Fragmentation and Re-assembly of the Golgi Apparatus in Vitro

A REQUIREMENT FOR PHOSPHATIDIC ACID AND PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE SYNTHESIS

Received for publication, May 22, 2001, and in revised form, November 7, 2001
Published, JBC Papers in Press, November 9, 2001, DOI 10.1074/jbc.M104639200

David A. Sweeney‡, Anirban Siddhanta¶, and Dennis Shields‡¶

From the ‡Department of Developmental and Molecular Biology and the ¶Department of Anatomy and Structural Biology Albert Einstein College of Medicine, Bronx, New York 10461

Recent work from our laboratory demonstrated that phosphatidic acid (PA) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$), are required to maintain the structural integrity of the Golgi apparatus. To investigate the role of these lipids in regulating Golgi structure and function, we developed a novel assay to follow the release of post-Golgi vesicles. Isolated rat liver Golgi membranes were incubated with [3H]CMP sialic acid to the release of post-Golgi vesicles. Isolated rat liver Golgi ture and function, we developed a novel assay to follow the role of these lipids in regulating Golgi struc-

Intracellular protein transport through the secretory pathway is mediated by membrane-bound carrier vesicles (1). In the past decade significant progress has been made in characterizing different classes of coat proteins, which function at various steps of the pathway (2). Transport vesicles interact with at least two distinct types of protein complex; those involved in vesicle formation and budding from donor organelles, e.g. coatamer; COP-I and -II; and those facilitating vesicle target-

ing to and fusion with the correct acceptor membranes (SNAREs)$^3$ (3). Several ancillary proteins, including members of the Rab family of small GTP-binding proteins interact with specific vesicles to regulate steps in the transfer process: targeting, docking, and fusion (4). Furthermore, recent theories have suggested that the Rab-related proteins in conjunction with SNAREs may play a key role in regulating the specificity of vesicle interactions (5). In addition to the Rabs, another family of GTP-binding proteins, ARFs (ADP-ribosylation factors) recruit COP-I and -II coat complexes as well as clathrin adaptors (6, 7) and a novel class of clathrin-binding proteins to the cytoplasmic face of the trans-Golgi network (8–11).

Considerable evidence has demonstrated that, in addition to the aforementioned components, inositol phospholipids play a key role in controlling vesicle transport from the Golgi apparatus and in regulating various steps in endocytosis in mammalian and yeast cells (12). Several laboratories have shown that ARFs may regulate vesicle trafficking, in part by effecting changes in phospholipid metabolism (6). Recently, it was demonstrated that ARF1 and -6 recruit phosphatidylinositol 4-kinase β to Golgi membranes and Type-Ia phosphatidylinositol-4-phosphate 5-kinase to the plasma membrane, respectively (13, 55). It is likely that different phosphatidylinositol kinase isoforms function in regulating the level of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) in the Golgi apparatus and plasma membrane as well as in other membranes. An initial link between phospholipid metabolism and ARFs came from earlier observations from several laboratories (6). It was shown that ARF1 activates a phospholipase D activity (14, 15), which in some cells associates with membranes of the Golgi apparatus (16, 17). Phospholipase D hydrolyzes phosphatidylincholine to generate phosphatidic acid (PA), which stimulates the synthesis of PtdIns(4,5)P$_2$ as well as promoting COP-I binding to membranes (6) and release of nascent secretory vesicles from the TGN (18). Additionally, our data demonstrated that PA stimulation of PtdIns(4,5)P$_2$ synthesis is required for maintaining the structural integrity and function of the Golgi apparatus (19). Treatment of growth hormone-secreting rat pituitary GH3 cells with 1-BtOH led to the synthesis of phosphatidyl butanol rather than PA as a result of the transphosphatidylation activity of PLD. Under these conditions hormone secretion was inhibited, in part, due to diminished PtdIns(4,5)P$_2$ synthesis, which resulted in quantitative fragmentation of the Golgi ap-
paratus. Upon alcohol removal, the Golgi apparatus structure was restored and hormone secretion resumed. In contrast to these results, other investigators have suggested that ARF-activated PLD does not function in mediating PtdIns(4,5)P₂ biosynthesis in the Golgi apparatus or in COP-I recruitment (13, 20, 21).

