Regulation of Constitutive Protein Transit by Phospholipase D in HT29-cl19A Cells*

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Phospholipase D (PLD) plays a central role in the control of vesicle budding and protein transit. We previously showed that in resting epithelial HT29-cl19A cells, PLD is implicated in the control of constitutive protein transit, from the trans-Golgi network to the plasma membrane, and that phorbol ester stimulation of protein transit is correlated with PLD activation (Auger, R.; Robin, P.; Camier, B.; Vial, G.; Rossignol, B.; Tenu, J.-P., and Raymond, M.-N. (1999) J. Biol. Chem. 274, 28652–28659). In this paper we demonstrate that: 1) PLD is not implicated in the earliest phases of protein transit; 2) PLD controls apical but not basolateral protein transit; 3) HT29-cl19A cells express PLD1b and PLD2a mRNAs and proteins; 4) the expression of a catalytically inactive mutant of PLD2 (mPLD2-K758R) significantly inhibited apical constitutive protein transit whereas expression of a catalytically inactive mutant of PLD1 (hPLD1b-K898R) prevented increases in the rate of apical transit as triggered by phorbol esters; 5) PLD2 appears to be located in a perinuclear region containing the Golgi whereas PLD1, which is scattered in the cytoplasm in resting cells, is translocated to the plasma membrane after phorbol ester stimulation. Taken together, these data lead to the conclusion that in HT29-cl19A cells, both PLDs regulate protein transit between the trans-Golgi network and the apical plasma membrane, but that they do so at different steps in the pathway.

Results from several laboratories have demonstrated that lipases are implicated in the regulation of intracellular protein transit in different cell types. As long ago as 1993, Stutchfield and Cockcroft (1) showed that phospholipase D (PLD) activity was correlated with secretion in HL60 cells. More recently, PLD and phosphatidic acid produced by the hydrolysis of phosphatidylcholine (PC) by PLD have been shown to stimulate the formation of secretory vesicles (2–5), to regulate the transport from the endoplasmic reticulum to the Golgi complex (6), and to control secretion (7–10).

To date, two different PLDs which catalyze the same reaction have been identified (for reviews, see Refs. 11 and 12). PLD1a is a 1074-amino acid enzyme; PLD1b is a splice variant that lacks a seemingly nonessential 38-amino acid region. PLD1 is activated in vitro by small GTPases such as ADP-ribosylation factor (ARF) and RhoA and by protein kinase C (PKC). A lipid cofactor, phosphatidylinositol 4,5-bisphosphate, is also required. PLD2 is a 933-amino acid enzyme that shares about 50% identity with PLD1. Three splice variants have been sequenced, but only the longest variant, PLD2a, has been functionally characterized. PLD2 exhibits high basal activity in the presence of phosphatidylinositol 4,5-bisphosphate but does not further respond to a PKC stimulation in vitro. The domain structures of PLD1 and PLD2 are very similar. They contain the four core domains I-IV; domain IV contains a relatively short sequence motif termed “HKD” which plays an important role in catalysis. The replacement of the lysyl residue of this motif (Lys-898 in PLD1, Lys-758 in PLD2) by an arginyl residue results in catalytically inactive enzymes (13).

Postconfluent epithelial HT29-cl19A cells are polarized and secrete different proteins including α1-antitrypsin (14, 8). We previously demonstrated that in those cells, PLD is implicated in the regulation of protein transit (8). To identify the PLD species responsible for the control of protein trafficking, we stably transfected HT29-cl19A cells with the catalytically inactive mutants of PLD1 and PLD2 (13) and analyzed protein secretion in those transfected cells.

Our present findings lead us to propose that in HT29-cl19A cells, PLD2 may control apical constitutive protein transit, potentially at the TGN level, whereas PLD1 is implicated more in the phorbol ester-triggered enhancement of the rate of apical secretion and functions potentially at the apical plasma membrane.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media were from Invitrogen SARL. [3H]Leucine (126–184 Ci/mmol, 4.66–6.81 TBq/mmol; 5 mCi/ml, 185 MBq/ml), [35S]Pro-mix (ratio Met/Cys = 70/30 and specific activity >1000 Ci/mmol), and Protein A-Sepharose were obtained from Amersham Biosciences, Inc. [3H]Myristic acid (49 Ci/mmol, 1.81 TBq/mmol; 1 mCi/ml, 37 MBq/ml) was purchased from PerkinElmer Life Science. PDBu, polyclonal antibody against α1-antitrypsin (A0409), monoclonal anti-Golgi 58K protein (G2404), FITC-conjugated anti-mouse IgG secondary antibody (F2266), and horseradish peroxidase-linked secondary antibody (A0412) were from Sigma. FITC-conjugated anti-rabbit IgG

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‡ The abbreviations used are: PLD, phospholipase D; ARF, ADP-ribosylation factor; TGN, trans-Golgi network; PDBu, phorbol 12,13-dibutyrate; FITC, fluorescein isothiocyanate; DMEM, Dulbecco’s modified Eagle’s medium; PEr, phosphatidylethanol; ER, endoplasmic reticulum; PC, phosphatidylcholine; PKC, protein kinase C.
secondary antibody (A-11034) was purchased from Molecular Probes. Reverse transcriptase and Taq polymerase were obtained from Promega. All solvents were from Prolabo.

