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Intracellular Localization of Phospholipase D1 in Mammalian Cells

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Phospholipase D (PLD) hydrolyzes phosphatidylcholine to generate phosphatidic acid. In mammalian cells this reaction has been implicated in the recruitment of coatomer to Golgi membranes and release of nascent secretory vesicles from the *trans*-Golgi network. These observations suggest that PLD is associated with the Golgi complex; however, to date, because of its low abundance, the intracellular localization of PLD has been characterized only indirectly through overexpression of chimeric proteins. We have used highly sensitive antibodies to PLD1 together with immunofluorescence and immunogold electron microscopy as well as cell fractionation to identify the intracellular localization of endogenous PLD1 in several cell types. Although PLD1 had a diffuse staining pattern, it was enriched significantly in the Golgi apparatus and was also present in cell nuclei. On fragmentation of the Golgi apparatus by treatment with nocodazole, PLD1 closely associated with membrane fragments, whereas after inhibition of PA synthesis, PLD1 dissociated from the membranes. Overexpression of an hemagglutinin-tagged form of PLD1 resulted in displacement of the endogenous enzyme from its perinuclear localization to large vesicular structures. Surprisingly, when the Golgi apparatus collapsed in response to brefeldin A, the nuclear localization of PLD1 was enhanced significantly. Our data show that the intracellular localization of PLD1 is consistent with a role in vesicle trafficking from the Golgi apparatus and suggest that it also functions in the cell nucleus.

INTRODUCTION

Inositol phospholipids play key roles not only in mediating signal transduction events but also in regulating intracellular vesicular transport (De Camilli *et al.*, 1996). The products of lipid hydrolysis act as potent messengers. Classical studies showed that phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2) generates diacylglycerol and inositol 1,4,5 triphosphate, which act as second messengers to activate protein kinase C and release intracellular calcium stores, respectively. Phospholipase D (PLD) is an enzyme that mediates the hydrolysis of phospholipids such as phosphatidylcholine to generate phosphatidic acid (PA). Recently, it has been shown that PA has multiple physiological functions in processes as diverse as exocytosis and endocytosis, cellular proliferation, senescence, and vesicular transport (Venable and Obeid, 1999; Liscovitch *et al.*, 2000).

Originally discovered in plants, cDNAs encoding several isoforms of PLD have been cloned in a wide array of species such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and mammalian species including humans. Two major isoforms of mammalian PLD—PLD1 and PLD2—have been characterized (Hammond *et al.*, 1995; Colley *et al.*, 1997). Furthermore, PLD1 splice variants exist where PLD1a possesses a 38 amino acid insert absent from PLD1b. In addition to phospholipid hydrolysis, members of the PLD superfamily engage in a transphosphatidylation reaction. In the presence of primary alcohols, the phosphatidyl group is transferred to the alcohol to generate phosphatidyl-alcohol, rather than PA. Although PA can be generated from various sources, including diacylglycerol kinase and glycerol 3-phosphate acyltransferase, transphosphatidylation is a reaction that appears unique to PLD. Studies from several laboratories, in-

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Abbreviations used: ARF, ADP-ribosylation factor; PA phosphatidic acid; BtOH, butanol; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate

cluding our own, have implicated PLD and PA synthesis in vesicle budding from the Golgi apparatus (Ktistakis *et al.*, 1996; Chen *et al.*, 1997; Siddhanta and Shields, 1998). Recent work from our laboratory has shown that treatment of endocrine cells with low concentrations of 1-butanol (1-BtOH) causes inhibition of secretion, in part as a result of the fragmentation of the Golgi apparatus (Siddhanta *et al.*, 2000). These studies suggest that PLD1 isoforms are associated with Golgi membranes. Indeed, earlier work demonstrated that isolated Golgi membranes possess PLD activity activated by the small GTP binding protein ADP-ribosylation factor 1 (ARF-1) (Ktistakis *et al.*, 1995). This reaction was implicated in Golgi vesicle trafficking through coatomer recruitment (Ktistakis *et al.*, 1996), although this role is somewhat controversial (Stamnes *et al.*, 1998).

In contrast to these studies, recent results suggested a paucity of ARF-activated PLD activity associated with hepatocyte Golgi membranes (Jones *et al.*, 2000). Furthermore, morphological experiments in which HA- or GFP-PLD chimeric proteins were overexpressed in various different cell types localized PLD1 to the ER, secretory granules, and the lysosomal/endosomal compartment (Brown *et al.*, 1998; Toda *et al.*, 1999). To resolve these apparent discrepancies in the intracellular localization of PLD1, we have further investigated the subcellular localization of endogenous PLD1 in cultured rat growth hormone-secreting GH_3 cells, NRK cells, and rat liver. Here we demonstrate that although endogenous PLD1 had a diverse distribution, it was present in the Golgi apparatus, and furthermore, significant levels of the enzyme were also evident in cell nuclei.

MATERIALS AND METHODS

Antibodies

A rabbit antibody to PLD1, designated P1–P4, was raised against a mixture of four unique peptides comprising different regions of PLD1: P1, ^{1}MSLKNEPRVNTSALQK^{16}; P2, ^{144}RRQNVREEPREMPS^{157}; P3, ^{967}DDPSEDIQDPVSDK^{981}; P4, ^{1027}KEDPIRAEEELKKI^{1040} (Marcil *et al.*, 1997). An independently generated rabbit polyclonal antibody directed against the C-terminal fragment of PLD1 (amino acids 712-1074) (Yamazaki *et al.*, 1999) was also used for some experiments. Rabbit antibody to TGN38 was a generous gift from Dr. Sharon Milgram (University of North Carolina, Chapel Hill, NC); mouse monoclonal antibody to mannosidase II (53FC3) was kindly provided by Dr. Brian Burke (University of Calgary, Calgary, Canada); rabbit anti-connexin43 was a gift of Dr. Eliot Hertzberg (Albert Einstein College of Medicine, Bronx, New York). Rabbit anti-rat lgp120 was a gift from Dr. Ira Mellman (Yale University Medical School, New Haven, CT). Mouse monoclonal antibody to Rab5 was purchased from Transduction Laboratories (Lexington, KY); Cy3-conjugated goat anti-rabbit secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA); FITC-conjugated goat anti-mouse secondary antibodies were from Cappel (Durham, NC). Mouse mAbs to HA were purchased from Boehringer Mannheim (Indianapolis, IN).

Immunofluorescence Microscopy

GH_3 and NRK cells were grown on poly-L-lysine–coated glass coverslips as described (Austin *et al.*, 1996; Lowe *et al.*, 2000). Cells were either untreated or pretreated with 5 μg/ml brefeldin A (BFA) for 40 min, 1.5% 1-butanol for 40 min, or 10 μM nocodazole for 4 h at 37°C, and fixed in 3% paraformaldehyde. Samples were incubated for 1 h at room temperature with primary antibodies diluted in solution I (0.5% BSA, 0.2% saponin, 1% fetal calf serum in PBS) before use. The samples were then treated with appropriate secondary antibodies also diluted in solution I. In some instances, cells were subsequently treated with 1 μg/ml Hoechst 33258 (Sigma Aldrich, St. Louis, MO) for 10 min to stain the cell nuclei. After extensive washing, the coverslips were mounted onto slides and examined using an Olympus (Melville, NY) IX 70 microscope with 60× N.A. 1.4 planapo optics using a Photometrics (Tucson, AZ) Censys cooled CCD camera. Z-series images were obtained through the depth of cells using a step size range of 0.1–0.4 μm and projected using the maximum pixel method. Deconvolution was performed with a Vaytek (Fairfield, IA) PowerHazeBuster running on a Macintosh G3, and maximum pixel projections were rendered with I.P. Lab Spectrum (Scanalytics, Fairfax, VA). Images were processed using Adobe Photoshop software at identical settings. Controls were imaged to rule out background fluorescence or bleed-through between Cy3 and FITC channels.

Transfection of NRK cells

Cells were grown on poly-L-lysine–coated coverslips and transfected with DNA encoding HA-tagged human PLD1 using Effectene transfection reagents (Qiagen, Valencia, CA) according to the manufacturer's specifications. After 24 h exposure to DNA, the medium was replaced, and the cells were observed after 48 h of recovery.

Cryo-immunogold Electron Microscopy

GH_3 cells were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde, 0.25 M HEPES, pH 7.4, and embedded in 10% gelatin. The cells were cryoprotected by infiltration with 2.3 M sucrose in PBS. After liquid nitrogen freezing, 90-nm sections were cut using a Leica (Nussloch, Germany) UCT cryoultramicrotome. Sections were placed on grids and immunolabeled with antibodies against PLD1 (P1–P4) followed by goat anti-rabbit IgG conjugated to 10-nm gold particles (Aurion, Wageningen, The Netherlands). Samples were then treated with 2% uranyl acetate, pH 7.0, and embedded in 0.75% methylcellulose. The grids were examined using a JEOL 1200 EX transmission electron microscope.

