane protein, TGN38/41 (Jones et al., 1993). Another is p200, a protein that was shown to bind reversibly and in a BFA-sensitive manner to Golgi membranes (Narula et al., 1992) and was localized by immuno-EM to the TGN and adjacent tubular and vesicular elements (Narula and Stow, 1995). It remains to be determined whether other protein coat subunits are associated with the p200-containing vesicles, although immuno-EM indicated that those vesicles do not carry β -COP (Narula and Stow, 1995).

Involvement of a PKC-like Molecule in Post-Golgi Vesicle Formation

We have previously shown that calphostin C, as well as a variety of other agents that specifically inhibit protein kinase C, inhibit post-Golgi vesicle formation in vitro and that the PKC activator TPA stimulates it (Simon et al., 1996). Using a PC12 membrane preparation, other authors (Xu et al., 1995) not only observed similar effects of PKC modulators on the production of post-Golgi vesicles containing the β -amyloid precursor protein, but they also obtained a stimulation by adding purified rat brain PKC. We now demonstrate that the modulators of PKC activity exert their effects on vesicle production equally well when endogenous ATP levels are reduced to concentrations much lower than the K_m of known PKC isozymes (Hannun and Bell, 1990; Kikkawa et al., 1982) and vesicle production is promoted only by GTP γ S. We had suspected that the phosphorylating activity of a protein kinase was not required for vesicle generation because this was not stimulated by the phosphatase inhibitors microcystin LR and okadaic acid (Simon et al., 1996). With the data presented here, we can firmly conclude that a PKC, but not its phosphorylating activity, is required for post-Golgi vesicle release, or that a protein that contains a diacylglycerol/phorbol ester-binding site similar to that in the regulatory domain of PKC, but is not a protein kinase, is also involved in this process. Several such proteins have been recently identified, including *N*-chimaerin (Ahmed et al., 1991, 1993) and the *Caenorhabditis elegans* Unc 13 protein (Ahmed et al., 1992). It is interesting that *N*-chimaerin is a GAP protein for the GTP-binding protein Rac1 and that its GAP activity is stimulated by TPA (Ahmed et al., 1993).

The capacity of a PKC to exert an effect independently of its kinase activity has been previously established for the activation of a membrane-associated PLD (Conricode et al., 1992; Singer et al., 1996), an enzyme that is highly enriched in Golgi membranes (Ktistakis et al., 1995). In that case, it was shown that in the presence of the phorbol ester, the regulatory domain of PKC α alone is sufficient to cause the stimulation of PLD. This suggests that a DAG/TPA-activated PKC interacts directly with PLD and activates it by an allosteric mechanism.

The participation of a phorbol ester-activated, calphostin C-sensitive protein without the requirement of a phosphorylating activity has previously been demonstrated for

ER-to-Golgi transport (Fabbri et al., 1994), where it is most likely necessary for the budding of ER-derived vesicles. The fact that protein seemed to act in a very early stage of budding, since kinetic studies in a permeabilized cell system (Fabbri et al., 1994) showed that ER-to-Golgi transport became resistant to calphostin C even before it became resistant to GTP γ S, which when incorporated into vesicles, prevents their uncoating and hence their subsequent fusion with acceptor membranes. Similarly, in our studies, calphostin C could inhibit vesicle formation only when added before the GTP γ S-promoted step of coat assembly. However, some of the properties of the phorbol ester-responsive protein involved in ER-to-Golgi transport differ significantly from those of the putative PKC that our work implicates in post-Golgi vesicle formation. Thus, the latter, although active when no phosphorylating activity could be manifested (i.e., in the absence of ATP), was nevertheless inhibited in its capacity to support vesicle generation, not only by calphostin C and *N,N*-dimethylsphingosine, agents that act at the DAG-binding regulatory site, but also by agents that act at or near the catalytic site; e.g., the mAb (clone 1.9) and the autoinhibitory pseudosubstrate peptide (Simon et al., 1996). Moreover, whereas the phorbol ester-sensitive protein required for ER-to-Golgi transport was a cytosolic component (Fabbri et al., 1994), we have shown that the putative PKC that participates in post-Golgi vesicle production is associated with Golgi membranes. The fact that agents that interact with PKC near or at its catalytic site inhibited post-Golgi vesicle production would suggest that they impede the direct interaction of PKC with its protein target.

In this regard, it should be noted that at least two different PKC isozymes have been found to be associated with the Golgi apparatus. A PKC ϵ was found to bind to Golgi membranes via its zinc finger domain, and overexpression of that domain in transfected NIH3T3 cells impaired the *trans*-Golgi-specific sulfation of glycosaminoglycans and their secretion (Lehel et al., 1995). A β II isozyme of PKC was also localized by immunocytochemistry to the Golgi apparatus of neurons within the cerebral cortex (Saito et al., 1989). We observed that Ca²⁺ chelation did not affect vesicle generation and, therefore, did not impair the function of the putative PKC. This would indicate that if the TPA-activated, calphostin C-sensitive molecule we detected is a PKC, then it must belong to the so called "novel" class of Ca²⁺-independent isozymes, such as PKC ϵ , and not to the conventional class, which includes PKC β (Nishizuka, 1992).

By What Mechanisms Could the Putative PKC Regulate Vesicle Production?