Cell Culture—HT29-c191A cells, which were derived from the parental HT29 cells after the induction of differentiation by treatment with butyric acid (15), were kindly donated by C. L. Laboisse. They were cultured as previously described (8). For all experiments, cells were seeded (about 0.25 x 10⁶ cells per filter) onto Falcon cell culture inserts (10.5-mm membrane diameter, 0.4-μm pore size, 1.0 x 10⁵ pores/cm²). Cells were used between 14 and 20 days following seeding, that is, at least 1 week after they had become confluent. The passage number of the cells used in this study varied between 18 and 30 for wild-type cells; transfected cells were used between passages 8 and 20 after transfection.

PCR Analysis—Total cellular RNA from HT29-c191A cells was isolated with Insta-Pure (Eurogentec) and 6 μg were reverse transcribed into cDNA using 200 units of Moloney murine leukemia virus-reverse transcriptase. Target cDNA was amplified using 1/5 of the reverse transcribed cDNA prepared, 0.2 mM dNTPs, 2 mM MgCl₂, 2.5 units of transcriptase. Target cDNA was amplified using 1/5 of the reverse transcribed cDNA prepared, 0.2 mM dNTPs, 2 mM MgCl₂, 2.5 units of Taq polymerase, and 100 pmol of primers in PCR buffer. The following primers were used: (i) to amplify PLD1a (expected 580 bp) and PLD1b (expected 466 bp); 5'-GGGATTCGCTGCGATGCTG (sense) and 5'-GATTACGTCGAATGAAATCAG-3' (antisense); (ii) to amplify PLD2a (expected 426 bp) and PLD2b (expected 395 bp); 5'-CCAGCCATGCG (sense) and 5'-GCAGCCAGCGAGATGCTG-3' (antisense). The mixture was amplified in a thermal cycler (iCycler Bio-Rad) under the following conditions: 94 °C (15 s), 55 °C (30 s), and 72 °C (30 s) for 30 cycles. 10-μl aliquots of the resulting PCR products were analyzed by electrophoresis on a 2% agarose gel.

Genomic DNA from transfected HT29-c191A cells was prepared according to the “Wizard genomic DNA purification” protocol (Promega). To detect in the genomic DNA samples the DNA sequences coding for the fusion proteins GFP-PLD1 and GFP-PLD2, respectively, the following primers, directed against the GFP-DNA were used: 5'-TGTTGTCGCCATCTCTGTTG (sense) and 5'-CCATCGGAGGAGTGATTCC-3' (antisense). A 680-bp band was expected. The mixture was amplified under the following conditions: 94 °C (15 s), 60 °C (30 s), and 72 °C (30 s) for 30 cycles. 10-μl aliquots of the resulting PCR products were analyzed by electrophoresis on a 2% agarose gel.

Plasmids and Transfections—The catalytically inactive variants of PLD1 and PLD2, hPLD1b-K898R and mPLD2-K758R were subcloned: they were seeded at 500 per ml into 25-cm² culture flasks. They were designated as internal dodecapeptide of PLD1 generated and reported by Dr. Min (17). To confirm integration of the entire construct, we conducted PCR analysis of genomic DNA using a catalytically inactive variant of mPLD2. Cells were immediately suspended in 5 ml of culture medium and 48 h later selection was begun with G418 at 800 μg/ml in culture medium. Transfected hPLD1b-K898R was not efficient and only a few cells survived; we thus did not isolate individual clones and designate those cells as P1 cells. Cells issuing from transfection with plasmid mPLD2-K758R were subcloned: they were seeded at 500 per ml into 130-mm dishes; isolated clones were individually transfected, subcultured, and amplified separately from 96-well dishes to 25-cm² culture flasks. They were designated as clone “P2x.” To confirm integration of the entire construct, we conducted PCR analysis of genomic DNA using GFP primers.

Metabolic Labeling and Measurement of Protein Secretion—For metabolic labeling with [3H]leucine, cells were treated as described by Auger et al. (8). Protein secretion measured after 4 h of incubation of the cells at 37 °C was expressed as the percentage of [3H]leucine-labeled proteins released into either the apical or basolateral medium (i.e. 100 × total amount of [3H]leucine-labeled proteins released in one medium/total amount of [3H]leucine-labeled proteins in the two media and in the cells). The secretion stimulation factor (SF) represents the ratio of protein secretion in the presence of PDUs to control protein secretion.