Determination of PLD Activity in Golgi Membranes Isolated from GH_3 Cells

Endogenous PLD activity was determined via transphosphatidylation using 1-butanol in an assay modified from Wakelam *et al.* 1995 (Siddhanta *et al.*, 2000). GH_3 cells were grown to ~70% confluency and radiolabeled with 6 μCi [9,10-^{3}H(N)]-oleic acid for 24–36 h, after which they were harvested. The radiolabeled cells were homogenized, and the homogenate was loaded onto a sucrose equilibrium density gradient that was centrifuged for 15 h at 150,000 × g_{av} (Xu and Shields, 1993; Austin and Shields, 1996). To determine PLD activity, each gradient fraction was incubated in the presence or absence of ARF-1 and 0.3% 1-butanol. The lipids were extracted with chloroform–methanol and analyzed by TLC (Chen *et al.*, 1997). Each gradient fraction was also assayed for the presence of TGN38 by immunoblotting (Austin and Shields, 1996).

Preparation of Rat Liver Golgi Membranes

Golgi membranes were purified by adaptation of the method of Slusarewicz *et al.* (1994) (to be described elsewhere) (Sweeney and Shields, unpublished observations) using a sucrose step gradient. After gradient centrifugation of the rat liver homogenate, each fraction was assayed for sialyl transferase activity (Xu and Shields, 1993) as well as by Western blotting for the presence of TGN38, Rab5, and connexin43, -TGN, early endosome, and plasma membrane marker proteins, respectively. Immunoblotting using the rabbit polyclonal antibody P1–P4 was used to detect PLD1 in each gradient fraction.

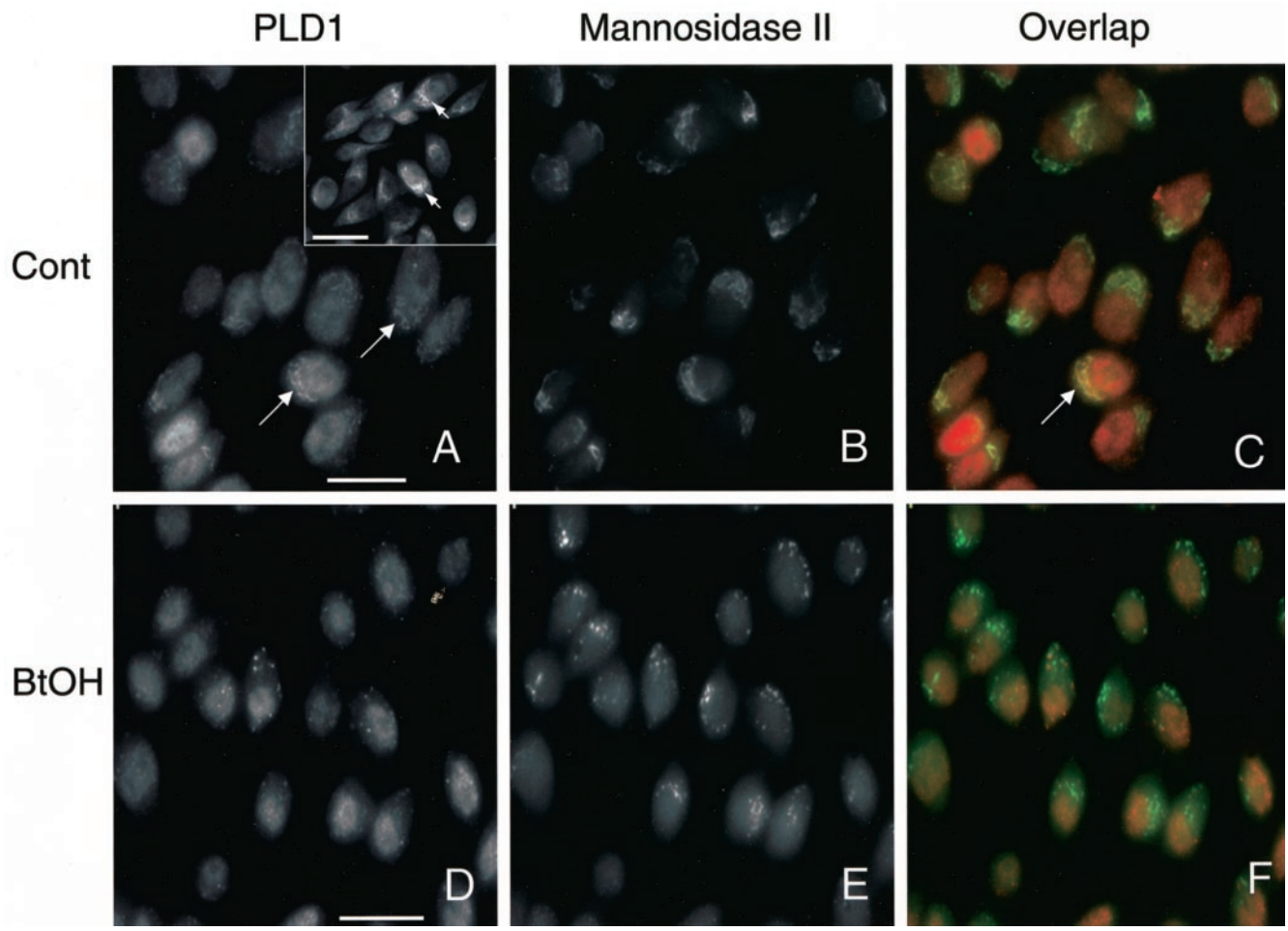

Figure 1. Colocalization of PLD1 with Golgi markers in GH_3 cells. GH_3 cells were incubated with media alone (A–C) or with 1.5% 1-BtOH (D–F) for 40 min at 37°C. After incubation, the cells were prepared for double immunofluorescence microscopy (MATERIALS AND METHODS) using a rabbit antibody to PLD1 (A and D) and monoclonal antibody 53FC3 to the *cis*/medial Golgi marker mannosidase II (B and E). Mannosidase II was visualized using FITC-conjugated goat anti-mouse IgG, and PLD1 was localized using Cy3 goat anti-rabbit IgG. Each sample is from the same field of cells. To demonstrate overlap of PLD1 and mannosidase II, the images were merged (C and F); yellow regions indicate complete localization. (C) The arrow indicates overlap between PLD1 and mannosidase II. All micrographs are projected Z-series images using a cooled CCD camera (MATERIALS AND METHODS). Bar, 10 μm. (A) Inset, single staining of GH_3 cells using antibody P1–P4 directed against PLD1 peptides; arrows indicate areas of PLD1 enrichment.

RESULTS

PLD1 Localizes to the Golgi Apparatus

Previous work from our laboratory demonstrated PLD-stimulated release of growth hormone-containing nascent secretory vesicles from permeabilized rat anterior pituitary GH_3 cells. It was likely that this PLD activity was associated with Golgi membranes (Chen *et al.*, 1997). Consequently, our initial experiments were designed to determine the intracellular localization of endogenous PLD1 in these endocrine cells using immunofluorescence microscopy. To that end, we used a rabbit polyclonal antibody directed against several unique PLD1 peptides, designated P1–P4 (Figure 1). Although PLD1 had a diffuse distribution including nuclear staining, the enzyme was also localized to distinct perinuclear regions of the cell corresponding to the Golgi apparatus, as evidenced by its colocalization with the medial Golgi marker enzyme, mannosidase II (Figure 1, A–C). It was possible that the apparent colocalization of PLD1 with mannosidase II resulted from overlap of the fluorescence signals between the Cy3 and the FITC channels. To exclude this possibility, GH_3 cells were treated with the anti-PLD1 antibody alone (Figure 1A, inset); these cells manifested an identical pattern of PLD1 staining. In addition, the fine, lace-like ribbon staining of the Golgi apparatus was similar to our previous observations using antibodies directed against a bona fide TGN marker protein such as TGN38 (Siddhanta *et al.*, 2000).

Although unlikely (Ktistakis *et al.*, 1996), it was possible that the Golgi localization of PLD1 was a property exclusive to endocrine cells. To exclude this possibility, we examined the immunolocalization of the enzyme in NRK cells, a rat

kidney epithelial cell line used extensively in the study of Golgi organization (Lowe *et al.*, 2000) (Figure 2). In these cells PLD1 also manifested a diffuse, reticular staining pattern; however, there was clear enrichment of PLD1 in the perinuclear Golgi region and considerable overlap with the lace-like staining of the *cis*-Golgi peripheral membrane protein GM130 (Figure 2, D–F) and the medial Golgi enzyme, mannosidase II (A–C). To control for the specificity of PLD1 localization, a second antiserum raised against the C-terminal 300 amino acids of PLD1 was used (Figure 2G). A similar pattern of PLD1 staining exhibiting diffuse perinuclear Golgi localization was also apparent. Most importantly, both antisera showed significant enrichment of PLD1-immunoreactive material in the Golgi region of the cell. As a control for the specificity of the P1–P4 antiserum, peptide competition experiments were performed. The antiserum was preincubated with increasing concentrations of the PLD1 peptides before immunofluorescence microscopy (Figure 2, H and I). Even in the presence of the lowest concentration of competitor peptides (1.3 μg), PLD1 immunostaining was abolished completely (Figure 2H) further demonstrating the fidelity of the antibody staining and its localization.

