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

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Mammalian phosphatidylinositol-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 Ca²⁺, 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 phosphatidylinositol 4,5-bisphosphate (PI-4,5-P₂), 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-P₂ 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 (Kitstakis 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 (Kitstakis 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 phosphatidylinositol, designated PLD1 (Hammond et al., 1995). PLD1 cDNA encodes a 1,072-residue polypeptide (M, = ~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-P_2 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 (Starnes 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 Sec7p (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-P_2, and this is mediated via a pleckstrin homology domain however, the GEF activity resides in the Sec7 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 NH_2-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**

[^3H]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 were purchased from Sigma Chemical Co. (St. Louis, MO). Proteinase K, guanosine-5’-O(3-thiotriphosphate) (GTPyS), and adenosine-5’-O(3-thiotriphosphate) (ATPyS) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Rabbit antiserum to the COOH-terminal peptides of GH and PRL have been described previously by Austin and Shields (1996) 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% CO_2, 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 x 10^6 cells were pulse labeled with [^35S]Promix 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 1 ml permeabilization buffer (90 mM KCl, 1 mM MgCl₂, 0.5 mM CaCl₂, 35 mM KACO₃, 110 mM KCl, and an energy regenerating system (ERS: 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, 80 mM MgCl₂, 20 mM Hepes, pH 7.2)) by determining the resistance of GH to proteolysis (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-But) in response to PLD activity. After incubation for 1 h at 37°C, samples were placed on ice and the lipids extracted with methanol and chloroform to give a final ratio of 1:1:0.8 (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, NY). 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/ethylecetate/acetic acid/water (50:10:20:100) (Liscovitch et al., 1994). The plate was air dried, treated with \( \text{EN}^\text{H} \) (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 proteinase K protection), samples were treated with 25#g/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 PLCB2, 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 using 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 NTPs 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 immunopurification (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 (Sung 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 IgG at 4°C for 1 h. Unbound material was removed by centrifugation and the resin washed extensively with cell lysis buffer (Hammond et al., 1995). PLD was eluted as described (Hammond et al., 1997) using 100 mM glycine, pH 3.0, containing 1 M KCl, instead of β-octyl-glucoside. 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 M₉ 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 (Sung 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 pre-bound 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 (Kitstakis 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

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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, 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 Mr. 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. 2 B) 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. 2 B).

**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. 3 B). Similar to the mammalian enzyme, stimulation of vesicle budding by cabbage PLD was also energy dependent (Fig. 3 A, lanes 7 and 8) and required ATP hydrolysis since there was minimal budding with GTP alone or in the presence of the nonhydrolyzable analogues ATPγS and GTP (Fig. 3 A, lanes 9 and 10, and 11 and 12, respectively). Heat inactivation or pretreating either cabbage 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 vesicles in response to PLD activity, we used a high speed centrifugation assay to pellet nascent vesicles (Fig. 4 A) or a protease protection assay (Fig. 4 B). 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. 4 A, lanes 7 and 4). Similarly, in response to plant PLD, approximately twofold more GH was protease resistant than in control permeabilized cells (Fig. 4 B, compare lanes 2 and 4); whereas in the presence of Triton X-100, the GH was degraded quantitatively (Fig. 4 B, 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

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**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 I1 and J2 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).
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).

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 (Fig. 6). 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 coatomer 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 PLD. We argued therefore, that if PLD hydrolysis resulted in vesicle budding from the TGN. We explored 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 secretory 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.
Ca^2+ and its addition to permeabilized PC12 cells stimulated vesicle budding in two stages of the late secretory pathway. Firstly, a transport at the nonpermissive temperature. Significantly, temperature-sensitive mutants of ARF (Bankaitis et al., 1989, 1990) and yeast cells expressing a temperature-sensitive allele of ARF-1 stimulated 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 phosphatidylycholine-specific PLD, designated PLD2 (Colley et al., 1997), stimulated vesicle release (data not shown). Secondly, 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), 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), this suggests that enzyme activity per se promotes vesicle release. 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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 (Siddhanta, A., A. Elgort, and D. Shields, manuscript in preparation). These results suggest that myristoylation may potentiate ARF action rather than being absolutely required for its function.

Consistent with our previous results (Chen and Shields, 1996), an “activated” ARF mutant (Q71L) that potently enhances vesicle budding, stimulated PLD maximally. In contrast, the JNT and T31N ARF mutants that do not stimulate vesicle budding had no significant effect on PLD activity. These data demonstrated a direct correlation between the ability of ARF to stimulate endogenous PLD activity and to enhance nascent secretory vesicle release from the TGN. It might be argued that endogenous ARF present in the insect cell lysate, rather than the human PLD1 activity, stimulated vesicle budding. However, this was not the case since the control insect extract had no effect on vesicle budding (Fig. 1).

In the absence of exogenous ARF, PLD was presumably activated by the low level of endogenous ARF present on the Golgi membranes (Donaldson and Klausner, 1994) or by other PLD-activating proteins such as Rho family members, Rac, cdc42, or PKC (Frohman and Morris, 1996). At present however, we do not know if ARF activation of PLD occurs by a direct interaction between these two polypeptides. Evidence from several laboratories has shown 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 Ras 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 phosphoinositol-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 neccesary 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.

D. Shields thanks A. Elgort for expert technical help and Drs. D. Wilson, M. Kielian, and P. Melançon for many helpful suggestions and discussions. This work was supported by National Institutes of Health (NIH) grants DK21860, and in part by a grant from the Juvenile Diabetes Foundation to D. Shields, and by NIH grants GM 50388 to A.J. Morris and HD 29758 to M.A. Frohman. Core support to D. Shields was provided by an NIH Cancer Center grant P50CA13330.

Received for publication 14 October 1996 and in revised form 12 June 1997.

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