Immunoblotting—Cells were lysed with 1% Triton X-100 in 50 mM Tris buffer, pH 7.4, in the presence of protease inhibitors (antipain, chymostatin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride). Proteins denatured by 4 μl urea in Laemmli buffer were separated by SDS-PAGE on 7% acrylamide gels were electroblotted onto nitrocellulose (Immobilon-NC, 0.45 μm pore, Millipore). The PLD isoforms were detected with the following antibodies: 1) antibody against the C-terminal dodecapeptide of PLD1 generated and reported by Dr. Min (17) (dilution 1/3000); 2) antibody against the residues 675-688 of human PLD1 (18) kindly provided by Dr. Liscovitch (dilution 1/2000); 3) antibody against PLD2 (dilution 1/2000) prepared by Dr. Geny according to the following procedure: MTATPESFLPTGDELC peptide was synthesized and coupled to keyhole limpet hemocyanine by Neosystem (Strasbourg, France). A fusion protein with GST containing the 124 N-terminal residues of hPLD2 was constructed and used as antigen and produced in Escherichia coli. Antiserum against hPLD2 peptide plus hPLD2 fusion protein was raised in rabbits and immunoadsorbed on a Sepharose column coupled to the relevant proteins; 4) antibody against PLD2 (number 58) raised against a peptide corresponding to residues 523-534 of human PLD2 and reported by Drs. Banno and Nozawa (18).

An horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody was used diluted 1/1000. Detection was performed using an ECL kit (PerkinElmer Life Sciences).

Immunoprecipitation and SDS-PAGE Analysis of α1-Antitrypsin—For these experiments, proteins were radiolabeled for 10 min with [35S]Pro-mix and then incubated with 1 ml of DMEM in both compartments. For the analysis of the PLD-dependent phases of the transit, 3% ethanol was added in the basolateral compartment. At different times after the incubation, cells were lysed in 1 ml of 0.1% Triton X-100 in 5 mM HEPES buffer, pH 7.5, in the presence of protease inhibitors. For the analysis of the α1-antitrypsin content in the apical and basolateral media, the cells were incubated with 1 ml of DMEM in both compartments for 4 h. When the effect of ethanol was tested, 3% ethanol was added in the basolateral compartment just after the pulse. At the end of the incubations both media were removed and Triton X-100 was added (final concentration, 0.1%). Cell lysates or media were then incubated at 4 °C with polyclonal antibody against α1-antitrypsin (dilution 1:25) for 2 h followed by incubation with protein A-Sepharose for 30 min. Immunoprecipitates were washed twice with 5 ml HEPES buffer, pH 7.5, containing 1% Triton X-100 and 1% SDS, once with 5 ml HEPES buffer, pH 7.5, containing 0.1% Triton X-100, once with 5 ml HEPES buffer, pH 7.5, and then denatured at 100 °C in Laemmli's sample buffer. Samples were separated by SDS-PAGE on 10% acrylamide gels. After fixation and staining with Coomassie Blue, the gels were dried and exposed to radiographic film quantified with a Storm 850 PhosphorImage (Molecular Dynamics).

PLD Activity—For determinations of PLD activity, we measured phosphatidylethanolamine (PEr), the non-metabolizable product of the transphosphatidyltransferation reaction. HT29-c191A cells grown for about 20 days on cell culture inserts were washed in serum-free DMEM and then labeled for 2 days with [3H]myristic acid in DMEM containing 1 mg/ml lipid-free bovine serum albumin. [3H]Myristic acid (49 Ci/mmol) was added to the basolateral side of the cultures the day before the experiments, the cells were washed with lipid-free bovine serum albumin in phosphate-buffered saline and preincubated for 15 min in 1 ml of DMEM in the presence of 3% ethanol; PDBu was then added for 45 min to the basolateral side. At the end of the experiment the cells were collected by cutting the filters from the filter cups and the cellular lipids were extracted as described previously (8). The amounts of PEr formed were expressed as percentages of the amount of total radiolabeled lipids.

Immunofluorescence Microscopy—The cells (either the WT cells or the cells obtained 3 days after electroporation) were grown on coverslips, washed with phosphate-buffered saline, fixed with methanol at -20 °C for 3 min, and washed again with phosphate-buffered saline. For all the following steps the coverslips were incubated in phosphate-buffered saline supplemented with 10% fetal bovine serum. The 1-h incubations with the primary antibody and secondary antibody are all performed at room temperature and the following dilutions were used: 1/100 for the antibody against PLD1 from Dr. Min, 1/100 for the antibody number 4 against a mixture of four peptides corresponding to different regions of PLD1 (1’′MLKNEPRVNTSAIQ68, 144RQVPNVRPEPMS262, 586DPSEDIQDPDS9K287, and 1027KDIPARAEELKK1066) from Dr. Bourgoin (19), 1/1000 for the antibody against PLD2 from Dr. Geny, 1/200 for the antibody against PLD2 from Drs. Banno and Nozawa, 1/100 for the antibody number 26 against a mixture of two peptides corresponding to residues 823-839 of human PLD2 and 483-499 of rat PLD2 prepared by Dr. Bourgoin (20), 1/100 for the antibody against ARF1 from Dr. Bourgoin (21), 1/25 for the antibody against the 58K Golgi-protein, 1/100 for the antibody against GFP, 1/1000 for the FITC-conjugated anti-mouse IgG secondary antibody, and 1/500 for the FITC-conjugated anti-rabbit IgG secondary antibody. The cells were examined with an epi-illumination Axioskop microscope (Zeiss) equipped with a ×83 oil immersion objective and the appropriate filter.
Regulation of Protein Transit by PLDs in HT29-c119A Cells