Given the pleiotropic functions of PLD (Liscovitch *et al.*, 2000), it was likely that the enzyme was present on other organelles in addition to the Golgi apparatus. To investigate this possibility, PLD1 localization to the ER, late endosomes/lysosomes, and plasma membrane/early endosomal compartments was determined using antibodies to BiP, lgp120, and the transferrin receptor, respectively (Figure 3). No significant overlap was observed between PLD1 and BiP when NRK cells were stained with antibodies to these two proteins. Unlike PLD1, BiP had a diffuse reticular pattern characteristic of the ER, suggesting that little PLD1 was present in this compartment (Figure 3, A–C). Similar results were obtained on quantitative analysis of EM gold-labeled cryoelectron micrographs, where PLD1 displayed little localization in the ER (Table 1). In contrast, staining with antibodies to lgp120, a membrane protein present in late endosomes and lysosomes, showed partial overlap between PLD1 in the perinuclear Golgi region but little colocalization in peripheral lysosomes (Figure 3, D–F). Similarly, there was overlap in localization between PLD1 and the transferrin receptor in the Golgi region, but little, if any, costaining of the plasma membrane or transferrin-containing endosomes (Figure 3, G and H). Together, these results suggest that in NRK cells, although endogenous PLD1 exhibits a diffuse staining pattern, it does localize to the perinuclear Golgi region and overlaps with proteins that exit and/or recycle through the *trans*-Golgi compartment.

The immunofluorescence data suggested that PLD1 was present in Golgi stacks. To confirm that PLD1 was localized throughout the Golgi apparatus in GH_3 cells, we used immunogold labeling of ultrathin cryosections incubated with the P1–P4 antibody (Figure 4). Gold particles were evident in the Golgi apparatus and on multiple saccules, indicating that PLD1 was present throughout the organelle and not enriched in particular cisternae. No staining was seen in the absence of either the P1–P4 antibody or secondary gold-conjugated antibodies. In agreement with the immunofluorescence data, gold particles were also evident in the nucleus (Figure 4B). Quantitative analysis of the immunogold distribution demonstrated that ~25% of PLD1 was associated with the Golgi apparatus and 28% with nuclei (Table 1). Most significantly, these data confirmed the immunofluorescence microscopy results and demonstrated that PLD1 is present on membranes of the Golgi apparatus.

Recent work from our laboratory demonstrated that during treatment with low concentrations of 1-BtOH, which inhibits production of phosphatidic acid, the Golgi apparatus undergoes complete fragmentation; this correlates with diminished PtdIns(4,5)P_2 synthesis (Siddhanta *et al.*, 2000). We argued that if PLD1 were tightly associated with Golgi membranes via this lipid, then on inhibition of PtdIns(4,5)P_2 synthesis, the enzyme would dissociate from the membrane. After treatment with 1-BtOH, the Golgi lace-like perinuclear staining of mannosidase II was completely disrupted, as was that of PLD1 (Figure 1, D–F). In agreement with our idea, although some PLD1 localized to the fragmented Golgi apparatus, much of the PLD1 and mannnosidase II colocalization was disrupted. This suggested that PLD may have dissociated from the membrane. It is noteworthy that the PLD1 nuclear staining was largely unaffected by alcohol treatment. Furthermore, the distribution of the ER markers calnexin, ribophorin I, and BiP was unaffected by 1-BtOH treatment (our unpublished results).

Brefeldin A and Nocodazole Affect PLD1 Localization

BFA disrupts the structure of the Golgi apparatus, leading to redistribution of Golgi enzymes into the ER (Lippincott-Schwartz *et al.*, 1989). In contrast, fragmentation of the Golgi in response to nocodazole, which depolymerizes microtubules, causes clumping of Golgi-derived vesicles at the cell periphery. It was of interest, therefore, to compare the localization of PLD1 when cells were treated with either of these drugs (Figure 5). In contrast to control cells, BFA-treated GH_3 cells exhibited little or no distinct perinuclear mannosidase II staining. Instead, they showed a very diffuse localization consistent with an ER localization, confirming previous observations (Lippincott-Schwartz *et al.*, 1989). Similarly, the Golgi localization of PLD1 was greatly diminished and also had a diffuse reticular appearance. Surprisingly, the nuclear localization of PLD1 was enhanced in response to BFA treatment (Figure 5A). Disruption of the Golgi apparatus by nocodazole resulted in the fragmentation of PLD1 immunoreactive material; however, unlike treatment with 1-BtOH, PLD1 exhibited tight colocalization with mannosidase II-containing Golgi fragments (Figure 5, D–F). In contrast to BFA treatment, there was no enhancement of nuclear PLD1 localization in nocodazole-treated cells (compare A and D). Taken together, these data strongly suggest that although PLD1 manifests a diffuse staining pattern in cells, a significant fraction of the enzyme is tightly associated with the Golgi apparatus.

Subcellular Fractionation

To confirm that PLD1 was associated with Golgi membranes, subcellular fractionation was used. Two different cell types were used to separate the Golgi apparatus from other organelles: namely, the hormone-producing GH_3 cells and rat liver cells, which possess regulated and constitutive secretory pathways, respectively. When the homogenate from GH_3 cells was fractionated on an equilibrium density gradi-

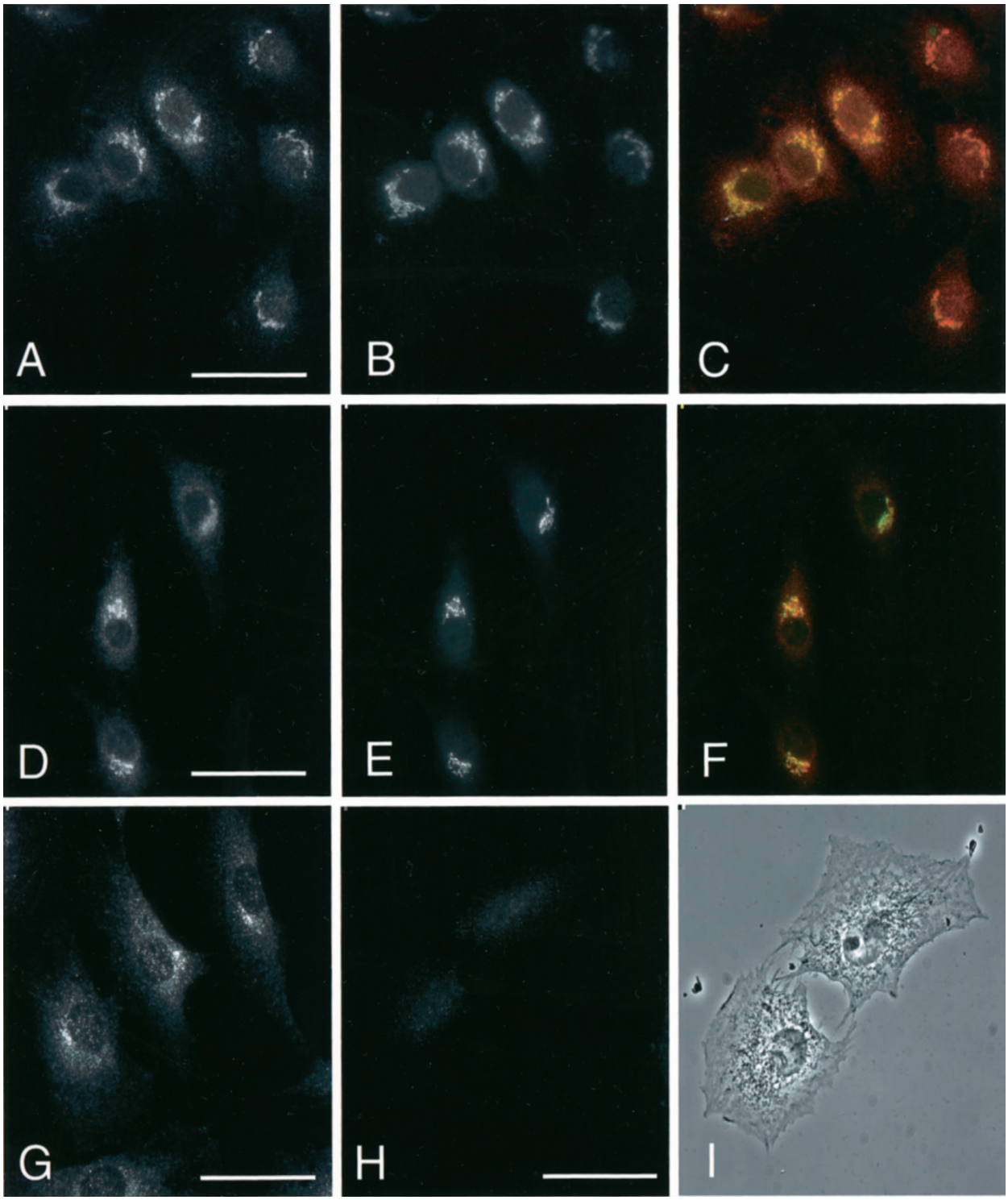

Figure 2. Immunolocalization of PLD1 in rat NRK cells. Cells were stained with rabbit antibodies directed against PLD1 peptides P1–P4 (A and D). These cells were costained with either mAbs 53FC3 directed against mannosidase II (B) or GM130 (E). C and F show the overlaping regions between PLD1 and the Golgi marker proteins. (G) Cells were also stained with an independently generated rabbit antibody to the C-terminal of PLD1 (MATERIALS AND METHODS). (H) Preincubation of the P1–P4 antibody with 1.3 μg of antigenic peptide before immunolocalization; (I) corresponding phase-contrast image. Images are from projected Z-series. Bar, 10 μm.