To understand further the mechanism by which polyphosphoinositide synthesis functions in regulating the structural organization of the Golgi apparatus, we have now utilized a novel assay system to follow the release of radiolabeled endogenous glycoproteins into post-Golgi vesicles in vitro. To this end, we exploited previous observations (22, 23) that specific nucleotide sugar transporters and sialyl transferases, which localize to late Golgi cisternae and the TGN, can incorporate radiolabeled nucleotide sugars into endogenous cargo molecules efficiently (22, 24). Our rationale was to use purified rat liver Golgi membranes labeled with radioactive sialic acid to follow the release of soluble and membrane glycoproteins from the TGN into post-Golgi vesicles in response to changes in PA and PtdIns(4,5)P₂ synthesis. In agreement with our earlier findings, vesicle budding was stimulated by exogenously added bacterial PLD and addition of 1-butanol resulted in complete fragmentation of Golgi cisternae (18, 19). Strikingly, following alcohol washout, PA and PtdIns(4,5)P₂ synthesis resumed, and the fragmented Golgi reformed into its characteristic flattened cisternae in vitro. Most significantly, the re-assembled Golgi apparatus was able to support nascent vesicle release. Our results demonstrate that inositol phospholipid synthesis is essential for maintaining the structure and function of the Golgi apparatus.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The following antibodies were used: monoclonal antibody 1D9 against ARF1 was provided by Dr. R. Kahn. A rabbit antibody to PLD1, designated P1–P4, was generated using four peptides specific to human PLD1 and has been described previously (17, 25). A monoclonal antibody to TGN38 was purchased from Affinity Bioreagents, Golden, CO; monoclonal anti-Rab5 was obtained from Transduction Laboratories, Lexington, NY. Rabbit antibodies were as follows: to calnexin were from Santa Cruz Biotechnology in Cruz, CA; to α₁-antitrypsin (from Zymed Laboratories Inc., San Francisco, CA); and to transferrin (from Cappel, Aurora, OH). Rabbit anti-connexin43 was a gift of Dr. Elliot Hertzberg, Albert Einstein College of Medicine; rabbit anti-Tom20 was a gift from Dr. Gordon Shore, McGill University. Bacterial PLD (Streptomyces chromofuscus) anti-Tom20 was a gift from Dr. Gordon Shore, McGill University. Bacterial PLD (Streptomyces chromofuscus) anti-Tom20 was a gift from Dr. Gordon Shore, McGill University. Bac-to transferrin (from Cappel, Aurora, OH). Rabbit anti-connexin43 was a gift of Dr. Elliot Hertzberg, Albert Einstein College of Medicine; rabbit anti-Tom20 was a gift from Dr. Gordon Shore, McGill University.

**Preparation of Rat Liver Golgi Membranes**—Rat liver Golgi membranes were isolated using a modification of the procedure of Slusarek et al. (26). All steps were performed at 4°C; four to six livors from rats that had been fasted overnight were homogenized using a loose-fitting Potter homogenizer in 3 volumes of 0.5M sucrose containing 50 mM phosphate buffer, pH 6.8, 2.5 mM MgCl₂ and a mixture of protease inhibitors. A post-nuclear supernatant was obtained by centrifugation at 1000 × g for 5 min; this fraction was diluted to 0.25 mM sucrose and transferred to Beckman SW28 Ultracentrifuge tubes. 12 ml of the supernatant was underlayered with 7, 9, and 7 ml of 0.5, 0.8, and 1.3 mM sucrose, respectively, in the same buffer and centrifuged at 28,000 rpm (100,000 × g, max) for 90 min. Material floating at the 0.5/0.8/1.3 mM sucrose interface, which was enriched in Golgi membranes (Fig. 1), was diluted to 0.25 mM sucrose and centrifuged at 7000 rpm in the SW28 rotor for 30 min onto a 100-μl cushion of 1.3 mM sucrose. This step was repeated, and the membrane pellet was resuspended in 2–3 ml of 0.25 mM sucrose-containing buffer. Isolated Golgi membranes were stored at ~80°C until use.

**Preparation of Rat Brain Cytosol**—All steps were preformed at 4°C. Four rat brains were homogenized using a loose-fitting Potter homogenizer in 3 volumes of 0.25% sucrose containing 20 mM Tris-HCl, pH 7.4, 50 mM KCl, 2 mM MgCl₂, and a mixture of protease inhibitors. A post-nuclear supernatant was obtained by centrifugation at 10000 × g for 10 min followed by centrifugation of the supernatant at 100,000 × g for 3 h. The high speed supernatant was dialyzed overnight against 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM MgCl₂, and a mixture of protease inhibitors and concentrated using a Centricon-10 concentra-

**Determination of Sugar Transferase Activities**—Golgi membranes and samples from the in vitro membrane budding and vesiculation reactions were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and post-fixed with 1% osmium tetroxide followed by 1% uranyl acetate. The samples were then dehydrated through a series of graded ethanol concentrations and embedded in LX112 resin (LADD Research Industries, Burlington, VT).
Ultra thin sections were cut on a Reichert Ultracut E ultramicrotome, stained with uranyl acetate followed by lead citrate, and viewed on a JEOL 1200EX transmission electron microscope at 80 kV.

**RESULTS**

**Characterization of Isolated Golgi Membranes—**Purified Golgi membranes were isolated from rat liver according to methods used previously (26). Following centrifugation, the gradient was fractionated, aliquots of alternate fractions were analyzed by SDS-PAGE and Western blotting using antibodies to Golgi, ER, mitochondria, endosomal, and plasma membrane marker proteins (Fig. 1A). TGN38 a trans-Golgi marker, co-fractionated on the gradient with an opaque band of material at the interface of 0.5 and 0.86 M sucrose (fractions 23-30) and plasma membrane (fractions 35-39; Fig. 1A). Fractions 17 and 18 were enriched in both ARF1 and PLD1, although most ARF1 was recovered in the cytosol. Surprisingly, little PLD2 immunoreactivity was detected in fractions corresponding to the load zone (fractions 1-12). Our recent work demonstrated that in several different cell types PLD1 was detected in the perinuclear Golgi region and bound to Golgi membranes (17). Consequently, it was of interest to identify which fractions contained this enzyme: Most of the PLD1-immunoreactive material was present in two regions of the gradient; those corresponding to the plasma membrane (31). In contrast to PLD1, most of the PLD2-immunoreactive material was present in the endosomal, Golgi, and lighter membrane fractions. Material collected from fractions 24-29, corresponding to Golgi membranes, was pooled and used for subsequent experiments.