PKC has been found to play a role in the binding of ARF and β -COP to Golgi membranes (De Matteis et al., 1993), and considerable evidence indicates that heterotrimeric G proteins control transport through the Golgi via the regulation of the Golgi-associated guanine nucleotide dissociation stimulator, or exchange factor, that activates ARF (see Bomsel and Mostov, 1992). Based on the fact that PKC depletion or inhibition led to the failure of fluoroaluminate, a heterotrimeric G protein activator, to promote ARF and β -COP binding to Golgi membranes, it was pro-

posed that the action of a PKC is required to activate the heterotrimeric G protein that controls ARF function (De Matteis et al., 1993). In fact, it has been reported that some heterotrimeric G proteins are substrates for protein kinase C (Sagi-Eisenberg, 1989). The scheme proposed for PKC action in intra-Golgi vesicle formation (De Matteis et al., 1993), however, could not apply to the formation of post-Golgi vesicles, since we found that calphostin C did not prevent the ARF-dependent assembly of their coat, but only blocked scission of these vesicles from the donor membrane. Density shift analysis and EM showed that Golgi membranes pretreated with calphostin C and then incubated with cytosolic proteins and GTP γ S at 20°C acquired a protein coat and developed coated buds and attached vesicles to the same extent as untreated controls, but these membranes failed to release any post-Golgi vesicles in subsequent incubations with cytosol at 37°C.

Because calphostin C did not prevent coating, this inhibitor and other PKC modulators suppressed or enhanced vesicle production only when added before the priming phase of coat assembly was completed. The most straightforward interpretation of these observations is that the PKC-like target of the modulators is activated early in the process of vesicle formation, probably before coat assembly, but its effects are required only for the vesicle scission that takes place in the second phase. Alternatively, it is conceivable that once the coat has been assembled, the PKC-like protein becomes inaccessible to the modulating agents or acquires a conformation that does not permit their binding.

Another possible mechanism by which PKC or a PKC-like molecule could play a role in vesicular transport is through the activation of PLD, an enzyme that is enriched in Golgi membranes (Ktistakis et al., 1995) and is activated in cells treated with the phorbol ester (see Whatmore et al., [1993]) and references therein). PLD hydrolyzes phospholipids to generate phosphatidic acid (PA) and the free head groups, and it has been suggested that PA plays a role in the membrane remodeling that must take place during membrane fusion (Kahn et al., 1993; Liscovitch and Cantley, 1995). Partially purified PLD has been shown to be activated by three regulatory proteins that act synergistically: the GTP-binding proteins ARF (Brown et al., 1993; Siddiqi et al., 1995), Rho (Brown et al., 1993; Siddiqi et al., 1995; Singer et al., 1995), and PKC α (Singer et al., 1996). It has been convincingly demonstrated that PLD stimulation by PKC does not require the phosphorylating activity of the kinase (Conricode et al., 1992; Singer et al., 1996). Indeed, the regulatory domain of PKC α , when activated by TPA, was sufficient to cause stimulation of PLD (Singer et al., 1996). This kinase-independent TPA stimulation of PLD parallels our observations with the post-Golgi vesicle-generating system and suggests that the activation of PLD could play an important role in vesicle formation and, in particular, in the vesicle scission step. We speculate that the direct interaction of PKC with PLD can take place at 20°C, during the priming/coat assembly phase of vesicle generation, but that the phospholipase, whose activity is regulated by phospholipids (Liscovitch et al., 1994) and by ARF, can only manifest its activity at the higher temperature at which lipids are more mobile.

The discovery that ARF is also a stimulator of PLD (Brown et al., 1993) indicated that this GTP-binding protein may have a dual role in vesicular transport, and it was speculated that in addition to promoting coat assembly, after vesicle docking, it served to activate PLD in the acceptor membrane (Liscovitch and Cantley, 1995). The resulting PA would stimulate a PtdIns(4)P 5-kinase (Jenkins et al., 1994) to generate PtdIns(4,5)P₂, a key lipid activator of PLD (Liscovitch et al., 1994). This positive feedback in PLD activation, leading to a high local production of PA and PIP₂, was proposed to create membrane microdomains enriched in acidic phospholipids that could promote membrane fusion. One could equally envisage that activation of PLD at the budding site in the TGN resulting from the synergistic action of ARF, PKC, and a cytosolic factor, possibly RhoA, results in a similar cascade of events, creating a high local concentration of negatively charged phospholipids. The ensuing remodeling of the cytoplasmic leaflet of the membrane would, in turn, alter the luminal face of the bilayer leading to membrane fusion and its consequent vesicle scission.

What could be the source of the DAG required to activate the PKC-like protein that is needed for vesicle production? One possibility is that it results from the activation of a phospholipase C-linked receptor that could be located either at the plasma membrane, in which case it would respond to an extracellular ligand, or in intracellular membranes (e.g., the TGN), where it would be expected to bind a luminal ligand, possibly a protein needing to be transported. In addition, DAG could be produced locally by the phosphorylation of phosphatidic acid generated by PLD, which would involve another positive feedback loop operating during vesicle generation.

Further biochemical studies with the *in vitro* system used in this work should allow the identification and characterization of the cytosolic proteins that are required for the assembly of the coat of post-Golgi vesicles and for the subsequent vesicle scission. It remains to be established experimentally whether PLD plays a critical role in post-Golgi vesicle formation and, in particular, in the vesicle scission step, and whether it is the target of a Golgi-associated PKC-like molecule.

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