**RESULTS**

PLD Independence of the Early Phases of Protein Transit—We previously showed that protein secretion in the apical medium was reduced by ethanol in a dose-dependent manner. Since in the presence of ethanol PLD catalyzes a transphosphatidylation reaction leading to an inhibition of the production of phosphatidic acid, we concluded that PLD and phosphatidic acid were implicated in the regulation of the secretory process and we speculated that a TGN (or post-TGN) step of protein transit was involved (8). To analyze a possible PLD regulation of the ER to Golgi transport, we took advantage of the structural features of α₁-antitrypsin, the main protein secreted by HT29-c119A cells (8). This protein contains three N-linked long carbohydrate side chains (22). We first followed the ER to Golgi transit of this radiolabeled glycoprotein by SDS-PAGE. In wild-type cells, a shift in the electrophoretic mobility of α₁-antitrypsin during the chase was observed (Fig. 1A). Two forms of α₁-antitrypsin were detected; the high molecular weight form is endo H resistant whereas the other is sensitive to endo H digestion (data not shown). As glycoproteins became endo H-resistant when they have reached the Golgi apparatus, we concluded that the high molecular weight form corresponds to the Golgi-matured α₁-antitrypsin and that, after a 1-h chase, the ER to Golgi transit is nearly achieved. We then followed the maturation of α₁-antitrypsin in the presence of ethanol or butanol. Fig. 1B shows that the rate of acquisition of the whole glycosylated motif was not modified when the cells were chased in the presence of 3% ethanol, indicating that the duration of protein transit from the ER to Golgi was not modified. Identical results were obtained when the cells were incubated with 0.8% 1-butanol (data not shown). Taken together, these results indicate that in HT29-c119A cells, PLD is not implicated in the early steps of α₁-antitrypsin transit.

**PLD Dependence of the Apical Secretory Pathway—α₁-Antitrypsin was shown to be secreted in both the apical and basolateral media (8). To analyze the potential involvement of PLD in the apical and basolateral secretory pathways, we first quantified the radiolabeled proteins secreted in the two media obtained from HT29-c119A cells treated or not with primary alcohols (3% ethanol, 0.8% 1-butanol) or tertiary alcohol (0.8% 3-butanol) (Fig. 1, C and D). After 6 h of incubation, ethanol and 1-butanol were shown to induce a reduction of about 30% in the amount of apically secreted radiolabeled proteins. In contrast, when 3-butanol was present during the incubations, radiolabeled proteins were secreted apically with the same efficiency as control cells. Moreover, neither primary nor tertiary alcohols modified the amount of radiolabeled proteins secreted basolaterally. Even more convincing are the results obtained after immunoprecipitation of the radiolabeled α₁-antitrypsin. Fig. 1E shows that the amount of α₁-antitrypsin (expressed in arbitrary units) secreted in the apical medium after 4 h of incubation is reduced from 146 (in control cells) to 56 (in ethanol-treated cells); this represents a decrease of about 60%. Under the same conditions, the amount of basolaterally secreted α₁-antitrypsin (about 38 in control cells) was not affected by the ethanol treatment. To see if the reduction of the amount of apically secreted α₁-antitrypsin was due to a decrease in the rate of apical transit, we performed longer incubations. In control conditions, the amount of radiolabeled α₁-antitrypsin released in the apical medium slightly increased from 146 after 4 h of incubation to 158 after 8 h whereas with ethanol-treated cells, this amount rose from 56 to 106. The amount of radiolabeled α₁-antitrypsin found in the basolateral medium was not increased when the time of incubation was lengthened (about 38 and 36, respectively, after 4 and 8 h of incubation performed in the absence or presence of ethanol). Thus the rate of apical α₁-antitrypsin transit is reduced in the presence of ethanol but α₁-antitrypsin is not reoriented to the basolateral membrane. The analysis of the α₁-antitrypsin content of control cell lysates or ethanol-treated cell lysates confirms those results; after 4 h and even 8 h of incubation, more α₁-antitrypsin remained in the ethanol-treated cells than in the control cells (data not shown). Taken together, these results indicate that in HT29-c119A cells, PLD is implicated only in the regulation of the apical secretory pathway.

Identification of the PLD Isoforms Expressed in HT29-c119A Cells—To identify the PLD species that could be responsible for the control of the intracellular transit of protein, we examined the expression of PLD mRNAs in HT29-c119A cells by reverse transcriptase-PCR. The sequences of the primers were based on the published DNA sequences for hPLD1 (23) and hPLD2 (24) and were designed to discriminate between the different PLD splice variants. As shown in Fig. 2A, lane 1, two bands are detected, a major one corresponding to the 466-bp fragment expected for PLD1b and a minor one corresponding to the 580-bp fragment expected for PLD1a. In lane 2, two bands are

