Phospholipase D Stimulates Release of Nascent Secretory Vesicles from the trans-Golgi Network

Ye-Guang Chen,* Anirban Siddhanta,* Cary D. Austin,* Scott M. Hammond,§ Tsung-Chang Sung,§
Michael A. Frohman,§ Andrew J. Morris,§ and Dennis Shields*‡

*Department of Developmental and Molecular Biology, ‡Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461; and §Department of Pharmacology, and Institute for Cell and Molecular Biology, State University of New York, Stony Brook, New York 11794

Abstract. Phospholipase D (PLD) is a phospholipid hydrolyzing enzyme whose activation has been implicated in mediating signal transduction pathways, cell growth, and membrane trafficking in mammalian cells. Several laboratories have demonstrated that small GTP-binding proteins including ADP-ribosylation factor (ARF) can stimulate PLD activity in vitro and an ARF-activated PLD activity has been found in Golgi membranes. Since ARF-1 has also been shown to enhance release of nascent secretory vesicles from the TGN of endocrine cells, we hypothesized that this reaction occurred via PLD activation. Using a permeabilized cell system derived from growth hormone and prolactin-secreting pituitary GH3 cells, we demonstrate that immuno-affinity-purified human PLD1 stimulated nascent secretory vesicle budding from the TGN approximately twofold. In contrast, a similarly purified but enzymatically inactive mutant form of PLD1, designated Lys898Arg, had no effect on vesicle budding when added to the permeabilized cells. The release of nascent secretory vesicles from the TGN was sensitive to 1% 1-butanol, a concentration that inhibited PLD-catalyzed formation of phosphatidic acid. Furthermore, ARF-1 stimulated endogenous PLD activity in Golgi membranes approximately threefold and this activation correlated with its enhancement of vesicle budding. Our results suggest that ARF regulation of PLD activity plays an important role in the release of nascent secretory vesicles from the TGN.

Mammalian phosphatidylycholine-specific phospholipase D (PLD) has been implicated in a wide range of physiological responses including metabolic regulation, cell proliferation, mitogenesis, oncogenesis, inflammation, secretion, and diabetes (Exton, 1994). PLD catalyzes the hydrolysis of phospholipids to generate phosphatidic acid (PA) and the corresponding free polar head group. PA can itself be converted to the second messenger diacylglycerol or lysophosphatidic acid which activates various downstream signaling events. Consistent with a role in cell signaling, PLD can be activated by small G proteins, intracellular Ca\textsuperscript{2+}, protein kinase C (PKC), and protein–tyrosine kinases (Liscovitch and Cantley, 1995). In mammalian cells, at least two classes of PLD can be differentiated by their susceptibility to regulation by G proteins, or requirements for the phospholipid phosphatidyl 4,5-bisphosphate (PtdIns-4,5-P	extsubscript{2}) and fatty acids (Massenburg et al., 1994). One of the small G proteins that stimulates PLD activity is ADP ribosylation factor (ARF), which is an ~20-kD GTP binding protein that is a member of the Ras superfamily (Donaldson and Klausner, 1994). Interestingly, ARF, Rho, and PKC stimulation of PLD activity requires PtdIns-4,5-P	extsubscript{2} as an essential cofactor (Liscovitch et al., 1994; Pertile et al., 1995). PLD is present in Golgi membranes and its activity can be stimulated by ARF in vitro (Ktistakis et al., 1995). Furthermore, the ARF-stimulated PLD activity enhanced the binding of the β-COP subunit of coatomer to isolated Golgi membranes, suggesting that changes in the membrane lipid composition influences coat recruitment (Ktistakis et al., 1996). It has been suggested that ARF stimulation of PLD plays a role in membrane trafficking (Brown et al., 1993; Kahn et al., 1993; Cockcroft et al., 1994; Boman and Kahn, 1995; Liscovitch and Cantley, 1995; Bednarek et al., 1996) although to date, this has not been demonstrated directly.
Recently, three laboratories cloned an open-reading frame encoding PLD activity from Saccharomyces cerevisiae (Rose et al., 1995; Ella et al., 1996; Waksman et al., 1996); this cDNA encodes a polypeptide of 1,683 amino acids (predicted mol wt 195,000). Based on the sequence of the yeast enzyme, one of our laboratories also cloned a cDNA encoding a human PLD specific for phosphatidylincholine, designated PLD1 (Hammond et al., 1995). PLD1 cDNA encodes a 1,072-residue polypeptide (Mr = ~120,000) that is predominantly membrane associated, but unlike other phospholipases involved in signal transduction, it lacks SH2, SH3, or pleckstrin domains (Hammond et al., 1995). Similar to the PLD activity that has been widely studied, PLD1 requires PtdIns-4,5-P₂ as a co-factor and is inactive unless stimulated by members of the ARF and Rho families of small G proteins or protein kinase C (Hammond et al., 1995, 1997). PLD1 localizes to the ER, Golgi apparatus, and endosomes suggesting that it may play a role in vesicular trafficking (Colley et al., 1997). In contrast, PLD2, the second mammalian PLD cloned, is not activated by ARF; it localizes to the plasma membrane, and has been proposed to play a role in agonist-induced actin rearrangement or receptor-mediated recycling (Colley et al., 1997).