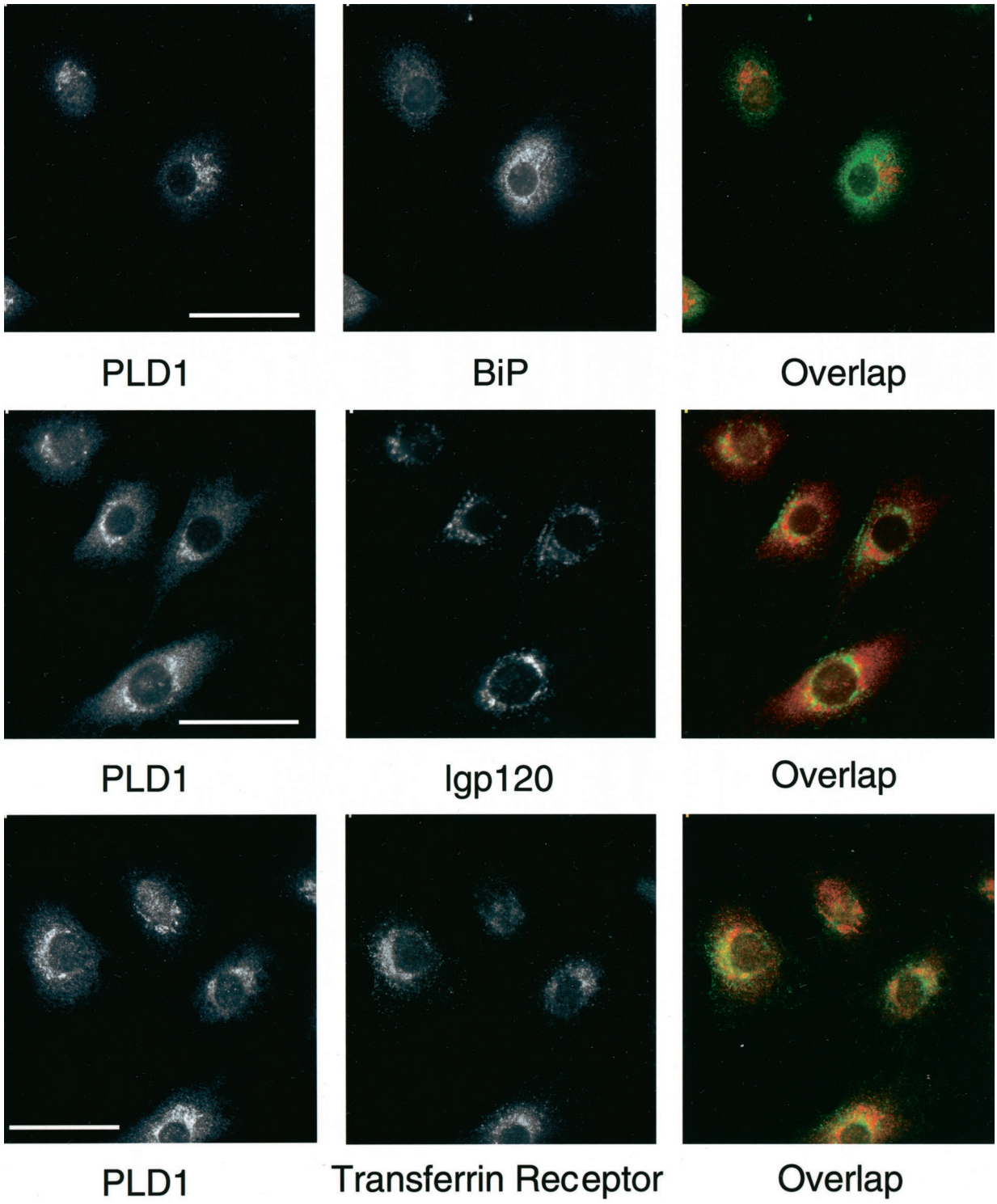

Figure 3. PLD1 localization with different organelles in rat NRK cells. Cells were prepared for immunofluorescence microscopy and incubated with rabbit anti-P1–P4 antibodies to PLD1 and costained with mAbs to the ER protein BiP, with the late endosome/lysosome marker lgp 120, or with a marker for the plasma membrane and early endosomes, transferrin receptor. Note the partial overlap between PLD1 with lgp120 and transferrin receptor but only minimal colocalization with BiP. Bar, 10 μm.

Table 1. Distribution of immunoreactive PLD1 gold particles in GH_3 cells

Golgi apparatus	Nucleus	Plasma membrane	Endoplasmic reticulum	Other
24.7 ± 2.4%	28.4 ± 7.9%	13.2 ± 5.2%	4.4 ± 0.5%	29.3 ± 9.6%

The subcellular distribution of PLD1 immunoreactive gold particles was quantitated from 14 cells. The percentage of total gold particles within each organelle was calculated. The designation "Other" refers to mitochondrial and vesicular cytosolic structures.

ent, which separates the ER from the Golgi apparatus, PLD enzyme activity was present in those fractions containing Golgi marker enzymes, and its specific activity was enriched ~10-fold over the homogenate (Figure 6, fractions 2 and 3). PLD activity was also detected near the load zone of the gradient and in fraction 7; the latter corresponds to plasma membrane material (Austin *et al.*, 1996); however, the specific activity of this enzyme was <10% of the Golgi membrane fractions. Rat liver Golgi membranes were isolated by gradient centrifugation, and fractions were assayed for activity of the *trans*-Golgi enzyme sialyl transferase and markers of early endosomes (Rab5), plasma membrane (connexin43), as well as endogenous PLD by Western blotting (Figure 7). Rab5 was present mostly at the top of the gradient (fractions 1–10), and no significant levels were detected in the Golgi membrane fractions. Sialyl transferase activity was enriched in fractions 23–29, which also had the highest levels of TGN38 immunoreactivity (fractions 27–29). Approximately 26–30% of total PLD1-immunoreative material was also present in these Golgi fractions that lacked connexin43, an integral membrane protein of hepatocyte gap junctions (Figure 7). Additionally, a significant level of PLD1 was present in the connexin43-enriched plasma membrane fractions 31–39, consonant with its activity in multiple or-