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Fig. 1. Ethanol inhibits protein transport neither from the ER to the Golgi apparatus nor toward the basolateral membrane. Cells were pulse-labeled with [35S]Pro-mix for 10 min and chased for the indicated times in the absence or presence of 3% ethanol. Radiolabeled α₁-antitrypsin was immunoprecipitated from cell lysates, analyzed by SDS-PAGE and quantified with a PhosphorImager. **A,** autoradiography showing the maturation profile of α₁-antitrypsin in wild-type cells. **B,** kinetics of appearance of the Golgi-matured form of α₁-antitrypsin in wild-type and ethanol-treated cells. The Golgi form was expressed as percent of total (sum of ER and Golgi forms). Values are mean ± S.D., n = 2. **C** and **D,** secretion of radiolabeled proteins: cells were labeled with [3H]leucine and chased in the presence or absence of 3% ethanol, 0.8% 1-butanol (but-1), or 0.8% of 3-butanol (but-3). The radiolabeled proteins were quantified in the apical and basolateral media after 6 h of incubation. C represents the % of apical radiolabeled proteins relative to the total radiolabeled proteins. D represents the % of basolateral radiolabeled proteins relative to the total radiolabeled proteins. Values are mean ± S.D., n = 3 to 5. **E,** secretion of α₁-antitrypsin in apical and basolateral media obtained from cells treated or not with 3% ethanol for 4 h. Values are mean ± S.D., n = 4.
also detected, a major one corresponding to the 426-bp fragment expected for PLD2a and a minor one corresponding to the 393-bp fragment expected for PLD2b. Thus, HT29-c19A cells express mainly PLD1b and PLD2a. The presence of both PLD1 and PLD2 in HT29-c19A cells was confirmed by Western blot analysis using several different antibodies raised against PLD peptides (Fig. 2B).

**Cellular Localization of PLDs**—To localize PLD1 and PLD2 in HT29-c19A cells we used different antibodies that all recognize PLD1 or PLD2 but that exhibited different degrees of nonspecific labeling in Western blot analysis: Dr. Min’s antibody against ARF1 or with the antibody against the 58K Golgi-protein (G), Dr. Bourgoin’s antibodies against PLD1 and PLD2. Fig. 3, A and C, shows that PLD1 appears distributed in very small dots throughout the cytoplasm suggestive of small vesicles. PLD2 presents a different localization (Fig. 3, D-F): patches organized in crescent-like structures are found close to the nuclei. When, in the case of the anti-PLD2 from Dr. Bourgoin, the antibody is preincubated with its peptide antigen, the perinuclear labeling disappears (data not shown). Similar crescent-shape structures appear to be decorated with an anti-ARF1 antibody (Fig. 3G) and with the antibody against the 58K Golgi-protein (Fig. 3H). Thus, even if the cytoplasm is very scant in HT29-c19A cells, these results likely indicate a Golgi localization for PLD2. Neither anti-PLD1 nor anti-PLD2 antibodies appeared to stain the plasma membrane in resting cells; nevertheless, when the cells were incubated for 1 h in the presence of PDBu, plasma membrane localization was observed for PLD1 (Fig. 3B). Under the same conditions, PLD2 labeling was not modified (data not shown). Thus, after phorbol ester treatment, PLD1 translocates to the plasma membrane.

**Role of PLD1 in the Secretory Process**—To explore the role of PLD1 in protein transit, we transfected HT29-c19A cells with the hPLD1b-K98SR plasmid that encodes the catalytically inactive form of hPLD1b. As protein secretion must be analyzed on confluent polarized cells grown on filters for at least 14 days after seeding and as the usual transfection methods are relatively inefficient with epithelial intestinal cells (23), transient transfections were not applicable for our purpose. We thus decided to establish cell lines stably expressing the inactive PLD.

In the cells transfected with the plasmid encoding hPLD1b-K98SR (P1 cells) the cellular distribution of the GFP-tagged inactive PLD1 resembled that of the endogenous enzyme (Fig. 4A) and stably transfected cells were successfully generated (Fig. 4B). We then determined PLD activity in the P1 cells. PLD1 is activated by PKC (11, 12) and we previously reported that the PDBu-triggered PLD activity measured in HT29-c19A cells by phospholipase C (PLC) is identified in wild-type and P1 cells. Nevertheless, when the P1 cells are treated with PDBu, the PDBu-stimulated increase in the rate of secretion was about 60% inhibited (the secretion stimulation factor was 1.37 in those cells compared with 1.93 in wild-type cells). Thus, a decrease in PDBu-enhanced secretion correlates with the decrease in PDBu-triggered PLD activity. Finally Fig. 4E shows that in P1 cells, the amount of radiolabeled protein released in the basolateral medium is close to that measured for wild-type cells indicating that the sorting of proteins was not significantly modified in those transfected cells. These results dem-
PLD activity was determined in the P2 cell lines. Unstimulated PLD activity is very low in HT29-cl19A cells and no significant difference in secretory activity was observed between wild-type and PLD1 knockdown cells. Nevertheless, when the transfected cells are treated with PDBu, the secretion stimulation factor (1.67 and 1.78 for P2r and P2j cells, respectively) was close to that obtained for wild-type cells (1.91). Basolateral secretion was also determined and found for both cell lines to be close to that obtained for wild-type cells (Fig. 5F). α1-Antitrypsin being the main protein released in apical medium (8), we analyzed by immunoprecipitation the distribution of this protein in the two media in wild-type cells and in P2j and P2r cells. In wild-type cells about 79% of the secreted protein are, respectively, found in the apical medium whereas in clones P2j and P2r only about 55 and 30% of the secreted protein are, respectively, found in the apical medium. Taken together these results indicate that PLD2 appears to be involved only in the regulation of constitutive protein transport to the apical membrane.