**Vesicle Budding from Isolated Golgi Membranes—**Our initial goal was to radiolabel endogenous glycoproteins in the late
Golgi/TGN and follow their release into post-Golgi vesicles in vitro. Isolated Golgi membranes were incubated with [3H]CMP sialic acid, and its incorporation into N-linked glycoproteins was determined (Fig. 2). Consistent with earlier observations (22) the translocation of [3H]CMP sialic acid across Golgi membranes and its incorporation into endogenous glycoprotein acceptors was cytosol- and energy-independent (Fig. 2A). Measurement of the trichloroacetic acid precipitable radioactivity across the sucrose gradient ([3H]sialic acid-labeled or [3H]-galactosylated polypeptides; Fig. 1A) revealed only background radioactivity in fractions corresponding to the cytosol (fractions 1–12) demonstrating incorporation was dependent on the presence of Golgi membranes. Analysis of the total radiolabeled material by SDS-PAGE revealed a heterogeneous distribution of polypeptides ranging in size from ~200 to 40 kDa (Fig. 2B, lanes 7 and 8). To determine the efficiency of vesicle budding from the TGN, we measured the release of [3H]sialic acid-labeled cargo molecules into a medium speed supernatant fraction (Fig. 2, B and C). Samples were incubated in the presence and absence of an energy regeneration system (EGS) with and without 2 mg/ml rat brain cytosol as indicated. Following incubation, samples were centrifuged at 12,000 × g for 1 min and the supernatant (S) and pellet (P) were analyzed by SDS-PAGE. C, quantitation of vesicle release from B; % vesicle release = S/(S + P) × 100. Data are the average of two separate experiments.

**Fig. 2.** Incorporation of [3H]sialic into endogenous Golgi glycoproteins. A, 10 μg of isolated Golgi membranes was incubated with 1 μM [3H]CMP sialic acid at 20 °C (■) or 37 °C (▲) and in the presence (△) or absence (▲) of Mn2+ for the indicated times or in the presence of 1% 1-BtOH (●). Aliquots were removed and spotted onto Whatman 3MM discs, and the radioactivity was determined by liquid scintillation counting. B, isolated Golgi membranes were incubated at 20 °C or 37 °C for 45 min in the presence of an energy generating system (EGS) with and without 2 mg/ml rat brain cytosol as indicated. Following incubation, samples were centrifuged at 12,000 × g for 1 min and the supernatant (S) and pellet (P) were analyzed by SDS-PAGE. C, quantitation of vesicle release from B; % vesicle release = S/(S + P) × 100. Data are the average of two separate experiments.

**Fig. 3.** Identification of sialylated Golgi glycoproteins. A, Golgi membranes were incubated with [3H]CMP sialic acid under vesicle budding conditions. One-tenth of the sample was prepared for SDS-PAGE directly (lanes 1–4). The remainder of the samples was treated sequentially (see “Experimental Procedures”) with rabbit anti-α1-antitrypsin (lanes 5, 6), rabbit anti-TGN38 (lanes 7, 8), and rabbit anti-TfR (lanes 9) and the immunoprecipitates analyzed by SDS-PAGE. B, time course of vesicle budding: [3H]CMP sialic acid-labeled Golgi membranes were incubated under budding conditions for the indicated times. Following incubation, samples were centrifuged briefly, and the pellets and supernatants were incubated with rabbit antibodies to transferrin or TGN38 (‘Experimental Procedures’), and the immunoprecipitates were resolved SDS-PAGE. C, quantitation of vesicle release: the intensity of bands corresponding to transferrin (■), TGN38 (▲) and total trichloroacetic acid-precipitable radiolabeled protein (●) was quantitated by densitometry. The ordinate corresponds to the percentage of the total [3H]-labeled material released into the supernatant fraction; % vesicle release = 100 × 100. Data are the average of two separate experiments.

Identification of [3H]sialic Acid-labeled Polypeptides—Hepatocytes synthesize numerous specific proteins. We therefore used antibodies to two such soluble secreted glycoproteins, α1-antitrypsin and transferrin (Fig. 3A, lanes 5, 6, 9, 10; M, 51,000 and 78,000, respectively) as well as to the Golgi integral membrane protein TGN38 (~90 kDa) to identify these molecules by immunoprecipitation following vesicle release from the TGN (Fig. 3, A and B). All three polypeptides were evident, although their relative distribution in the vesicle (S) and residual Golgi (P) fractions was significantly different. In particular, the release of transferrin-containing vesicles was higher (~20%) than that of TGN38 (~14%; Fig. 3, B and C) and more efficient than release of the total acid-precipitable radioactivity, suggesting selective packaging of cargo molecules in vitro. Whether this represents formation of separate vesicle populations containing different cargo molecules or the differential “filling” of transferrin and TGN38 into the same vesicle remains to be determined. Additionally, two major transferrin-
immunoreactive bands were evident in the residual Golgi (P) fraction (Figs. 3A, lane 10, and 3B) whereas a faster migrating species was released into the vesicle fraction (S) (Fig. 3A, lanes 9 and 10). The latter transferrin molecule may correspond to post-Golgi processing of transferrin in the released vesicles.

If vesicles were released from the Golgi membranes, then cargo molecules should be resistant to exogenously added proteases. Conversely, if the cargo molecules had leaked into the incubation medium from ruptured membranes or were present as a result of nonspecific effects on membranes, the radiolabeled polypeptides would be protease-sensitive. To distinguish between these possibilities, supernatant and pellet fractions were digested with proteinase K in the absence and presence of detergent (Fig. 4). In the absence of detergent, virtually all the polypeptides were protease resistant (Fig. 4, A). These data further demonstrate that release of vesicles from isolated Golgi membranes did not result from leakage of endogenous cargo molecules from the organelle (see below).