In the past few years, genetic and biochemical studies have shown that ARF-1 plays an essential role in mediating intracellular vesicular transport (Donaldson and Klausner, 1994). Numerous studies using the fungal metabolite brefeldin A (BFA), a drug that perturbs ARF function by inhibiting GTP–GDP exchange, or studies in which mutant forms of ARF were expressed in cells have demonstrated that ARF-1 is involved in: (a) maintaining the structural integrity of the Golgi apparatus; (b) transport from the ER to the Golgi apparatus; and (c) endosome trafficking. In vitro binding studies showed that ARF in its GTP-bound form recruits the β-COP subunit of coatomer (COP-I) to Golgi membranes, suggesting that it regulates the formation of coated transport vesicles. ARF-1 also functions in the late Golgi apparatus; where it facilitates binding of the γ-adaptin subunit of the AP1 clathrin adaptor complex to isolated Golgi membranes in vitro (Stamnes and Rothman, 1993; Traub et al., 1993; Liang and Kornfeld, 1997). Recently, several ARF-specific guanine nucleotide exchange factors (GEFs) have been characterized from yeast and mammalian cells (Chardin et al., 1996; Morinaga et al., 1996; Peyroche et al., 1996; Tsai et al., 1996). These appear to fall into two classes, one of high molecular weight, that is BFA sensitive, and the other of ~47–55 kD that is BFA insensitive. Interestingly, several of these GEFs possess a domain that is very similar to a motif present in yeast Sec 7p (Chardin et al., 1996), a high molecular weight protein involved in ER to Golgi and intra-Golgi vesicular trafficking (Franzusoff et al., 1991). GTP–GDP exchange activity is enhanced by inositol phospholipids, particularly PtdIns-4,5-P₂, and this is mediated via a pleckstrin homology domain however, the GEF activity resides in the Sec 7 domain (Chardin et al., 1996). A Golgi-localized, 49-kD ARF GTPase activating protein (GAP) has been purified from rat liver cytosol and its cDNA sequence determined (Cukierman et al., 1995). Recently, an ARF-1, and -3–binding protein, designated arfaptin, was identified by the yeast two-hybrid system (Kanoh et al., 1997); this protein, which is Golgi localized, only binds to ARF in its GTP-bound form. Together these studies suggest that ARF and its accessory proteins function in mediating vesicular trafficking by the recruitment of specific coat proteins to membranes. However, although ARF is clearly central to vesicular transport, its exact role is still unclear.

Using a permeabilized cell system derived from rat anterior pituitary growth hormone (GH)- and prolactin (PRL)-secreting GH3 cells, we demonstrated that recombinant human ARF-1 stimulates the release of nascent secretory vesicles from the TGN approximately two- to threefold (Chen and Shields, 1996). In contrast, mutant forms of ARF unable to exchange GDP for GTP or one lacking the NH₂-terminal 17 residues did not stimulate vesicle budding. In light of observations that ARF can regulate Golgi-localized PLD activity and recruitment of coatomer to Golgi membranes (Ktistakis et al., 1995, 1996) and the increasing evidence that phospholipid-modifying enzymes play a role in membrane trafficking (Liscovitch and Cantley, 1995; De Camilli et al., 1996), we hypothesized that PLD could be a link between ARF and secretory vesicle release. The availability of human PLD (Hammond et al., 1995) and a permeabilized cell system that supports efficient secretory vesicle budding from the TGN (Xu and Shields, 1993) has enabled us to test this hypothesis directly. Here, we demonstrate that addition of human PLD to permeabilized endocrine cells stimulated budding of nascent secretory vesicles from the TGN. Furthermore, ARF activation of endogenous PLD activity present in the Golgi apparatus correlated closely with its ability to potentiate nascent secretory vesicle formation. Our data suggest that ARF can regulate vesicle budding from the TGN by modulating PLD activity.

Materials and Methods

Materials

[35S]Pro-mix (>1,000 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL), [9,10-3H(N)]oleic acid (14 Ci/mmol) was purchased from DuPont-NEN (Boston, MA). Cabbage phospholipase D extract (type V), peanut phospholipase D extract (type III), and neomycin phosphates were purchased from Sigma Chemical Co. (St. Louis, MO). Proteinase K, chymotrypsin, and granzyme K, guanosine-5'-O-(3-thiotriphosphate) (GTPγS), and adenosine-5'-O-(3-thiotriphosphate) (ATPγS) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Rabbit antisera to the COOH-terminal peptides of GH and PRL have been described previously by Austin and Shields (1996a) and Xu and Shields (1993), respectively. The expression, maturation, and purification of recombinant ARF-1 was performed exactly as described previously (Chen and Shields, 1996).

Cell Culture

GH3 cells were grown in Ham’s F10 medium, supplemented with 15% horse serum, 2.5% bovine fetal serum, 2 mM glutamine, 25 U/ml penicillin, and 25 U/ml streptomycin at 37°C with 5% CO₂, as previously described (Stoller and Shields, 1988).

Release of Nascent Secretory Vesicles in Permeabilized Cells

The preparation of permeabilized cells and release of nascent secretory vesicles from the TGN (vesicle budding assay) was described previously (Xu and Shields, 1993; Chen and Shields, 1996). Approximately 2×10⁶ cells were pulse labeled with [35S]Pro-mix for 12 min, and then chased for 2 h.
at 19°C to accumulate radiolabeled GH and PRL in the TGN. The cells were permeabilized at 4°C by incubation in swelling buffer for 5 min, the buffer was aspirated and replaced with a buffer containing 0.1 M KCl and 0.5 mM Hepes, pH 7.2, after which the cells were broken by scraping with a rubber policeman. The cells were centrifuged at 800 g for 5 min, washed in 3–5 ml of breaking buffer, and resuspended in 5 vol of breaking buffer. This procedure resulted in >95% of cell breakage, evaluated by staining with trypan blue. The permeabilized cells were incubated at 37°C for 90 min in a 100-μl reaction containing the following reagents: 10 mM Hepes, pH 7.2, 2.5 mM MgCl₂, 0.5 mM CaCl₂, 35 mM KOAc, 110 mM KCl, and an energy regenerating system (ERS: 1 mM AMP, 0.2 mM GTP, 10 mM creatine phosphate, 50 μg/ml creatine phosphate kinase). After incubation, the samples were centrifuged at 13,000 g for 15–30 s. The supernatant containing nascent secretory vesicles (S) and cell lysates (P; residual permeabilized cells) were treated sequentially with anti-GH antibodies, followed by anti-PRL antibodies. The immunoprecipitated polypeptides were analyzed by SDS-PAGE and the intensity of each band quantitated using a computing densitometer (model 300A; Molecular Dynamics, Inc., Sunnyvale, CA). Nascent secretory vesicle budding efficiency was calculated as GH-or PRL-immunoreactive material in the supernatant divided by the total GH or PRL material in the pellet and supernatant.