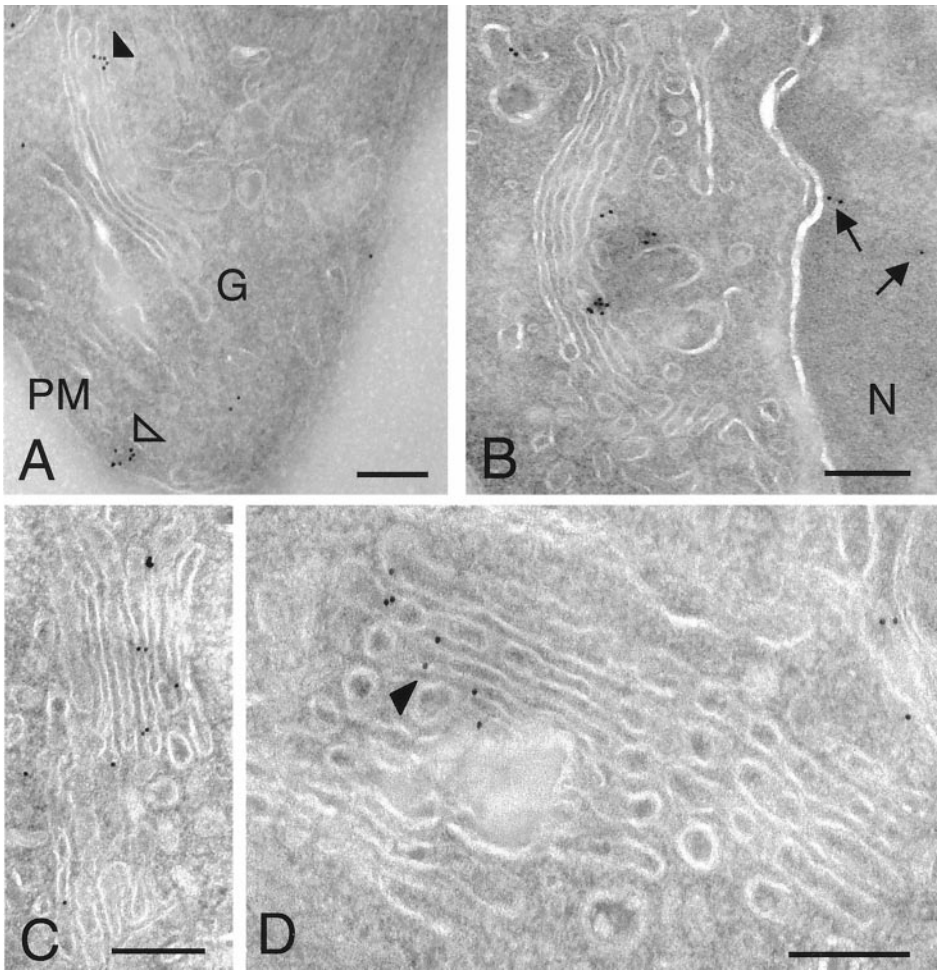

Figure 4. Localization of PLD1 by cryo-immunoelectron microscopy. GH_3 cells were prepared for cryo-immunoelectron microscopy (MATERIALS AND METHODS). Sections were labeled with polyclonal antibodies directed against PLD1 (P1–P4) followed by secondary antibodies conjugated to 10-nm gold particles. Staining revealed PLD1 localization to the plasma membrane (A, PM, open arrowhead) and Golgi apparatus (G, indicated by filled arrowheads in A and D), as well as throughout the Golgi cisternae (B–D) and nuclei (B, N, arrows). Bars, 0.2 μm (B–D); 0.5 μm (A).

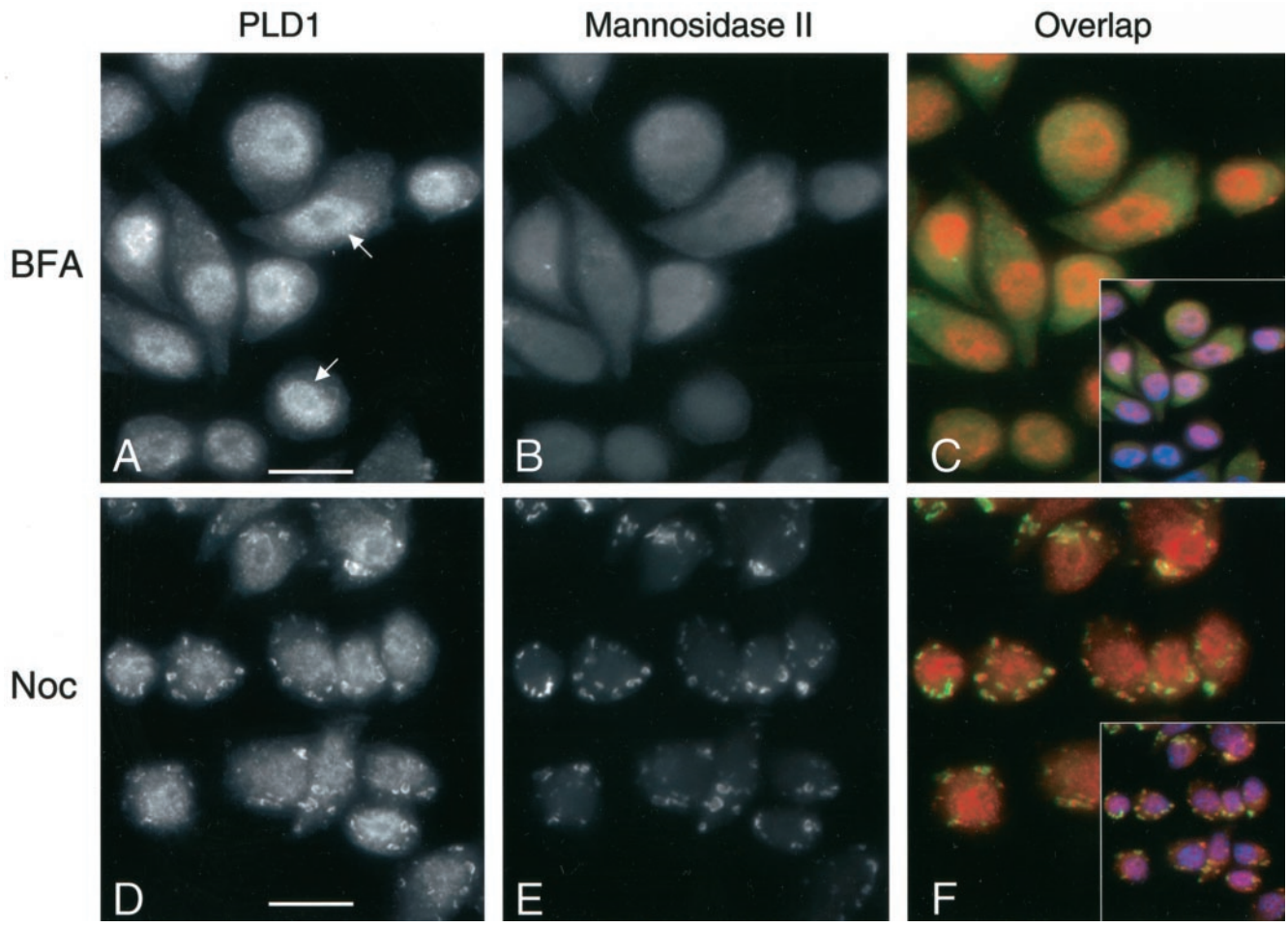

Figure 5. Brefeldin A and nocodazole alter the localization of Golgi-associated PLD1. GH_3 cells were incubated with 5 μg BFA/ml for 40 min (A–C) or with 10 μM nocodazole for 4 h at 37°C (D–F). After incubation, the cells were prepared for microscopy using the rabbit PLD1 antibody (P1–P4) or the 53FC3 monoclonal antibody to mannosidase II. PLD1 and mannosidase II were visualized using appropriate anti-rabbit and anti-mouse IgG antibodies (Figures 1 and 2). (C and F) Inset, nuclear staining with Hoechst 33258 dye. Note overlap between cell nuclei and PLD1. Bar, 10 μm. (A) Arrow indicates PLD1 enrichment in cell nuclei.

ganelles (Liscovitch *et al.*, 2000). These biochemical data support the immunolocalization results and together suggest that a significant fraction of PLD1 is associated with Golgi membranes in cells with either constitutive or regulated secretory pathways.

Overexpression of PLD1 Leads to Its Mislocalization

Previous studies (Brown *et al.*, 1998; Toda *et al.*, 1999) in which epitope-tagged forms of PLD1 were overexpressed in several different cell types suggested that the enzyme is present in lysosomes, endosomes, and secretory granules but largely absent from the Golgi apparatus. On the basis of the foregoing data, we hypothesized that overexpression of PLD1 may result in its mislocalization to post-Golgi compartments. To test this idea, an expression plasmid encoding HA-tagged PLD1 (Colley *et al.*, 1997) was transiently transfected into NRK cells, and the localization of total PLD1 was compared with that of the exogenously expressed enzyme (Figure 8). High levels of overexpressed PLD1 resulted in the absence of significant Golgi region staining (Figure 8, D–F). Instead, PLD1-immunoreactive material was evident in a heterogeneous population of small and large peripheral vesicular structures; however, in cells that expressed relatively low levels of the HA-tagged enzyme (Figure 8, A–C), immunoreactive PLD1 was evident in the perinuclear Golgi region (Figures 1 and 2). These results demonstrated that overexpression of PLD1 resulted in significant mislocalization of the enzyme from the perinuclear Golgi region to a heterogeneous class of vesicular structures.