By exploiting the transphosphatidylation activity of PLD, we showed previously that primary but not secondary or tertiary alcohols inhibited vesicle budding from the Golgi apparatus due to lack of PA synthesis (18, 19). To determine if treatment of isolated Golgi membranes with alcohol might inhibit Golgi function, isolated membranes were incubated with [3H]sialic acid in the presence of 1-BtOH (Fig. 2A, diamond). Under these conditions 1-BtOH had no effect on [3H]sialic acid incorpora-

**Fig. 4. Vesicle budding is stimulated by bacterial PLD.** A, protease protection and stimulation by PLD activity: [3H]CMP sialic acid-labeled Golgi membranes were incubated under vesicle budding conditions in the presence of energy and cytosol. Following incubation at 37 °C for 45 min, samples were incubated with 50 μg/ml proteinase K (PK) at 4 °C for 30 min (lanes 3–6, 15, 16) in the absence or presence of 1% Triton X-100 (TX) (lanes 5, 6, 9–12). Following incubation, samples were either centrifuged at 12,000 × g for 1 min or at 100,000 × g for 5 min as indicated. Lanes 13–16, radiolabeled Golgi membranes incubated under vesicle budding conditions with 5 units of bacterial PLD/ml. Lanes 17–20, radiolabeled Golgi membranes incubated under vesicle budding conditions in the presence of 1% 1-BtOH (lanes 17, 18) or 1% t-BtOH (lanes 19, 20). B, budding of TGN38-containing vesicles. [3H]CMP sialic acid-labeled Golgi membranes were incubated under vesicle budding conditions (“Experimental Procedures”) and centrifuged at 12,000 × g for 1 min. The pellets and supernatants were digested with proteinase K after which the protease was inactivated with 1 mM phenylmethylsulfonyl fluoride; the samples were then treated with 1% Triton X-100 and incubated with rabbit anti-TGN38 antiserum (“Experimental Procedures”). The immunoprecipitable material was analyzed by SDS-PAGE followed by fluorography.
Fig. 5. Morphology of released Golgi vesicles. Golgi membranes were incubated under vesicle budding conditions in the presence of cytosol and energy; the samples in B were also incubated with 1% t-BtOH. Following incubation, samples were either centrifuged at 100,000 g for 5 min or at 12,000 g for 1 min. The high speed pellets (A and B) and the 12,000 g pellet (C) and supernatant (D) material were prepared for electron microscopy. E, quantitation of membrane profiles: Data are derived from micrographs similar to those shown in Figs. 1, 5, and 6. The levels of total membrane profiles per treatment; two separate fields were counted in each micrograph. Error bars represent the standard deviation. The data are derived from micrographs similar to those shown in Figs. 1C, 5A, 6B, and 6C. Gray bar, stacked Golgi (>2 cisternae); white bar, individual cisternae; black bar, vesicles. A–C: arrows, Golgi cisternae; arrowheads, multivesicular bodies (MVBs). Insets (B and D) show material at higher magnification.

Fragmentation and Re-assembly of the Golgi apparatus in Vitro

The data are derived from micrographs similar to those shown in Figs. 1C, 5A, 6B, and 6C. Gray bar, stacked Golgi (>2 cisternae); white bar, individual cisternae; black bar, vesicles. A–C: arrows, Golgi cisternae; arrowheads, multivesicular bodies (MVBs).Insets (B and D) show material at higher magnification.

Fig. 5. Morphology of released Golgi vesicles. Golgi membranes were incubated under vesicle budding conditions in the presence of cytosol and energy; the samples in B were also incubated with 1% t-BtOH. Following incubation, samples were either centrifuged at 100,000 × g for 5 min or at 12,000 × g for 1 min. The high speed pellets (A and B) and the 12,000 × g pellet (C) and supernatant (D) material were prepared for electron microscopy. E, quantitation of membrane profiles: Data are derived from micrographs similar to those in Figs. 5 and 6. The levels of total membranes present in Golgi stacks, individual cisternae, and vesicles were quantified according to Misteli (Ref. 28; “Experimental Procedures”). Data are derived from 20–30 separate micrographs of each treatment; two separate fields were counted in each micrograph. Error bars represent the standard deviation. The data are derived from micrographs similar to those shown in Figs. 1C, 5A, 6B, and 6C. Gray bar, stacked Golgi (>2 cisternae); white bar, individual cisternae; black bar, vesicles. A–C: arrows, Golgi cisternae; arrowheads, multivesicular bodies (MVBs). Insets (B and D) show material at higher magnification.