**Determination of Phospholipase D Activity**

Endogenous PLD activity was measured by its transphosphatidylation activity using 1-butanol. The assay was performed according to Wakelam et al. (1995) with the following modifications. GH3 cells grown to 70% confluence were radiolabeled with 6 μCi/ml [9,10-3H(N)]oleic acid for 24–36 h after which the cells were harvested, homogenized, and Golgi membranes prepared using a sucrose equilibrium density gradient (Xu and Shields, 1993). The Golgi-enriched fractions were incubated in the presence or absence of native or mutant ARF-1, as indicated, under conditions that promote secretory vesicle budding from the TGN. Incubations contained 0.3% 1-butanol to measure the formation of phosphatidylbutanol (Ptd-Bu) in response to PLD activity. After incubation for 1 h at 37°C, samples were plated on ice and the lipids extracted with methanol and chloroform to give a final ratio of 1:1.08 (methanol/chloroform/water). After vortex mixing, the organic phase containing phospholipids was separated by centrifugation, and dried under vacuum using a Speedvac (Savant Instruments, Farmingdale, UK). The dried samples were resuspended in chloroform/methanol (19:1) and the phospholipids resolved on Whatman LK5DF TLC plates (Whatman Inc., Clifton, NJ) by developing with an organic phase consisting of 2,2,4-trimethylpentane/ethylacetate/acetic acid/water (50:10:20:100) (Liscovitch et al., 1994). The plate was air dried, treated with ENHANCE (DuPont-NEN) and exposed to a Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY).

**Budding of Nascent Secretory Vesicles**

The integrity of the nascent secretory vesicles formed in the permeabilized cells was measured one of two ways: (a) by determining the resistance of GH to proteolysis (Xu and Shields, 1993); or (b) by a vesicle sedimentation assay (Chen and Shields, 1996). After the vesicle budding incubation (for protease K protection), samples were treated with 25 μg/ml proteinase K and 4 mM tetracaine on ice for 30 min in the absence or presence of 1% Triton X-100. PMSF was added to a final concentration of 1.3 mM to inhibit further proteolysis, and the products were incubated with appropriate antibodies followed by resolution upon SDS-PAGE. After incubation (for sedimentation), permeabilized cells were separated by centrifugation (13,000 g for 30 s) from the vesicle-containing supernatant. The 13,000 g supernatant fraction was centrifuged in a Beckman airfuge (150,000 g for 10 min; Beckman Instruments, Inc., Fullerton, CA) to pellet nascent vesicles from supernatant material. All samples were treated with anti-GH antibodies and analyzed by SDS-PAGE.

**Human PLD1 Expression in Insect Cells**

A cDNA encoding human PLD1a was expressed in insect Sf-9 cells using a baculovirus expression system from which a cytosolic extract was prepared (Hammond et al., 1995). Briefly, monolayers of Sf-9 cells infected with recombinant vectors encoding human PLD1 or as a control PLC-β2, were scraped in 10 mM Tris-HCl, pH 7.5, 1 mM EGTA containing a cocktail of protease inhibitors, and the suspension sonicated to lyse the cells. The cell lysate was centrifuged at 2,000 g for 10 min at 4°C, the supernatant adjusted to 500 mM KCl, incubated on ice for 1 h, and centrifuged at 100,000 g for 30–60 min. The high speed supernatant was dialyzed briefly against 100 mM KCl, 2.5 mM MgCl₂, 20 mM Hepes, pH 7.2, and PLD activity determined by measuring the release of [3H]phosphatidylcholine-containing liposomes (Brown et al., 1993; Hammond et al., 1995). Aliquots of this postribosomal supernatant were flash frozen in liquid nitrogen and stored at −80°C until used. The PLD activity for human PLD1, determined by using [3H]phosphatidylcholine as substrate in the presence of GTPγS and ARF (Brown et al., 1993), was 5.1 nmol hydrolyzed/mg per min and was 0.1 nmol hydrolyzed/mg per min for the control or the PLD1 mutant K898R (see below). It is likely that endogenous insect cell activators of PLD (e.g., small G proteins or PKC) are present in this crude extract, because the basal activity of the enzyme dramatically decreases with immunofluoinity purification (Hammond et al., 1997).