DISCUSSION

Different forms of PLD have been implicated in mediating various key processes in cell metabolism, including cell division, signal transduction, and vesicle trafficking (Lisco-

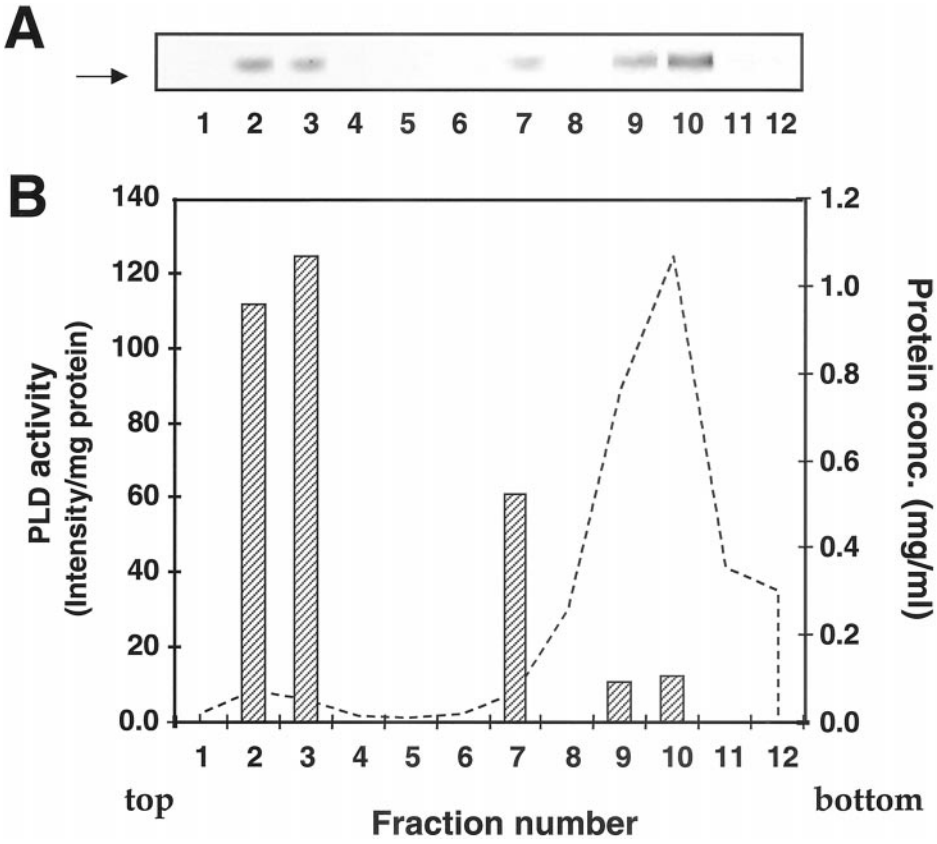

Figure 6. PLD1 enzyme activity cofractionates with the Golgi apparatus in endocrine cells. GH_3 cells were incubated with 10 μCi/ml ^{3}H-oleic acid for 24 h to radiolabel phospholipids, after which the cells were homogenized. The homogenate was fractionated on a floatation gradient designed to separate the Golgi apparatus from total microsomes (MATERIALS AND METHODS). (A) An aliquot of each gradient fraction was assayed for ARF-1–stimulated PLD activity in the presence of 0.3% 1-BtOH, and the products were analyzed by TLC followed by fluorography. The arrow indicates the mobility of PtdBtOH. (B) The TLC plate was scanned using a densitometer, and the band intensities corresponding to PtdBtOH were quantitated using the Image Quant program (Siddhanta *et al.*, 2000). An aliquot of each gradient fraction was also assayed for protein concentration. Hatched bars, PLD activity expressed as pixel intensity per milligram protein. Dashed line, distribution of total protein (mg/ml). TGN38 is concentrated in fractions 2 and 3 (Austin and Shields, 1996).

vitch *et al.*, 2000). To date, three forms of human PLD have been characterized: PLD1a is a cytosolic enzyme that is tightly bound to membranes, and a shorter splice variant, PLD1b, has similar properties (Hammond *et al.*, 1997). PLD2, which is regulated by different signaling molecules from PLD1, is associated with the plasma membrane (Colley *et al.*, 1997). Both overexpressed forms of PLD1 and PLD2 have been localized to the Golgi apparatus, endosomes, lysosomes, and secretory granules; the presence of PLD in secretory granules suggests a possible function in regulated exocytosis (Brown *et al.*, 1998). A number of laboratories, including our own, have demonstrated the presence of an ARF-1–stimulated PLD enzymatic activity associated with Golgi membranes (Ktistakis *et al.*, 1995; Chen *et al.*, 1997). Additionally, these studies also showed that the product of PLD-mediated PtdCho hydrolysis, PA, functions in coat protein recruitment to Golgi membranes (Ktistakis *et al.*, 1996) and in the budding of nascent secretory vesicles from the TGN (Chen *et al.*, 1997). Consequently, localization of PLD to the Golgi apparatus would be consistent with a function in regulating vesicle trafficking in the late secretory pathway; however, the localization of endogenous PLD1 to Golgi membranes or the mechanism by which different PLD isoforms are recruited to specific membranes has not been determined, in part because of the low levels of the enzyme in many cells. Furthermore, in contrast to the above results, several recent reports suggest that PLD1 is absent from the Golgi apparatus. To address the first question and resolve the controversy over its intracellular localization, we have used highly specific antibodies directed against several PLD1 epitopes together with immunoelectron and fluorescence microscopy to localize the endogenous enzyme in endocrine and nonendocrine cells.

Our data demonstrated that although PLD1 had a diffuse distribution in pituitary GH_3 cells and NRK cells, both cell types exhibited areas of enhanced perinuclear staining that overlapped with Golgi markers, a result consistent with localization to the Golgi apparatus (Figures 1–4). In agreement with the immunofluorescence data, immunogold cryoelectron microscopy (Figure 4) confirmed the presence of PLD1 on Golgi membranes, on the plasma membrane, and in nuclei. Quantitative analysis suggested that PLD1 was present throughout the Golgi apparatus, where ~30% was associated with cisternal rims (our unpublished results). It is noteworthy that in analyzing the overall cellular distribution of PLD1-immunoreactive gold particles, ~25% was present on the Golgi apparatus of GH_3 cells (Table 1), a value very close to that found for the rat liver enzyme that cofractionated with Golgi membranes on sucrose gradient centrifugation (Figure 7). Although similar levels of PLD1 were present in the Golgi apparatus, its plasma membrane distribution was different in GH_3 cells and rat liver (Figure 7 and Table 1). Given that PLD1 is a downstream effector of diverse signal transduction events (Liscovitch *et al.*, 2000), it is not surprising that its association with and activity on membranes would be dynamic and vary considerably in response to different stimuli and in different cells. Most

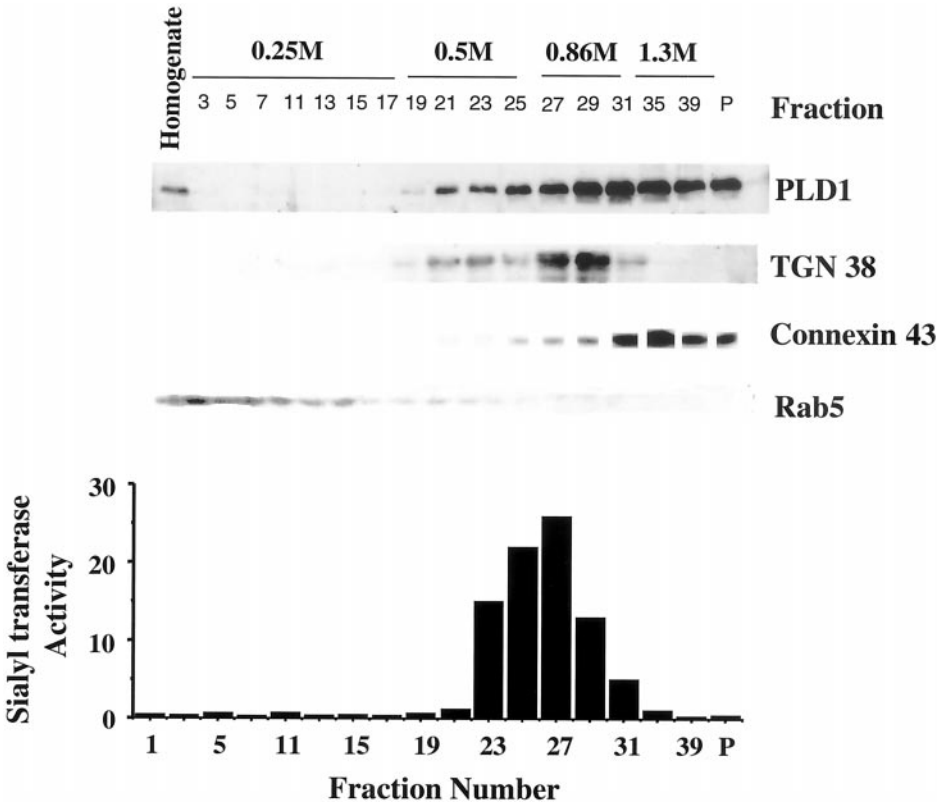

Figure 7. PLD1 cofractionates with the Golgi apparatus and plasma membrane in rat hepatocytes. A postnuclear supernatant from a rat liver homogenate was loaded onto a step gradient containing the indicated concentrations of sucrose (Slusarewicz *et al.*, 1994). Gradients were centrifuged in a Beckman SW28 rotor for 2 h at 100,000 × *g*. One milliliter fractions were collected, and an aliquot of each was analyzed by SDS-PAGE followed by transfer to PVDF membranes that were immunoblotted with antisera to PLD1 (P1–P4), TGN38, connexin43, or Rab5. An aliquot of each fraction was also assayed for sialyl transferase activity (filled bars) (Xu and Shields, 1993). Ordinate, Percent of total sialyl transferase activity in each fraction.

importantly, the similarity of PLD1 Golgi distribution in pituitary GH_3 cells and rat liver and its determination by independent experimental procedures further strengthens our conclusions that the enzyme is associated with the Golgi apparatus.