Morphology of Golgi Membranes—To characterize the Golgi elements during in vitro incubation, we compared the morphology of the Golgi membranes and post-Golgi vesicles following vesicle budding (Fig. 5, A, C, and D). Golgi membranes were incubated under vesicle budding conditions and separated into supernatant and pellet fractions that were prepared for transmission electron microscopy. The starting material (see Fig. 1C) contained numerous Golgi stacks with 2 or 3 characteristic flattened cisternae (arrows) as well as cisternal elements and multivesicular bodies (MVBs; arrowheads, Figs. 1C, 5A, and 5C). Following incubation under budding conditions total membranes were sedimented at 100,000 × g, and their morphology was examined (Fig. 5A). Under these conditions abundant individual Golgi cisternae and MVBs were evident, although there were correspondingly fewer Golgi stacks and cisternae present than in the starting material (compare Fig. 1C with Fig. 5, A, C, and D). Furthermore, upon medium speed centrifugation (12,000 × g for 1 min) the residual Golgi cisternae and MVBs were present predominantly in the pellet fraction (Fig. 5C), consistent with the data from the budding assay (Figs. 2 and 3). In contrast, the supernatant fraction contained mostly a heterogeneous population of vesicles ranging in diameter from ~50 to ~350 nm (Fig. 5D). Although individual Golgi cisternae were present following incubationunder vesicle budding conditions (Fig. 5, A and C), no further changes in morphology were seen with longer incubation times (data not shown). Together with the vesicle budding results, our morphological data further demonstrated that release of material into the supernatant fraction was not a consequence of nonspecific fragmentation of the Golgi apparatus.
Recent data from our laboratory using rat pituitary GH3 cells demonstrated that, in the absence of PA synthesis, induced by treatment with primary alcohols, the Golgi apparatus became fragmented in vivo (19). To determine if this also occurred in the isolated organelle, control and 1-BtOH-treated samples were analyzed using electron microscopy (cf. Figs. 5A and 6B). Our previous data showed that 1% t-BtOH did not affect Golgi structure or secretion in whole cells; however, to control for possible nonspecific effects of the alcohol, we examined the effect of t-BtOH on the morphology of the isolated membranes (Fig. 5B). In contrast to control post-Golgi vesicles or those treated with t-BtOH in which some cisternae were still present, samples incubated with 1-BtOH consisted of a relatively uniform population of small vesicles (~50- to 100-nm diameter); most significantly, no Golgi cisternae were evident (compare Fig. 5, A and B, with Fig. 6B). The absence of individual cisternae suggested complete fragmentation of the Golgi apparatus (Fig. 6B).

Our previous data (19) showed that, in vivo, wash out of 1-BtOH led to reformation of the Golgi apparatus and restoration of secretion. To determine if alcohol-induced fragmentation of the Golgi apparatus could be reversed in vitro, 1-BtOH-treated Golgi membranes were isolated by centrifugation and resuspended in fresh buffer lacking 1-BtOH (Fig. 6A); this was repeated to remove traces of the alcohol. The washed vesicles were incubated with cytosol and energy after which the samples were prepared for electron microscopy (Fig. 6C). Numerous structures containing one or two putative elongated cisternae were evident, suggesting partial re-assembly of the Golgi apparatus, although fewer bone fide Golgi stacks were present than in the starting material (Fig. 6C, inset). Little, if any Golgi re-assembly was observed in the absence of both ATP and GTP or cytosol.

**Morphometry of Golgi Membranes**—To quantitate the relative distribution of Golgi stacks, individual cisternae, and vesicles, the method of Misteli was used (28). Analysis of the starting Golgi material showed that it consisted of predominantly stacked cisternae (70% of the total membrane) with only ~20% of the membrane present as vesicles (Fig. 5E, “Start”). Following incubation under budding conditions the amount of total membranes present in vesicles increased ~3-fold (~17% to 50%) compared with the starting Golgi material, and there was a concomitant decrease in cisternae to ~35% of total (Fig. 5E, “Budding”). These data were consistent with the aforementioned protease protection experiments (Fig. 4) and suggested that nascent vesicles were released into the supernatant fraction. As evident from the electron micrographs, after 1-BtOH treatment only membrane vesicles were present; no cisternae or stacks were detected. Indeed, quantitation demonstrated virtually complete vesiculation of the Golgi apparatus (Fig. 5E, “1-butanol”). Most significantly, upon removal of the alcohol, the amount of membrane present in vesicles decreased to ~50% of the total membrane population and ~45% of the membranes were now in Golgi cisternae and stacks (Fig. 5E, “Washout”).

**Phosphoinositide Synthesis on Golgi Membranes**—Our previous work also showed that the fragmentation of the Golgi apparatus correlated with decreased PtdIns(4,5)P₂ synthesis (19). We expected that Golgi membrane vesiculation would be a consequence of diminished PtdIns(4,5)P₂ synthesis. To test this idea, Golgi membranes were incubated with and without cytosol (as a source of phosphatidylinositol-4-phosphate 5-kinases), [γ-³²P]ATP, in the absence or presence of either 1- or t-BtOH and the radiolabeled inositol phospholipids analyzed (Fig. 7). Inclusion of cytosol resulted in robust PtdIns(4,5)P₂ and PA synthesis (Fig. 7A, lane 2), whereas when 1-BtOH was...
present, the synthesis of both lipids, particularly PtdIns(4,5)P2, was inhibited dramatically (Fig. 7A, lane 4). As expected, t-BtOH had no effect on the synthesis of either lipid and their levels were indistinguishable from controls (lanes 5, 6). These results further demonstrate a strong correlation between the synthesis of PtdIns(4,5)P2 and the structural integrity of the Golgi apparatus.

We argued that, if t-BtOH treatment led to fragmentation of the Golgi apparatus via diminished PtdIns(4,5)P2 synthesis, then alcohol removal (washout) should allow re-synthesis of this lipid. Following alcohol washout (Fig. 6A) the 1-BtOH-derived vesicles were incubated in the presence of cytosol, ATP, and GTP and analyzed: (i) using TLC to assay the synthesis of PtdIns(4,5)P2 (Fig. 7B) or (ii) for their ability to support vesicle release (Fig. 8). Significantly, following removal of the alcohol, PtdIns(4,5)P2 synthesis was restored (Fig. 7B, lanes 4 and 5). This result, which was consistent with our earlier observations (19), demonstrated a strong correlation between the synthesis of PtdIns(4,5)P2, and maintenance of Golgi structure.