**Immunopurification of Native and Mutant Forms of Human PLD1**

Affinity-purified antibodies generated against two peptides corresponding to residues 1–15 and 525–541 of PLD1 were covalently coupled to protein A-Sepharose CL4B, as previously described (Hammond et al., 1997). This resin was used to immunopurify a native and a mutant form of human PLD1. Mutation of Lys 898 to Arg (K898R), which is a lysine conserved amongst all mammalian, yeast, plant, and bacterial PLD enzymes (Hammond et al., 1995), abolishes PLD activity in human PLD1, PLD2, and the yeast enzyme Spo14 (Sun et al., 1997). Postribosomal supernatants from Sf-9 cells infected with recombinant baculoviruses encoding native or the K898R mutant were prepared as outlined above and incubated with the immunopurified resin at 4°C for 1 h. Unbound material was removed by centrifugation and the resin washed extensively with cell lysis buffer (Hammond et al., 1997) using 100 mM glycine, pH 3.0, containing 1 M KCl, instead of β-mercaptoethanol. The eluate was neutralized immediately, dialyzed against incubation buffer, and assayed for PLD activity (Brown et al., 1993). The immunopurified PLD migrated as a doublet of ~110,000 Mₙ (Fig. 2, B, inset) on our gel system. PLD1 prepared in the absence of detergent was quite labile and all assays were performed within 24 h of purification. Freshly prepared native PLD1 had an ARF-stimulated specific activity of 260 nmol phosphatidyl choline (PC) hydrolyzed/mg per min, whereas the activity of the K898R mutant was undetectable. The mutant PLD1 K898R is completely inactive in vivo but appears to fold correctly and exhibits correct subcellular localization when expressed in fibroblasts (Sun et al., 1997).

**Results**

**Human PLD Stimulates Budding of Nascent Secretory Vesicles from the TGN**

Using a permeabilized system derived from rat pituitary GH3 cells, which secrete GH and PRL, our previous data showed that ARF-1 stimulated nascent secretory vesicle budding from the TGN almost threefold (Chen and Shields, 1996). The stimulation of vesicle release occurred in the absence of exogenously added cytosol, which suggested that either ARF might function by recruiting prebound coat components or less likely, independently of coat recruitment. Since PLD was suggested to be a downstream effector of ARF (Brown et al., 1993; Cockcroft et al., 1994), and PLD activity was shown to effect β-COP binding to Golgi membranes (Kistakis et al., 1996), we reasoned that enhanced vesicle budding might be a consequence of PLD activation. If this were correct, then direct addition of PLD to the permeabilized cells should also stimulate vesicle budding. Of the two cloned mammalian PLDs, only PLD1 localizes to the Golgi and is activated by ARF (Colley et al., 1997). Consequently, our initial experiments used a postribosomal supernatant fraction isolated from insect cells expressing human PLD1. This was added
to the permeabilized cells and its effect on nascent vesicle formation determined (Fig. 1). As previously observed (Xu and Shields, 1993; Chen and Shields, 1996), in control permeabilized cells vesicle budding was energy dependent and ~30% efficient (Fig. 1, lanes 1–4). Significantly, PLD1 stimulated vesicle budding approximately twofold (compare Fig. 1, lanes 3 and 4 with 5 and 6) in a reaction that required ATP and GTP (Fig. 1, lanes 7 and 8). Control incubations in which an extract from insect cells expressing a different phospholipase, phospholipase C-β2, was added to the system, did not stimulate vesicle budding above basal levels (Fig. 1, lanes 9 and 10). The stimulated budding of nascent secretory vesicles was dependent on the concentration of added PLD1-containing extract (Fig. 1 B) and addition of as little as 3 μg cell extract per ml enhanced vesicle release up to twofold. Increasing the concentration of the Sf-9 extract containing recombinant human PLD1 stimulated release of GH- or PRL-containing vesicles slightly to a level of ~60% efficiency (Fig. 1 B). Most significantly, these data demonstrated that PLD stimulated vesicle budding in a concentration- and energy-dependent reaction.

It was possible that overexpression of human PLD1 in insect Sf-9 cells modified the cell extract such that vesicle release was a consequence of the modification rather than the enzyme activity per se. To exclude this possibility, a highly purified preparation of native PLD1 was added to the permeabilized cells (Fig. 2). Human PLD1 was immunopurified to apparent homogeneity from a postribosomal supernatant of Sf-9 cells (Fig. 2 B, inset). As a control, we added an identically purified but inactive mutant form of PLD1, K898R, possessing Arg at position 898 instead of Lys (the latter residue is conserved in human, yeast, plant, and bacterial PLD enzymes) (Fig. 2 A and B). Both preparations of PLD1 migrated on SDS-PAGE as a doublet of ~110,000 M₀. Before use, each purified enzyme preparation was assayed for its ability to hydrolyze PC in a reconstituted liposome assay (Brown et al., 1993); whereas na-
tive PLD was highly active (PC hydrolysis was 260 nmol/mg per min; see Materials and Methods), the K898R mutant was inactive (Sung et al., 1997; and data not shown). Addition of increasing concentrations of native PLD1 stimulated release of nascent secretory vesicles about twofold (Fig. 2B) and maximal vesicle budding occurred with 0.6–1 μg PLD1/ml, equivalent to ~5 nM enzyme. Most significantly, addition of the enzymatically inactive mutant form of PLD1 to the permeabilized cells did not stimulate vesicle budding above control levels even at the highest concentration used (Fig. 2B).

Plant PLD Stimulates Vesicle Release

PLD has been isolated from several plant sources and shares similarity to the human and yeast enzymes (Hammond et al., 1995). We argued that if PLD1 stimulated vesicle budding as a result of its activity then PLD enzymes from other species (including plant) should have a similar effect (Fig. 3). PLD isolated from cabbage (type V) or peanut (type III) was added to the permeabilized cell system and their effect on GH- and PRL-containing vesicle budding determined. Both species of plant PLD stimulated vesicle release from the TGN approximately twofold (Fig. 3B). Similar to the mammalian enzyme, stimulation of vesicle budding by cabbage PLD was also energy dependent (Fig. 3A, lanes 7 and 8) and required ATP hydrolysis since there was minimal budding with GTP alone or in the presence of the nonhydrolyzable analogue ATPγS and GTP (Fig. 3A, lanes 9 and 10, and 11 and 12, respectively). Heat inactivation or pretreatment either with cabbag or peanut PLDs with proteinase K inhibited their ability to stimulate vesicle formation above background levels (data not shown) demonstrating that increased vesicle release was dependent on an active enzyme.