Inhibition of PA synthesis by treatment with low concentrations of primary alcohols leads to diminished PtdIns(4,5)P_2 synthesis and fragmentation of the Golgi apparatus (Siddhanta *et al.*, 2000). We exploited this observation to demonstrate further the Golgi association of PLD1 (Figure 1). When cells were treated with 1-butanol, the Golgi apparatus was fragmented completely, and most of the immunoreactive PLD1 dissociated from the mannosidase II-localized membrane fragments. A possible interpretation of these observations is that in addition to PtdIns(4,5)P_2 being a cofactor for PLD enzyme activity (Pertile *et al.*, 1995), it mediates the enzyme's membrane association via a putative PH domain (Hodgkin *et al.*, 2000). Consequently, in the presence of diminished PtdIns(4,5)P_2 levels, PLD1 binding would be weakened and dissociate from the Golgi membranes. In contrast, when cells were treated with nocodazole, which causes fragmentation of the Golgi apparatus via depolymerization of microtubules (Yang and Storrie, 1998), the localization of PLD1 mirrored that of the disrupted Golgi fragments (Figure 5). This suggests that microtubules per se are not required for PLD membrane binding.

In agreement with Ktistakis *et al.* (1995), during sucrose gradient centrifugation, PLD was localized to and its specific activity was enriched in fractions corresponding to Golgi membranes (Figures 6 and 7). Together, these observations confirm previous results from our laboratory and others demonstrating PLD activity in isolated Golgi membranes (Liscovitch *et al.*, 1999). Most importantly, the data are consistent with a function of PLD in Golgi vesicle budding. To exclude possible artifacts of cell fractionation, we used different gradient centrifugation techniques to isolate Golgi membranes from distinctly different cell types: a rat pituitary somatomammotrope cell line and rat liver. In both cases, immunoreactive PLD1 was evident in the Golgi fractions, and PLD enzymatic activity was also present in Golgi membranes isolated from pituitary GH_3 cells. When rat liver was used, our cell fractionation data showed significant levels of PLD1 in both the Golgi fractions and plasma membrane (Figure 7). Our present and earlier results (Ktistakis *et al.*, 1995; Chen *et al.*, 1997) differ from those of Jones *et al.* (2000), who reported only minor levels of ARF-1–stimulated PLD activity in Golgi membranes; most likely these discrepancies are related to the procedures used for organelle isolation.

In contrast to the above findings, when GFP- or HA-tagged PLD1 were overexpressed, the enzyme was localized to several organelles, including lysosomes and secretory granules; little if any was detected in the Golgi apparatus (Brown *et al.*, 1998; Toda *et al.*, 1999). Our data confirmed these findings when HA-tagged PLD1 was overexpressed in NRK cells and demonstrated that the distribution of the enzyme was significantly different from endogenous PLD1 (Figure 8). On the basis of our data, we speculate that transient overexpression of PLD

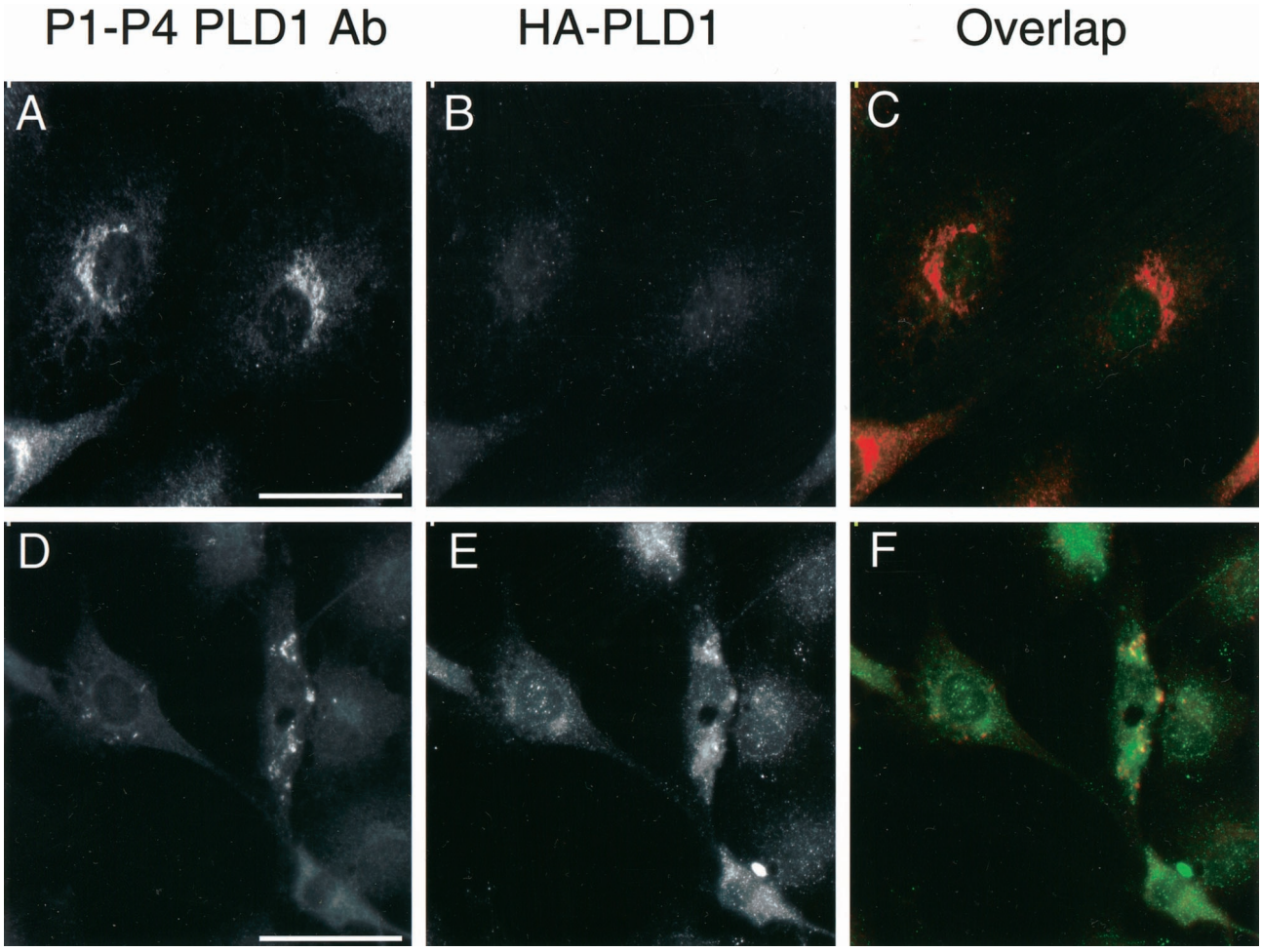

Figure 8. Overexpression of wild-type PLD1 in NRK cells disrupts PLD1 perinuclear localization. Cells were transiently transfected with DNA encoding N-terminally HA-tagged PLD1 and stained with P1–P4 PLD1 antibodies (A and D) and monoclonal anti-HA antibodies (B and E). Top row, low level of HA-PLD1 expression; bottom row, high level of HA-PLD1 expression. Bar, 10 μm.

chimeras most likely saturated membrane binding sites, leading to mislocalization of the enzyme. It is possible that PLD overexpression was toxic, resulting in high levels of PA synthesis in inappropriate organelles. Such cells might compensate by degrading the enzyme via the lysosomal compartment. In agreement with this idea, both Brown *et al.* (1998) and Toda *et al.* (1999) found significant levels of PLD1 colocalized with lysosomes. In this context, it is noteworthy that it has been particularly difficult to generate stable cell lines expressing exogenous PLD, possibly as a consequence of PA toxicity (Frohman, unpublished observations).