Restoration of Vesicle Budding—If Golgi cisternae had reassembled following removal of the alcohol, then, upon incubation under vesicle budding conditions, radiolabeled cargo molecules should be released into the supernatant fraction in an energy- and cytosol-dependent reaction. To test this prediction, Golgi membranes radiolabeled with [3H]sialic acid were treated with 1-BtOH, the alcohol was removed, and the residual membranes were incubated under vesicle budding conditions (Fig. 8). As above (Fig. 2), addition of 1-BtOH resulted in elevated levels of cargo molecules released into the 12,000 × g supernatant fraction, presumably resulting from fragmentation of the organelle (Fig. 8A, lane 3, 4). Similar to control Golgi membranes, upon removal of the alcohol little material was released into the supernatant in the absence of either cytosol and/or energy (lanes 5–10). Strikingly, in the presence of cytosol and energy vesicle budding was restored albeit at ~50% of the efficiency of control untreated Golgi membranes (Fig. 8A; lanes 11, 12; cf. Fig. 2). Together, these results (Figs. 6–8) suggest that upon alcohol washout functional Golgi cisternae reformed that were able to support vesicle release.

**DISCUSSION**

Numerous factors cause disruption of the Golgi apparatus; these include pathological conditions, overexpression of Golgi-associated proteins, and pharmacological agents (32). In several neurological diseases, including amyotrophic lateral sclerosis, the Golgi apparatus in spinal cord neurons is fragmented (33) and resembles that of cells treated with nocodazole, which disrupts microtubule organization. During mitosis the Golgi stacks fragment into vesicle clusters and tubules, which partition into daughter cells, and several factors mediating this process have been characterized in detail previously (34–37).
Treatment of cells with the sponge metabolite ilumiquinone induces a similar Golgi phenotype to that observed during mitosis (38–40). More recently, it has been shown that, during apoptosis, the Golgi apparatus fragments into vesicles that are reminiscent of those present during mitotic breakdown (54); in this case fragmentation, which is irreversible, involves the action of several caspases that cleave a Golgi-associated matrix protein (41).

Treatment of cells with the fungal metabolite brefeldin A (BFA) causes collapse of the Golgi apparatus into tubulo-vesicular clusters that fuse with the ER (42). BFA prevents GTP-GDP exchange on several high molecular weight ARF guanine nucleotide exchange factors (GEF) (7). By maintaining ARF in a GDP-bound state in a complex with its GEF, GTP exchange is inhibited resulting in coat (COP-I) dissociation from the membrane and collapse of the Golgi structure (43). In this context, overexpression of a GDP mutant form of ARF1 (44) resulted in a BFA-like phenotype with respect to Golgi morphology. Similarly, overexpression of the low molecular weight ARF GEFs, ARNO-1 or -3 (7), which are relatively BFA-insensitive, also caused fragmentation of the Golgi apparatus and redistribution of molecules to the ER (45). Because ARF1 has been shown to regulate the activity and/or recruitment of several Golgi-associated enzymes involved in phospholipid metabolism (6, 13), it is possible that the above-mentioned effects resulted from changes in Golgi phospholipid metabolism. In this context, overexpression of a dominant negative mutant form of phosphatidylinositol-4-phosphate kinase β, whose Golgi association appears to be ARF-dependent, caused fragmentation of the Golgi apparatus (13). Treatment of cells with inhibitors of phospholipase A2 or overexpression of the enzyme led to marked disruption of the Golgi architecture further suggesting a role for phospholipid-modifying enzymes in maintaining Golgi structure (46, 47).

In this present study, we used isolated rat liver Golgi membranes and a novel vesicle budding assay to further understand the fragmentation and reassembly of the Golgi apparatus in response to changes in phosphoinositide synthesis. By utilizing the Golgi sialyl transferase activity to radiolabel endogenous glycoprotein cargo molecules, we demonstrated that release of post-Golgi vesicles was dependent on energy, cytosol, temperature, and time (Figs. 2 and 3). The release of radiolabeled cargo did not result from leakage of proteins from the Golgi membranes, because the molecules were resistant to exogenously added proteases and released into a high speed supernatant fraction only upon solubilization of the membranes with detergent suggesting their presence in sealed vesicles (Fig. 4). Furthermore, release of cargo into nascent vesicles was selective as evinced by the differential kinetics of transferrin and TGN38 release (Fig. 3).

Phospholipase D and Post-Golgi Vesicle Release—The data presented here confirm earlier studies from our and other laboratories, which showed that a pool of PLD1 is associated with Golgi membranes (16, 17). In this present study, addition of bacterial PLD enhanced the selective release of glycoprotein cargo molecules into post-Golgi vesicles (Fig. 4). This observation was consistent with our previous studies in which plant PLD added to permeabilized cells stimulated the budding of nascent secretory granules from the TGN (18). It is noteworthy that the pattern of 3H-labeled sialic acid-containing proteins released into the nascent vesicle fraction was different from that found upon fragmentation of the Golgi in response to alcohol treatment (Fig. 4) further demonstrating the specificity of vesicle release. At present the mechanism whereby PLD enhances the packaging of specific hepatocyte molecules into post-Golgi vesicles is unknown.