**DISCUSSION**

Previous reports have demonstrated the role of phosphatidic acid and PLD in the regulation of vesicle budding and protein transport. Bi et al. (6) and Boisgard and Chanat (10) showed that PLD was required for influenza hemagglutinin glycoprotein and caseins transport from the ER to the Golgi complex in...
Chinese hamster ovary and mammary cells, respectively. On the other hand we demonstrated here, exploiting the transphosphatidylation activity of PLD, that \( \alpha_1 \)-antitrypsin in HT29-c119A cells is transported from the ER to Golgi in a PLD-independent manner (Fig. 1B). Such a result was also obtained for whey acidic protein in mammary cells (10). We can thus hypothesize that the transport of those proteins from the ER to the Golgi apparatus would be by bulk flow whereas the packaging of hemagglutinin and caseins would be via a selective PLD-mediated mechanism. Concerning the exit from the TGN, it is now clearly established that secretory vesicle formation and secretion is PLD-dependent in many cell-types (2–5 and 7–9). Our findings (Fig. 1, C-E) extend these proposed models by showing that in the constitutive secretory pathway, apical but not basolateral transit is regulated by PLDs. This finding will be discussed further.

Two isoforms of PLD have been described; it was thus important to determine which isofrom is implicated in the control of protein trafficking. We transfected HT29-c119A cells with the catalytically inactive mutants of PLD1 and PLD2. These mutants have been previously shown to be devoid of activity in vivo and in vitro using PC as a substrate (13); they have been recently successfully used to determine the PLD isoforms implicated in different physiological processes (26–29).

Our results concerning PLD activity measurements indicate that PDBu-stimulated PLD activity is reduced in the cells transfected with the inactive PLD1 whereas in the cells expressing the inactive PLD2, the PDBu-stimulated PLD activity remains close to that of the wild-type cells. These data indicate that in HT29-c119A cells, PLD2 is not implicated in the PDBu-triggered PLD activation. The 25% inhibition of PDBu-stimulated PLD activity in P1 cells appears low, but since the PLDs are thought to be essential enzymes for physiological functioning of the cells, reducing PLD1 activity beyond this point might have led to inviability. The amount of this enzyme may be strictly controlled, since recent data reported that overexpression of active PLD1 is generally toxic (28, 30).

The results obtained regarding apical protein secretion were very striking. Constitutive secretion in P1 cells is not affected by transfection with the inactive PLD1, but the PDBu-stimulated increase in secretion observed in wild-type cells is about 60% blocked in those transfected cells. On the other hand, in P2j and P2r cells transfected with the inactive PLD2, constitutive secretion toward the apical pole of the cells is 55 to 70% reduced whereas the PDBu-stimulated increase in secretion is close to that determined for wild-type cells.

Taken together, these results indicate that both PLDs control apical protein transit: in resting cells constitutive secretion appears mainly regulated by PLD2 but, subsequent to phorbol ester stimulation, the increase in secretion is PLD1-dependent. These results are consistent with the known properties of the two isoforms of the enzyme, PLD2 has been described to be constitutively active to varying extents in cells whereas PLD1 requires activation, one mechanism of which is through the action of PKC (11, 12).

In contrast with apical secretion, basolateral secretion is scarcely modified in either type of transfected cells. These results strengthen the data presented in Fig. 1, D and E, which showed that ethanol had no effect on basolateral secretion of proteins. Thus, basolateral secretion does not appear to be regulated by PLDs.

The results concerning the sorting and the transit of proteins toward apical membranes are interesting: we showed that in wild-type cells about 79% of the secreted \( \alpha_1 \)-antitrypsin is transported to the apical surface. This finding suggests that \( \alpha_1 \)-antitrypsin is sorted in the TGN via a positive signal. This signal can be either a N-glycan-dependent signal as proposed by Schellefele et al. (31) or a proteinaceous apical sorting signal as suggested for the corticosteroid-binding protein, a glycoprotein that belongs to the same serpin family as \( \alpha_1 \)-antitrypsin (32, 33). In cells transfected with inactive PLD2, secretion of \( \alpha_1 \)-antitrypsin and all the proteins apically released is reduced whereas basolateral transit is not modified. We can thus hypothesize that PLD2 could be implicated in the regulation of the machinery that addresses proteins to the apical membrane. These results would suggest that the mechanisms controlling apical and basolateral vesicle formation and (or) fusion are not dependent on the same enzymes. Such a conclusion had been drawn by Pimplikar and Simons (34) who showed that in polarized Madin-Darby canine kidney cells, protein kinases A and C selectively stimulate apical transport.