It might be argued that the stimulation of nascent vesicle budding in response to human or plant PLDs resulted from membrane lysis or leakage of content proteins from the Golgi apparatus or secretory vesicles in response to the increased concentration of negatively charged lipids. To exclude this possibility and to demonstrate the release of intact, sealed GH- and PRL-containing vesicle budding in response to PLD activity, we used a high speed centrifugation assay to pellet nascent vesicles (Fig. 4A) or a protease protection assay (Fig. 4B). After treatment with human PLD1, significantly more GH was recovered in the high speed pellet (corresponding to nascent secretory vesicles) than from the control incubations (Fig. 4A, lanes 2 and 4). Similarly, in response to plant PLD, approximately twofold more GH was protease resistant than in control permeabilized cells (Fig. 4B, compare lanes 2 and 4); whereas in the presence of Triton X-100, the GH was degraded quantitatively (Fig. 4B, lanes 5 and 6). Together, these results indicated that incubation of permeabilized cells with either human or plant PLD stimulated the release of intact membrane-bound secretory vesicles.

ARF-1 Stimulates Endogenous Golgi-associated PLD Activity

The above results, together with the observation that ARF can activate PLD (Brown et al., 1993; Cockcroft et al., 1994; Ktistakis et al., 1995, 1996), suggested that ARF stimulation of nascent vesicle budding might occur via PLD activation. If this hypothesis was correct, then endogenous Golgi PLD activity should be enhanced under conditions that promote vesicle budding from the TGN as a result of active ARF. To test this idea, GH3 cells were incubated with [3H]oleic acid to radiolabel phospholipids and a Golgi membrane fraction was isolated; this was incubated in the absence or presence of native and mutant ARF-1 under vesicle budding conditions (Fig. 5). Since transphosphatidylation activity is a diagnostic property of PLD activity (Wakelam et al., 1995), we included low concentrations of 1-butanol (0.3%) in the incubations and determined the Golgi-associated PLD response to native and mutant ARF polypeptides by measuring the formation of PtdBut using TLC (Wakelam et al., 1995). Under basal vesicle budding conditions, i.e., in the presence of energy

Figure 3. Plant PLDs stimulate nascent vesicle budding from mammalian TGN. (A) Permeabilized cells were incubated in the absence (lanes 1–4) or presence (lanes 5–12) of 40 U/ml cabbage PLD type V or peanut PLD type III (lanes 13 and 14) without (−) or with (+) ATP and GTP as indicated. Samples in lanes 11 and 12 were incubated in the presence of 1 mM ATPγS plus GTP. After incubation, samples were separated into pellet (P) and nascent secretory vesicles (S) by brief centrifugation and the fractions treated with anti-GH or anti-PRL (not shown) antibodies. (B) For the quantitation of vesicle budding in response to plant PLDs, permeabilized cells were incubated with the indicated concentrations of plant PLD and the release of nascent GH (○, ●) and PRL-containing secretory vesicles (□, ■) determined. Data are the average of three experiments. ○, Cabbage PLDV-GH vesicle budding; □, cabbage PLDV-PRL vesicle budding; ●, peanut PLDIII-GH vesicle budding; ■, Peanut PLDIII-PRL vesicle budding. Vesicle budding efficiency was calculated as GH or PRL immunoreactive material in the supernatant (S) divided by the total GH or PRL material (pellet + supernatant, P + S).
Figure 4. Human and plant PLDs stimulate budding of intact nascent secretory vesicles. (A) Sedimentation of nascent secretory vesicles containing GH. Permeabilized cells incubated in the absence (−) or presence (+) of 15 μg/ml of Sf9 cell extract expressing human PLD1. After incubation, the permeabilized cells (lanes 1 and 3; CP) were separated by centrifugation (13,000 g for 20 s), from the vesicle-containing supernatant. This was further centrifuged in a Beckman airfuge (150,000 g, 10 min) to pellet nascent vesicles (lanes 2 and 4; SP). All the samples were treated with anti-GH antibodies and analyzed by SDS-PAGE. (B) Resistance of GH-containing vesicles to proteolysis. After the budding assay performed without (lanes 1, 2, 5, and 6) or with cabbage PLD (lanes 3 and 4), samples were incubated with 25 μg/ml proteinase K at 4°C for 30 min in the absence (lanes 1–4) or presence of 1% Triton X-100 (lanes 5 and 6). The pellet (P) and supernatant (S) fractions were separated by brief centrifugation and treated with anti-GH antibodies; identical results were obtained for PRL containing vesicles (data not shown).

(Fig. 5 A, lane 2). PtdBut was generated and its formation was stimulated nearly twofold upon incubation with myristoylated ARF (Fig. 5, A and B, lanes 2 and 4). Most significantly, those ARF polypeptides that have previously been shown (Chen and Shields, 1996) to stimulate vesicle budding, i.e., the “activated” ARF mutant Q71L (Fig. 5 A, lanes 5 and 10), which hydrolyzes GTP inefficiently, or native ARF (Fig. 5 A, lane 7) stimulated PLD activity approximately threefold (Fig. 5 B). By contrast, PLD activity was enhanced only slightly above basal levels when samples were incubated with an ARF–GDP mutant T31N or one lacking the NH2-terminal 17 amino acids ΔNT (comparing Fig. 5 A, lane 3 with 8 and 9). Control incubations demonstrated that the PtdBut generated was a consequence of PLD activity since this material was greatly diminished when: (a) an energy system was excluded from the incubation (lane 1); (b) neomycin was present in the incubation (lane 11) consistent with previous work demonstrating that low concentrations of neomycin inhibit PLD activity by binding PtdIns-4,5-P2, an essential cofactor for PLD activity (Liscovitch et al., 1994; Whatmore et al., 1994; Pertile et al., 1995); and (c) no PtdBut was formed in the absence of butanol (lane 12). Taken together, these data demonstrated that under conditions where native ARF enhances vesicle budding approximately twofold (Chen and Shields, 1996; Fig. 5 A, lanes 4 and 5) there is also a concomitant stimulation of endogenous Golgi localized PLD activity.