Data from our laboratory demonstrated that the PLD activity associated with Golgi membranes regulates PtdIns(4,5)P2 in the Golgi apparatus (19). In part, PtdIns(4,5)P2 functions to release nascent secretory vesicles and in maintaining the structure of the Golgi apparatus, although the mechanism of how this occurs remains to be determined. Protein kinase C regulates PLD activity and stimulates nascent vesicle budding (48). In this context, incubation of isolated Golgi membranes with calphostin C, an inhibitor of protein kinase C, (49) and PLD (50) also led to fragmentation of the Golgi apparatus similar to that observed following butanol treatment. Most significantly, the calphostin C-induced Golgi fragmentation occurred in the absence of its effect on protein kinase C, because other inhibitors of the enzyme had no effect on Golgi morphology (49). We speculate that Golgi fragmentation in response to calphostin C occurred via its inhibition of PLD activity.

PtdIns(4,5)P2 and Formation of Golgi Cisternae—In contrast to other studies (20), our data showed that inhibition of PA synthesis by treatment with a primary alcohol (1-BtOH) but not t-BtOH led to a dramatic decrease in Golgi PtdIns(4,5)P2 synthesis with little effect on the synthesis of PtdIns(4)P. This correlated with the in vitro fragmentation of the Golgi cisternae into a relatively uniform population of 50- to 100-nm diameter vesicles (Fig. 6). A likely explanation for the discrepancy between our present results and those reports may be due to differences in cell fractionation that could lead to diminished recovery of Golgi membranes. It is possible that the PtdIns(4,5)P2 synthesis and its inhibition by 1-BtOH that we observed in isolated Golgi membranes (Fig. 7) resulted from contamination by plasma membrane-derived vesicles. However, even the plasma membranes proteins in our Golgi fractions (Fig. 1), this seems unlikely. The present data further support our and other earlier observations that PA stimulates type I phosphatidylinositol-4-phosphate 5-kinase, the final enzyme involved in PtdIns(4,5)P2 synthesis (19, 51, 56). Together, these data suggest that PA and PtdIns(4,5)P2 synthesis are required for maintaining the structure and function of the Golgi.

Strikingly, following washout of 1-BtOH from Golgi membranes in vitro PtdIns(4,5)P2 synthesis was restored as was the formation of Golgi cisternae, albeit inefficiently (Figs. 6 and 7). Most significantly, the reassembled Golgi cisternae were capable of supporting limited vesicle release (Fig. 8). Together, these data strengthen the correlation of PtdIns(4,5)P2 synthesis and the maintenance of Golgi structure and function. It might be argued that the effects of 1-BtOH on Golgi morphology were independent of phospholipid synthesis. Although such an explanation is possible, several observations suggest it is unlikely. First, treatment of Golgi membranes with t-BtOH (up to 3.5%) had no effect on Golgi structure in vitro (Fig. 5) or in vivo (19). Second, Golgi fragmentation was rapidly reversible in vivo (19) and in vitro following incubation with cytosol and energy, and this correlated with renewed PtdIns(4,5)P2 synthesis (Figs. 6 and 7). If the alcohol were extracting lipids or protein from the membrane nonspecifically, it is unlikely this would be a readily reversible reaction. Finally, only primary but not secondary or tertiary alcohols inhibit the production of phosphatidic acid (31).

Although re-assembly of mitotic Golgi membrane fragments has been observed following incubation with an interphase cytosolic extract (35), this is the first observation that fragmentation of the Golgi apparatus in response to decreased phosphoinositide synthesis, was also reversible in vitro. This observation will enable us to isolate factors that restore both PtdIns(4,5)P2 synthesis and Golgi reassembly in vitro. At present it is unclear how decreased PtdIns(4,5)P2 synthesis results...
in fragmentation of the Golgi apparatus. Our working hypothesis is that specific structural proteins possessing a pleckstrin homology domain interact with this lipid thereby enhancing their recruitment and binding to the Golgi apparatus. A candidate for such a PtdIns(4,5)P$_2$-binding protein is a Golgi-specific isoform of spectrin, designated β-III spectrin (52). Indeed, Godi et al. (53) demonstrated that the binding of β-III spectrin to Golgi membranes is inhibited in vitro in the absence of PtdIns(4,5)P$_2$. We speculate that, in part, the fragmentation of the Golgi apparatus and its re-assembly in vitro may be a consequence of β-III spectrin binding to the membranes. Currently, we are testing this model.

Acknowledgments—We thank Frank Macaluso and Leslie Gunther for expert technical help with electron microscopy and Raymond Chiu for helpful suggestions on the manuscript. We thank Dr. John Bergeron for his help in the preparation of and for providing rat liver Golgi membranes in the initial stages of this work and Dr. Gordon Shore for anti-Tom20 antibodies.