We do not presently know the exact mechanism of action through which the inactive PLD1 and PLD2 isoforms inhibit specific steps in protein transit as demonstrated here. It is likely that those inactive enzymes, which have only one conservative amino acid substitution in their active pocket, have a structural conformation similar to that of the native enzymes; therefore, since the inactive PLDs localize to the same sites as the endogenous enzymes, they may compete with the endogenous active PLDs for binding to substrate, cofactor, or activator(s) at the immediate site of action. Furthermore, since we found that the levels of expression of the inactive enzymes are lower than those of the endogenous PLDs, it is not surprising that we only observed partial inhibitions of the phenomena under study. With regards to possible perturbations of some upstream PLD effectors by the inactive PLDs, this appears rather unlikely: in the cells expressing the inactive PLD1, apical constitutive protein transit (controlled by PLD2 as shown by our data) is normal. Those data indicate that in the transfected P1 cells: 1) the regulators of protein transit others than PLDs do not appear to be affected by the expression of the inactive PLD1; 2) the inactive PLD1 does not block endogenous PLD2 access to PC substrate, phosphatidylinositol 4,5-bisphosphate cofactor or potential PLD2 activators. Furthermore, in the transfected P2 cells, the PDBu-stimulated increase in the rate of protein transit (regulated by PLD1 as shown by our data) is not significantly modified. This indicates that the inactive PLD2 does not block access of endogenous PLD1 to PC, phosphatidylinositol 4,5-bisphosphate, or the PLD1 activators such as PKC.

Our finding showing an implication of PLD2 in the control of the final steps of apical protein transit would potentially suggest a TGN localization for PLD2. Nevertheless the results concerning the cellular localization of the PLDs remains confusing: ARF-regulated PLD activity was found in secretory vesicles in control neutrophils and was mobilized to the plasma membrane upon stimulation with fMet-Leu-Phe (35). Tagged-PLD1s were localized solely to perinuclear regions (the endoplasmic reticulum, Golgi apparatus, and late endosomes) in rat embryo fibroblasts (36), in secretory granules and lysosomes in unstimulated RBL-2H3 cells and in the plasma membrane after cellular stimulation (37), in the plasma membrane, in vesicular structures and in a perinuclear region which appears similar to endoplasmic reticulum and Golgi apparatus in fibroblastic 3Y1 cells (38), in late endosomes and lysosomes in NRK cells (39) in a perinuclear region in COS-7 cells (40). Very recently endogenous PLD1 was shown to be present in nuclei and enriched in the Golgi apparatus of GH3 and NRK cells (30) but associated with the plasma membrane in chromaffin cells (29). Our own results concerning the localization of the endogenous PLD1 are different as we showed that under phorbol ester stimulation, PLD1 translocates from small dots scattered...
in the cytoplasm to the plasma membrane. Those apparent discrepancies in the results reported in the literature may be due to the fact that an overexpression of tagged-PLD1 may result in its mislocalization in different vesicular structures (30) but it can also reflect physiological differences as many cell types were examined. Concerning PLD2, there is little data on its localization and relatively few cell types or tissues have been examined in this regard. In 3T3-L1 adipocytes, PLD2 was shown to be associated with intracellular membranes (41). The tagged-enzyme has been localized primarily to the plasma membrane in rat embryo fibroblasts (36), and in ruffling membranes formed upon epidermal growth factor stimulation of HeLa cells (42). Our own results which concern the localization of the endogenous enzyme are in favor of a Golgi localization for this PLD2 isozyme.

The question of the role of each of the PLDs in the control of protein traffic is far from clear. It has been proposed that PLDs could control secretory vesicle budding from the TGN or regulate the fusion of vesicles with the plasma membrane; we cannot exclude the possibility that they could be implicated at these two steps (or more) of the secretory pathway. hPLD1 added to permeabilized GH3 cells was found to stimulate the release of nascent secretory vesicles (3). Recently it has been shown in RBL-2H3 mast cells that ARF1-mediated exocytosis occurs via PLD (43). PLD1 has also been described to act at the plasma membrane translocation of PLD1 after phorbol ester stimulation. Nevertheless we have to be very cautious with those conclusions since, in many respects, secretion is a "circular" process that depends not only on the generation and outflow of vesicles, but also the return of membrane to the TGN; disruption of this process at any step by the catalytically inactive PLDs could decrease secretion.