Exogenous PLD Hydrolyzes Endogenous Golgi Phospholipids

The above experiments, using immunopurified native and mutant human PLD1, strongly suggest that PLD catalytic activity mediates vesicle release from the TGN. To determine if exogenously added human PLD hydrolyzed endogenous Golgi membrane phospholipids, GH3 cells were incubated with [3H]oleate to radiolabel phospholipids, Golgi membranes were isolated and treated with 1 M KCl to extract endogenous PLD1 from the membranes (Ham-
The salt-treated Golgi membranes were incubated with Sf-9 cell extracts under vesicle budding conditions in the presence of 0.3% 1-butanol. The ability of human PLD1 to hydrolyze endogenous phospholipids was then determined by measuring the formation of PtdBut. Incubation of the Golgi membranes with recombinant myristoylated ARF had no effect on lipid hydrolysis since the endogenous PLD had been effectively removed from the membranes by high salt treatment (Fig. 6, lane 1). Addition of the PLD1 containing Sf-9 extract (Fig. 6, lane 2) but not the control extract (Fig. 6, lane 5) hydrolyzed the phospholipids to generate PtdBut. As previously observed (Brown et al., 1993; Cockcroft et al., 1994; Hammond et al., 1995), PLD activity was enhanced in the presence of GTPγS and myrARF (Fig. 6, lanes 2 and 3). Most importantly, these results demonstrated that human PLD1 was active in hydrolyzing endogenous Golgi membrane phospholipids, and that under these conditions vesicle budding was enhanced.

**Vesicle Budding Requires the Product of PLD Activity**

Recent studies from Kitstakis et al. (1996) demonstrated that PLD activity mediated β-COP recruitment to isolated Golgi membranes and that coatamer binding was abrogated in the presence of low concentrations of primary alcohols (i.e., when formation of PA was prevented). Our preceding experiments demonstrated that the endogenous phospholipids in Golgi membranes were substrates for endogenous (Fig. 5) as well as exogenously added PLD (Fig. 6), and that this lipid hydrolysis correlated with vesicle budding from the TGN. We argued therefore, that if PLD hydrolysis of PC, to yield PA, was necessary for vesicle budding, then inhibition of PA production should also inhibit nascent vesicle release. To test this hypothesis, we exploited the observation that only primary alcohols but not secondary or tertiary alcohols participate in the PLD transphosphatidylation reaction. Permeabilized cells were incubated with 1% butanol, 2-propanol, and tertiary butanol and the effect on vesicle budding determined (Fig. 7, A and B). 1-Butanol inhibited vesicle release by ~50% (higher concentrations ≥1.5% inhibited vesicle budding quantitatively; however this was a nonspecific effect resulting from partial protein precipitation). Significantly, inhibition of vesicle budding with 1% 1-butanol led to production of PtdBut (Fig. 7, C) as the major product of PLD1 activity. In contrast, incubation in the presence of 2-propanol or t-butanol, which do not participate in the transphosphatidylation reaction, had no effect on vesicle budding (Fig. 7, B and C). Taken together, these data suggest that enzymatically active PLD1 and the PA product of PC hydrolysis are required for nascent secretary vesicle release from the TGN.

**Discussion**

Evidence from several laboratories has implicated phospholipid-modifying enzymes and inositol phospholipid metabolism in mediating various steps of intracellular vesicular transport (Boman and Kahn, 1995; Liscovitch and Cantley, 1995; De Camilli et al., 1996). Earlier work (Herman et al., 1992; Schu et al., 1993; Stack and Emr, 1994) demonstrated that the yeast VPS34 gene encodes a PtdIns-3-kinase that is essential for protein transport from the Golgi apparatus to the vacuole. In mammalian cells, phosphoinositol (PI)-3 kinases are involved in regulating endocytic trafficking of plasma membrane receptors and in transport of lysosomal proteases from the Golgi apparatus.

![Figure 6. Human PLD1 hydrolyzes Golgi membrane phospholipids. GH3 cells were incubated for 24 h with [3H]oleic acid and Golgi membranes isolated by sucrose density flotation on a sucrose gradient (Austin and Shields, 1996). The isolated membranes were incubated with 1 M KCl at 4°C for 10 min to remove endogenous PLD. The salt-treated membranes were pelleted by centrifugation at 150,000 g for 50 min and resuspended in incubation buffer. Equal aliquots of the salt-washed membrane suspension (~100,000 cpm) were incubated with 0.3% butanol to generate phosphatidylbutanol in the presence of ~5 μg/ml of the Sf-9 cell extract expressing human PLD1 (lanes 2–4) or the control extract (lane 5) in a reaction containing (lanes 2, 4, 5) or lacking (lane 3) recombinant myristoylated ARF-1 (m-ARF5). When present, ARF was used at a final concentration of ~1 μM and GTPγS at 30 μM.](Image 501x303)