REFERENCES

1. Palade, G. (1975) Science 189, 347–357
2. Springer, S., and Schekman, R. (1998) Science 281, 698–700
3. Schekman, R., and Orci, L. (1996) Science 271, 1526–1530
4. Zerial, M., and McBride, H. (2001) Curr. Opin. Cell Biol. 13, 353–359
5. Pelham, H. R. (2001) Trends Cell Biol. 11, 504–510
6. Roth, M. G. (1999) Science 285, 591–600
7. Siddhanta, A., Backer, J. M., and Shields, D. (2000) J. Biol. Chem. 245, 371–378
8. Dorer, C., and Balch, W. E. (1994) J. Biol. Chem. 269, 1437–1448
9. Montier, S., Chardin, P., Robineau, S., and Goud, B. (1998) J. Cell Sci. 111, 3427–3436
10. De Figureiredo, P., Dreickrah, D., Katzenellenbogen, J. A., Strang, M., and Brown, W. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8642–8647
11. Choukroun, G. J., Marshansky, V., Gustafson, C. E., McKee, M., Hajjar, R. J., Rosenweig, A., Brown, D., and Bonventre, J. V. (2000) J. Clin. Invest. 106, 605–618
12. Chen, E. J. (1999) Methods Enzymol. 318, 488–504
13. Liscovich, M., Czarny, M., Ficuci, G., and Tang, X. (2000) Biochem. J. 345, 415–418
14. Shields, D., and Arvan, P. (1999) Curr. Opin. Cell Biol. 11, 489–494
15. Gonatas, N. K., Gonatas, J. O., and Stieber, A. (1998) Histochem. Cell Biol. 109, 591–600
16. Godi, A., Santone, I., Pertile, P., Devarajan, P., Stabach, P. R., Morrow, J. S., Sesso, A., Fujiwara, D. T., Jaeger, M., Jaeger, R., Li, T. C., Monteiro, M. M., Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Angelica, E. C., Puertollano, R., Mullins, C., Aguilar, R. C., Vargas, J. D., Di Tullio, G., Polishchuk, R., Petrucci, T. C., Luini, A., and De Matteis, M. (1999) EMBO J. 18, 1241–1255
17. Freyberg, Z., Sweeney, D., Siddhanta, A., Bourgoin, S., Frohman, M. A., and Shields, D. (1997) J. Cell Biol. 138, 263–275
18. Chen, Y. G., Siddhanta, A., Austin, C. D., Hammond, S. M., Sung, T. C., Malhotra, V., John, K. M., Stabach, P. R., Devarajan, P., Morrow, J. S., and Lux, S. E. (1998) J. Cell Biol. 141, 1031–1042
19. Corden, A. E., Puertollano, R., Mullan, C., Aguilar, R. C., Vargas, J. D., Hartnell, L. M., and Bonifacio, J. S. (2000) J. Cell Biol. 149, 81–94
20. Boman, A. L., Zhang, C., Zhu, X., and Kahn, R. A. (2000) Mol. Biol. Cell. 11, 1241–1255
21. Hirst, J., Lui, W. W., Bright, N. A., Totty, N., Seaman, M. N., and Robinson, M. S. (2000) J. Cell Biol. 149, 67–80
22. De Camilli, P., Emr, S. D., McPherson, P. S., and Novick, P. (1996) Science 271, 1533–1539
23. Siddhanta, A., Backer, J. M., and Shields, D. (2000) J. Cell Biol. 138, 483–494
24. Perez, M., and Hirschberg, C. B. (1987) Methods Enzymol. 138, 709–715
25. Bourguin, S., Harbour, D., Desmarnais, Y., Takai, Y., and Beaulieu, A. (1995) J. Biol. Chem. 270, 3172–3178
26. Yusupov, P., Nilsson, T., Hui, N., Watson, R., and Warren, G. (1994) J. Cell Biol. 124, 485–493
27. Xu, H., and Shields, D. (1993) J. Cell Biol. 122, 1169–1184
28. Misteli, T., and Warren, G. (1994) J. Cell Biol. 125, 269–282
29. Bergeron, J. M., Brenner, M. B., Thomas, D. Y., and Williams, D. B. (1994) Trends Biochem. Sci. 19, 347–350
30. Rabouille, C., Misteli, T., Watson, R., and Warren, G. (1993) Science 260, 601–613
31. Liscovich, M., Czarny, M., Ficuci, G., and Tang, X. (2000) Biochem. J. 345, 415–418
32. Godi, A., Santone, I., Pertile, P., Devarajan, P., Stabach, P. R., Morrow, J. S., and Lux, S. E. (1998) J. Cell Biol. 141, 1031–1042
33. Siddhanta, A., Backer, J. M., and Shields, D. (1998) J. Biol. Chem. 273, 17995–17998
34. Stanewski, M. C., Tie, W. T., Peters, L. L., Ch’ng, Y., John, K. M., Stabach, P. R., Devarajan, P., Morrow, J. S., and Lux, S. E. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14358–14363
35. Godi, A., Santone, I., Pertile, P., Devarajan, P., Stabach, P. R., Morrow, J. S., Di Tullio, G., Polischuk, R., Petrucci, T. C., Luini, A., and De Matteis, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8607–8612
36. Seo, A., Fujisawa, D. T., Jaeger, M., Jaeger, R., Li, P. T., Monteiro, M. M., Correa, H., Ferreira, M. A., Schumacher, R. L., Belisario, J., Kachar, B., and Chen, E. J. (1999) Tissue Cell 31, 357–371
37. Honda, A., Nagami, M., Yokoseki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A., and Kanako, Y. (1999) J. Biol. Chem. 199, 521–532
38. Jenkins, G. H., Fisette, P. L., and Anderson, R. A. (1994) J. Biol. Chem. 269, 11547–11554
Fragmentation and Re-assembly of the Golgi Apparatus in Vitro: A REQUIREMENT FOR PHOSPHATIDIC ACID AND PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE SYNTHESIS

David A. Sweeney, Anirban Siddhanta and Dennis Shields

J. Biol. Chem. 2002, 277:3030-3039.
doi: 10.1074/jbc.M104639200 originally published online November 9, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104639200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 56 references, 33 of which can be accessed free at http://www.jbc.org/content/277/4/3030.full.html#ref-list-1