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REFERENCES
1. Stutchfield, J., and Cockcroft, S. (1993) Biochem. J. 293, 649–655
2. Kitakata, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C., and Roth, M. G. (1990) J. Cell Biol. 134, 295–306
3. Chen, Y.-G., Siddhanta, A., Austin, C. D., Hammond, S. M., Sung, T.-C., Frohman, M. A., Morris, A. J., and Shields, D. (1997) J. Cell Biol. 138, 495–504
4. Tüshner, O., Lorra, C., Bouma, B., Wirtz, K. W. A., and Huttner, W. B. (1997) FEBS Lett. 419, 271–275
5. Siddhanta, A., and Shields, D. (1998) J. Biol. Chem. 273, 17995–17998
6. Bi, K., Roth, M. G., and Kistakos, N. T. (1997) Curr. Biol. 7, 301–307
7. Williger, B.-T., Ho, W.-T., and Exton, J. H. (1999) J. Biol. Chem. 274, 735–738
8. Auger, R., Robin, P., Camier, B., Vial, G., Rousiguié, B., Tena, J.-P., and Raymond, M.-N. (1999) J. Biol. Chem. 274, 28652–28659
9. Siddhanta, A., Backer, J. M., and Shields, D. (2000) J. Biol. Chem. 275, 12025–12031
10. Boisvert, R., and Chanat, E. (2000) Biochem. Biophys. Acta 1495, 281–296
11. Frohman, M. A., Sung, T.-C., and Morris, A. J. (1999) Biochem. Biophys. Acta 1439, 175–186
12. Liscovitch, M., Czarny, M., Fiucci, G., and Tang, X. (2000) Biochem. J. 349, 401–415
13. Sung, T.-C., Roper, R. L., Zhang, Y., Rudge, S. A., Temel R., Hammond, S. M., Morris, A. J., Moss, B., Engebrecht, J., and Frohman, M. A. (1997) EMBO J. 16, 4319–4330
14. Jilving, T., and Kirk, K. L. (1996) J. Biol. Chem. 271, 4381–4387
15. Augeron, C., and Laboisse, C. L. (1994) Cancer Res. 44, 3981–3969
16. Battu, S., Beneytout, J.-L., Pairet, M., and Rigaud, M. (1998) FEBS Lett. 437, 165–172
17. Min, D. S., Cho, N. J., Yoon, S. H., Lee, Y. H., Hahn, S.-J., Lee, K.-H., Kim, M.-S., and Jo, Y.-H. (2000) J. Neurochem. 75, 274–281
18. Czarny, M., Fiucci, G., Lavie, Y., Banno, Y., Nosawa, Y., and Liscovitch, M. (2000) FEBS Lett. 467, 326–332
19. Marcelli, J., Harbour, D., Naccache, P. H., and Bourgin, S. (1997) J. Biol. Chem. 272, 20660–20664
20. Morash, S. C., Byers, D. M., and Cook, H. W. (2000) Biochem. Biophys. Acta 1487, 177–180
21. Londono, I., Marshansky, V., Bourgin, S., Vinay, P., and Bendayan M. (1999) Kidney Int. 55, 1407–1416
22. Megna, T., Lujan, E., and Yoshida, A. (1988) J. Biol. Chem. 255, 4067–4061
23. Hammond, S. M., Altshuller, Y. M., Sung, T.-C., Rudge, S. A., Rose, K., Engebrecht, J., Morris, A. J., and Frohman, M. A. (1995) J. Biol. Chem. 270, 29640–29645
24. Lopez, I., Arndt, R. S., and Lambeth, J. D. (1998) J. Biol. Chem. 273, 12846–12852
25. Brandsch, C., Friedl, P., Lange, K., Richter, T., and Mothes, T. (1998) Scand. J. Gastroenterol. 33, 833–838
26. Rizzo, M. A., Shome, K., Vasudevan, C., Andresen, B., and Romero, G. (2000) Endocrinology 141, 2290–2298
27. Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A., and Bader, M.-F. (2001) FEBS J. 270, 191–201
28. Lu, Z., Hornia, A., Joseph, T., Sukezane, T., Frankel, P., Zhong, M., Bychenok, B., Xu, L., Feig, L. A., and Foster, D. A. (2000) J. Biol. Chem. 275, 1415–1419
29. Vitale, N., Caumont-Primus, A.-S., Chasserot-Golaz, S., Xu, L., Wu, S., Scierra, V. A., Morris, A. J., Frohman, M. A., and Rader, M.-F. (2001) EMBO J. 20, 2424–2434
30. Freyberg, Z., Sweeney, D., Siddhanta, A., Bourgin, S., Frohman, M., and Shields, D. (2001) Mol. Biol. Cell. 12, 945–955
31. Schellfie, P., Peransen, J., and Simons, K. (1995) Nature 378, 96–98
32. Culley, W. C., Sung, T.-C., RolL, R., Jencco, J., Hammond, S. M., Altshuller, Y., Bar-Sag, D., Morris, A. J., and Frohman, M. A. (1997) Curr. Biol. 7, 191–201
33. Forwood, M. D., Thompson, N., Saqib, K. M., Clark, J. M., Powner, D., Thompson, N. T., Solari, R., and Wakelam, M. J. O. (1998) Curr. Biol. 8, 835–838
34. Kim, Y., Kim, J.-E., Lee, S. D., Lee, T. G., Kim, J. H., Park, J. B., Han, J. M., Jang, S. R., Suh, P.-G., and Rya, S. H. (1999) Biochim. Biophys. Acta 1436, 319–320
35. Toda, K., Nogami, M., Murakami, K., Kanah, Y., and Nakayama, K. (1999) FEBS Lett. 442, 221–225
36. Sung, T.-C., Zhang, Y., Morris, A. J., and Frohman, M. A. (1999) J. Biol. Chem. 274, 3659–3666
37. Miller, C. A., Jess, T. J., Saqib, K. M., Wakelam, M. J. O., and Gould, G. W. (1999) Biochem. Biophys. Res. Commun. 254, 734–738
38. Honda, A., Nogami, M., Yokozeki, T., Yanazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A., and Kanah, Y. (1999) Cell 99, 521–532
39. Way, G., O’Luanaih, N., and Cockcroft, S. (2000) Biochem. J. 346, 63–70
Regulation of Constitutive Protein Transit by Phospholipase D in HT29-cl19A Cells
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