![Figure 7. Primary alcohols suppress release of nascent vesicles from the TGN. (A) Radiolabeled, permeabilized GH3 cells were either untreated (lanes 1 and 2) or incubated with 1% butanol (lanes 3 and 4), 2-propanol (lanes 5 and 6), or tertiary butanol, (t-Butanol; lanes 7 and 8) under vesicle budding conditions (See Materials and Methods). After 1 h incubation at 37°C, samples were centrifuged briefly at 13,000 g and the pellet (P) and supernatant (S) fractions incubated with anti-GH antibodies. The GH immunoreactive products were analyzed by SDS-PAGE. (B) For the quantitation of vesicle budding, duplicate samples identical to those shown in A were analyzed by SDS-PAGE and the GH band intensity quantitated by densitometry (Materials and Methods). Percent budding was calculated as GH immunoreactive material in the supernatant (S) divided by the total GH material (pellet + supernatant, P + S). (C) TLC analysis of phosphatidylbutanol formation by endogenous Golgi PLD. Cells were incubated overnight with [3H]oleic acid and Golgi membranes isolated by sucrose density centrifugation. The radiolabeled membranes were incubated under vesicle budding conditions for 1 h at 37°C with the indicated alcohols (final concentration, 1%), after which the total lipids were extracted with chloroform–methanol and the products analyzed by TLC (Materials and Methods). PtdBut, migration of phosphatidyl butanol; the asterisk indicates the probable mobility of phosphatidic acid. (Note this spot is almost absent from samples incubated with 1-butanol.)](Image 308x255)
Ca²⁺ and its addition to permeabilized PC12 cells stimulated Sec14p-related protein was purified from rat PC12 cells in two stages of the late secretory pathway. Firstly, a mammalian homologue of Sec14p has been implicated in vesicle transport at the nonpermissive temperature. Significantly, temperature-sensitive mutants of Sec14p (Kaitis et al., 1989, 1990) and yeast cells expressing a temperature-sensitive allele are blocked in intra-Golgi transport at the nonpermissive temperature. Significantly, mammalian homologues of Sec14p have been implicated in both stages of the late secretory pathway. Firstly, a Sec14p-related protein was purified from rat PC12 cells and its addition to permeabilized PC12 cells stimulated Ca²⁺-mediated exocytosis in vitro (Hay and Martin, 1993). Secondly, a 34-kD protein purified from bovine adrenal medulla which possesses PI-TP activity is required for nascent secretory vesicle budding from the TGN in vitro (Ohashi et al., 1995); the yeast Sec14p could substitute for the mammalian PI-TP in both reactions.

Native but Not Mutant PLD Stimulates Vesicle Budding

PLD hydrolyzes PC at its terminal phosphodiester bond to produce PA and choline; PA itself can function as a second messenger. Several cytosolic factors that stimulate PLD activity have been purified; these include ARF (Brown et al., 1993; Cockcroft et al., 1994; Houle et al., 1995; Siddiqi et al., 1995), Rho A (Malcolm et al., 1994; Bourgoin et al., 1995; Kwak et al., 1995) and Cdc42 (for review see Frohman and Morris, 1996). The essential role ARF1 plays in vesicle trafficking (Donaldson and Klausner, 1994; Rothman, 1994), its regulation of PLD (Brown et al., 1993; Kahn et al., 1993; Cockcroft et al., 1994; Lisco-vitch and Cantley, 1995), the presence of an ARF-regulated form of PLD in Golgi membranes (Ktistakis et al., 1995) and the recent demonstration that ARF-activated PLD activity enhances coatamer binding to Golgi membranes (Ktistakis et al., 1996) suggested ARF may stimulate vesicle budding from the TGN via PLD activation. The data of Figs. 1–3 and 5 argue that this hypothesis is correct. A cytosolic extract obtained from insect SF-9 cells expressing human PLD1 stimulated vesicle budding (Fig. 1). It was possible, although unlikely, that other components of the extract rather than PLD activity per se caused vesicle release. To exclude this possibility, we used an immunopurified preparation of PLD1 and this stimulated vesicle budding potently with maximal activity at ~5 nM (Fig. 2). Given that the purified enzyme is very labile in the absence of detergent (most of its activity is lost by 24 h; Hammond, S.M., and A.J. Morris, unpublished observations), it is likely that the enzyme is considerably more potent in promoting vesicle release than our data suggest. The specificity of this reaction was further demonstrated by the use of a purified but enzymatically inactive point mutant of PLD1. This mutant possesses only a minor change in that a highly conserved lysine 898 (present in mammals, yeast, and plant PLDs) was changed to arginine. Addition of this mutant to the permeabilized cell system had no effect on vesicle budding (Fig. 2). Furthermore, a control SF-9 cell extract expressing an unrelated phospholipase, PLC-β2 specific for the hydrolysis of inositol phospholipids, also had no effect on vesicle budding (Fig. 1). Together, these results demonstrated that recombinant human PLD potently stimulated vesicle budding in a reaction that was dependent on enzyme concentration and energy.

Several controls, in addition to the point mutant, demonstrated that the stimulation of vesicle budding resulted from PLD enzymatic activity. Firstly, plant PLD (Fig. 3) and a second form of human phosphatidycholine-specific PLD, designated PLD2 (Colley et al., 1997), stimulated vesicle release (data not shown). Secondly, the human PLD1 enzyme was active in hydrolyzing endogenous Golgi phospholipids (Fig. 6); and thirdly, the transphosphatidylation reaction inhibited vesicle budding (Fig. 7). Thus, when 1-butanol, but not tertiary butanol or any secondary alcohols was present in the budding reaction, vesicle release was inhibited significantly, suggesting that the product of PC hydrolysis (PA) is required for vesicle release. In this context, addition of choline to the permeabilized cells had no effect on vesicle release (data not shown). Furthermore, since both human and plant PLDs stimulated vesicle budding (the latter are not ARF regulated) and the similarity between these enzymes is confined largely to their putative active sites (Hammond et al., 1995), this suggests that enzyme activity per se promotes vesicle release. Consistent with this idea, incubation of Golgi membranes with Streptomyces chromofuscus PLD, which is also not ARF regulated and the similarity between these enzymes is confined largely to their putative active sites (Hammond et al., 1995), suggests that enzyme activity per se promotes vesicle release. Consistent with this idea, incubation of Golgi membranes with Streptomyces chromofuscus PLD, which is also not ARF regulated, increased coatamer binding about fourfold (Ktistakis et al., 1996). Our data provide direct evidence that in addition to PI-TPs and PI-3 kinases, a third class of phospholipid modifying proteins, namely PLDs, can mediate vesicle trafficking (in this case budding from the TGN).