Strikingly, in GH_3 cells, PLD1-immunostaining was also evident in the nucleus (Figure 1). Although previous work has demonstrated PLD enzyme activity in cell nuclei (Balboa *et al.*, 1995; Baldassare *et al.*, 1997; Martelli *et al.*, 1999), its localization had not been shown. Interestingly, collapse of the Golgi apparatus in response to BFA altered PLD localization to the ER and significantly enhanced its nuclear staining (Figure 5). This observation contrasts with that of Toda *et al.* (1999), who reported little effect of BFA on the localization of HA-PLD1b in NRK cells. Nevertheless, in agreement with our results, the data of Toda *et al.* (1999) showed that in some cells there was redistribution of PLD1b to the nuclear envelope and nucleoplasm. It is unclear why nuclear PLD1 staining was enhanced after pretreatment of cells with BFA. The close proximity of the ER to the nucleus may foster association of PLD1 with the nuclear envelope or stimulate its nuclear translocation; however, given its large size (1074 amino acids) and propensity for membrane association, it is highly unlikely that PLD1 would diffuse into the nucleus. Interestingly, phosphoinositides have been detected in nuclei, and their synthesis uses enzymes that appear to be similar or identical to the cytoplasmic counterparts (Boronenkov *et al.*, 1998). These authors demonstrated that several phosphatidylinositol kinases and PtdIns(4,5)P_2 were associated with "nuclear speckles," which have been

implicated in pre-mRNA processing. Because type I PtdIns(4P) 5-kinase activities are stimulated by phosphatidic acid (Jenkins *et al*., 1994; Siddhanta *et al*., 2000), our observations of PLD1 immunoreactivity in nuclei suggests that this enzyme might function in mRNA processing via regulation of PtdIns(4,5)P_2 synthesis. Currently, we are investigating this possibility.

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REFERENCES

Austin, C.D., and Shields, D. (1996). Formation of nascent secretory vesicles from the trans-Golgi network of endocrine cells is inhibited by tyrosine kinase and phosphatase inhibitors. J. Cell Biol. *135*, 1471–1483.

Balboa, M.A., Balsinde, J., Dennis, E.A., and Insel, P.A. (1995). A phospholipase D-mediated pathway for generating diacylglycerol in nuclei from Madin-Darby canine kidney cells. J. Biol. Chem. *270*, 11738–11740.

Baldassare, J.J., Jarpe, M.B., Alferes, L., and Raben, D.M. (1997). Nuclear translocation of RhoA mediates the mitogen-induced activation of phospholipase D involved in nuclear envelope signal transduction. J. Biol. Chem. *272*, 4911–4914.

Boronenkov, I.V., Loijens, J.C., Umeda, M., and Anderson, R.A. (1998). Phosphoinositide. Signaling pathways in nuclei are associated with nuclear speckles containing pre-mRNA processing factors. Mol. Biol. Cell *9*, 3547–3560.

Brown, F.D., Thompson, N., Saqib, K.M., Clark, J.M., Powner, D., Thompson, N.T., Solari, R., and Wakelam, M.J. (1998). Phospholipase D1 localizes to secretory granules and lysosomes and is plasma-membrane translocated on cellular stimulation. Curr. Biol. *8*, 835–838.

Chen, Y.G., Siddhanta, A., Austin, C.D., Hammond, S.M., Sung, T.C., Frohman, M.A., Morris, A.J., and Shields, D. (1997). Phospholipase D stimulates release of nascent secretory vesicles from the trans-Golgi network. J. Cell Biol. *138*, 495–504.

Colley, W.C., Sung, T.C., Roll, R., Jenco, J., Hammond, S.M., Altshuller, Y., Bar-Sagi, D., Morris, A.J., and Frohman, M.A. (1997). Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. Curr. Biol. *7*, 191–201.

De Camilli, P., Emr, S.D., McPherson, P.S., and Novick, P. (1996). Phosphoinositides as regulators in membrane traffic. Science *271*, 1533–1539.

Hammond, S.M., Altshuller, Y.M., Sung, T.C., Rudge, S.A., Rose, K., Engebrecht, J., Morris, A.J., and Frohman, M.A. (1995). Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family. J. Biol. Chem. *270*, 29640–29643.

Hammond, S.M., Jenco, J.M., Nakashima, S., Cadwallader, K., Gu, Q., Cook, S., Nozawa, Y., Prestwich, G.D., Frohman, M.A., and Morris, A.J. (1997). Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C-alpha. J. Biol. Chem. *272*, 3860–3868.

Hodgkin, M.N., Masson, M.R., Powner, D., Saqib, K.M., Ponting, C.P., and Wakelam, M.J. (2000). Phospholipase D regulation and localization is dependent upon a phosphatidylinositol 4,5-biphosphate-specific PH domain. Curr. Biol. *10*, 43–46.

Jones, D.H., Morris, J.B., Morgan, C.P., Kondo, H., Irvine, R.F., and Cockcroft, S. (2000). Type I phosphatidylinositol 4-phosphate 5-kinase directly interacts with ADP-ribosylation factor 1 and is responsible for phosphatidylinositol 4,5-bisphosphate synthesis in the Golgi compartment. J. Biol. Chem. *275*, 13962–13966.

Jenkins, G.H., Fisette, P.L., and Anderson, R.A. (1994). Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. J. Biol. Chem. *269*, 1547–1554.

Ktistakis, N.T., Brown, H.A., Sternweis, P.C., and Roth, M.G. (1995). Phospholipase D is present on Golgi-enriched membranes and its activation by ADP ribosylation factor is sensitive to brefeldin A. Proc. Natl. Acad. Sci. USA *92*, 4952–4956.

Ktistakis, N.T., Brown, H.A., Waters, M.G., Sternweis, P.C., and Roth, M.G. (1996). Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi coated vesicles. J. Cell Biol. *134*, 295–306.

Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S., and Klausner, R.D. (1989). Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell *56*, 801–813.

Liscovitch, M., Czarny, M., Fiucci, G., Lavie, Y., and Tang, X. (1999). Localization and possible functions of phospholipase D isozymes. Biochim. Biophys. Acta *1439*, 245–263.

Liscovitch, M., Czarny, M., Fiucci, G., and Tang, X. (2000). Phospholipase D: molecular and cell biology of a novel gene family. Biochem. J. *345*, 401–415.

Lowe, M., Gonatas, N.K., and Warren, G. (2000). The mitotic phosphorylation cycle of the *cis*-Golgi matrix protein GM130. J. Cell Biol. *149*, 341–356.

Marcil, J., Harbor, D., Naccache, P.H., and Bourgoin, S. (1997). Human phospholipase D1 can be tyrosine-phosphorylated in HL-60 granulocytes. J. Biol. Chem. *272*, 20660–20664.

Martelli, A., Bortul, R., Bareggi, R., Tabellini, G., Grill, V., Baldini, G., and Narducci, P. (1999). The pro-apoptotic drug camptothecin stimulates phospholipase D activity and diacylglycerol production in the nucleus of HL-60 human promyelocytic leukemia cells. Cancer Res. *59*, 3961–3967.

Pertile, P., Liscovitch, M., Chalifa, V., and Cantley, L.C. (1995). Phosphatidylinositol 4,5-bisphosphate synthesis is required for activation of phospholipase D in U937 cells. J. Biol. Chem. *270*, 5130–5135.

Siddhanta, A., Backer, J.M., and Shields, D. (2000). Inhibition of phosphatidic acid synthesis alters the structure of the Golgi apparatus and inhibits secretion in endocrine cells. J. Biol. Chem. *275*, 12023–12031.

Siddhanta, A., and Shields, D. (1998). Secretory vesicle budding from the trans-Golgi network is mediated by phosphatidic acid levels. J. Biol. Chem. *273*, 17995–17998.

Slusarewicz, P., Hui, N., and Warren, G. (1994). Purification of rat liver Golgi stacks. In: Cell Biology: A Laboratory Handbook, Vol. 2, ed, J. E. Celis, San Diego: Academic Press, 509–516.

Stamnes, M., Schiavo, G., Stenbeck, G., Sollner, T.H., and Rothman, J.E. (1998). ADP-ribosylation factor and phosphatidic acid levels in Golgi membranes during budding of coatomer-coated vesicles. Proc. Natl. Acad. Sci. USA *95*, 13676–13680.

Toda, K., Nogami, M., Murakami, K., Kanoho, Y., and Nakayama, K. (1999). Colocalization of phospholipase D1 and the GTP-binding-defective mutant of the ADP-ribosylation factor 6 to endosomes and lysosomes. FEBS Lett. *442*, 221–225.

Venable, M.E., and Obeid, L.M. (1999). Phospholipase D in cellular senescence. Biochim. Biophys. Acta *1439*, 291–298.

Wakelam, M.J., Hodgkin, M., and Martin, A. (1995). The measurement of phospholipase D-linked signaling in cells. In: Signal Transduction Protocols, ed, D. A. Kendall and S. J. Hill, Totowa, NJ: Humana Press, 271–278.

Xu, H., and Shields, D. (1993). Prohormone processing in the *trans*-Golgi network: endoproteolytic cleavage of prosomatostatin and formation of nascent secretory vesicles in permeabilized cells. J. Cell Biol. *122*, 1169–1184.

Yamazaki, M., *et al*. (1999). Interaction of the small G protein RhoA with the C terminus of human phospholipase D1. J. Biol. Chem. *274*, 6035–6038.

Yang, W., and Storrie, B. (1998). Scattered Golgi elements during microtubule disruption are initially enriched in *trans*-Golgi proteins. Mol. Biol. Cell *9*, 191–207.