**Figure 8.** Possible role of PLD in nascent secretory vesicle budding. (A) Hydrolysis of phosphatidycholine (PC) in the TGN mediated by ARF-activated PLD leads to a high local concentration of phosphatidic acid (PA). This could alter the composition of the lipid bilayer transiently, perhaps changing its physical properties. These putative lipid microdomains, enriched in PA, facilitate enhanced coat recruitment, possibly the Golgi-specific AP-1 adaptor complex, resulting in budding of nascent secretory vesicles from the TGN in a reaction requiring ATP and GTP. (B) PA is rapidly hydrolyzed to a number of possible intermediates which may be converted to other metabolites that could initiate a signaling cascade, the end product of which (X) mediates coat recruitment.

**ARF-1 Stimulates PLD Activity**

Both recombinant myristoylated and nonmyristoylated ARF-1 stimulated the endogenous PLD activity present in Golgi membranes about two- to threefold (Fig. 5). This was somewhat unexpected since the myristoylation of ARF appears necessary for several of its functions including coat binding (Stamnes and Rothman, 1993; Traub et al., 1993) and guanine nucleotide exchange activity (Morinaga et al., 1996; Liang and Kornfeld 1997). However, myristoylation is not an absolute prerequisite for ARF interaction.
function; for example, although myristate facilitates binding of ARF–GDP to membrane phospholipids, and this enhances GDP–GTP exchange, the interaction between ARF and its GEF was not myristate dependent (Franco et al., 1996). In addition, GAP-stimulated hydrolysis of GTP bound to ARF is independent of myristoylation (Ding et al., 1996) and most recently, the binding of arfaptin to ARF-3 was also shown to be independent of myristoylation (Kanoh et al., 1997). Furthermore, although PLD activation by ARF-5 and -6 required myristoylation, there was much less difference between the ability of myristoylated and nonmyristoylated ARF-1 to enhance PLD activity (Massenburg et al., 1994). Similarly, our results suggest that ARF stimulation of PLD can occur in the absence of myristate; however, our preliminary data suggest that native myristoylated ARF stimulates PLD at about a fivefold lower concentration than non-myristoylated ARF (Siddiqi et al., 1995). Similarly, our results suggest that ARF and PLD co-chromatograph through several purification steps suggesting a direct interaction (Brown et al., 1993; Siddiqi et al., 1995) and ARF and purified PLD do interact in phospholipid micelles (Hammond et al., 1997). Interestingly, during v-SRC activation of PLD, PLD appears to interact directly with the Ral GTPase (Jiang et al., 1995a,b). Given these results, we speculate that ARF and PLD might interact directly on the TGN membrane to effect nascent secretory vesicle release. Although PLD activity per se can be stimulated by native ARF and an activated ARF-1 mutant in the presence of GTP (Fig. 5), this was not sufficient to promote vesicle budding from the TGN. ATP hydrolysis was also required (Figs. 1 and 3) and since ARF stimulation of PLD did not occur in the presence of ATP alone (data not shown), ATP may therefore be required for other steps in the budding reaction. ATP does however, potentiate GTPγS activation of PLD (Geny and Cockcroft, 1992; Dubyak et al., 1993) and recent evidence suggests that ATP hydrolysis is required by phosphoinositid-4-phosphate-5-kinase (Per- tile et al., 1995) in the final step of PtdIns-4,5-P2 synthesis. Thus, in part ATP may be necessary to generate the PtdIns-4,5-P2 cofactor that activates PLD. In addition, ATP hydrolysis might be necessary to provide the energy for vesicle scission which releases the nascent vesicle from the TGN membrane.

At present, we have not determined whether PLD-stimulated vesicle release occurs by a change in the Golgi membrane lipid composition or by activating a signaling cascade. In the first case, it is possible that increased PA levels, generated as a consequence of PLD activity, could transiently alter the lipid composition and local charge of the outer bilayer leading to recruitment of coat proteins, e.g., γ-adaptin and clathrin to result in vesicle budding (Fig. 8). In the second scenario, PLD would function as a signal transducer by producing PA (Song et al., 1994; Briscoe et al., 1995; Jiang et al., 1995a). The PA produced in this reaction could then function as a second messenger by being hydrolyzed to a number of possible intermediates such as diacylglycerol which could further trigger intracellular signaling events (e.g., activation of PKC). Consistent with this idea, PKC itself has been shown to stimulate vesicle budding (Xu et al., 1995; Singer et al., 1996), and recently PKC inhibitors were shown to prevent vesicle budding from isolated Golgi membranes (Simon et al., 1996). Significantly, ARF-1 was also implicated in phosphotyrosine-mediated vesicle budding (Austin and Shields, 1996); whether this occurs by activation of phospholipase D or by another mechanism is currently under investigation. Most significantly, our results have identified a novel mechanism for vesicle budding in which a Golgi phospholipid modifying enzyme, PLD can be activated by input from multiple signals, and enhances release of nascent secretory vesicles from the TGN.

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