A Role for Phospholipase D in GLUT4 Glucose Transporter Translocation*

Masahiro Emoto‡, Jes K. Klarlund‡, Steve B. Waters§, Vivian Huś, Joanne M. Buxton, Anil Chawla, and Michael P. Czech¶

From the Program in Molecular Medicine and Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655 and Metabolex Inc., Hayward, California 94545

Based on recent studies showing that phospholipase D (PLD)1 is associated with intracellular membranes and promotes membrane budding from the trans-Golgi, we tested its possible role in the membrane trafficking of GLUT4 glucose transporters. Using immunofluorescence confocal microscopy, expressed Myc epitope-tagged PLD1 was found to associate with intracellular vesicular structures by a mechanism that requires its N-terminal pleckstrin homology domain. Partial co-localization with expressed GLUT4 fused to green fluorescent protein in both 3T3-L1 adipocytes and Chinese hamster ovary cells was evident. Furthermore, microinjection of purified PLD into cultured adipocytes markedly potentiated the effect of a submaximal concentration of insulin to stimulate GLUT4 translocation to cell surface membranes. Insulin stimulated PLD activity in cells expressing high levels of insulin receptors but no such insulin effect was detected in 3T3-L1 adipocytes. Taken together, these results are consistent with the hypothesis that PLD1 associated with GLUT4-containing membranes acts in a constitutive manner to promote the mechanism of GLUT4 translocation by insulin.

Physiological glucose homeostasis in humans is largely dependent on the actions of the hormone insulin, particularly its ability to enhance glucose transport into fat and muscle. Insulin exerts this effect primarily through a process whereby sequestered, intracellular GLUT4 glucose transporter proteins are rapidly redistributed to cell surface membranes where they can catalyze glucose uptake into cells (1–3). In both the basal and insulin-stimulated states, GLUT4 proteins appear to cycle between intracellular membrane and plasma membrane locations, but exocytosis is relatively slow in the absence of insulin (4). The action of insulin to stimulate this process appears dependent on the activity of p85/p110-type phosphatidylinositol 3-kinase (PI)1 3-kinase because it is blocked by inhibitors of this enzyme (5, 6) or by expression of dominant inhibitory constructs of the p85 regulatory subunit (7). Insulin receptor tyrosine kinase activity activates PI 3-kinase through recruitment of Src homology 2 domains of the p85 subunit to phosphotyrosine sites on insulin receptor substrate proteins (8). The concept that the 3'-polyphosphoinositide products of insulin-stimulated PI 3-kinase are key mediators of insulin action on GLUT4 translocation is consistent with extensive data supporting a major role of these lipids in membrane trafficking (9).

Much effort has been expended attempting to identify downstream targets of the PI 3-kinase products, phosphatidylinositol 3-phosphate (PtdIns(3)P), PtdIns(3,4)P2, and PtdIns(3,4,5)P3, that might connect insulin receptor signaling to GLUT4 glucose transporter trafficking (1). Some evidence (10, 11) has implicated Akt/protein kinase B, which is thought to be activated in the presence of 3'-polyphosphoinositide through the actions of PDK1 and another unknown protein kinase (12), in GLUT4 regulation. However, other data do not support this view but instead favor a role for protein kinase C isozymes (13, 14). Recent studies in our laboratory identified another type of downstream effector for PtdIns(3,4,5)P3, GRP1, which catalyzes guanine nucleotide exchange on ARF proteins through a Sec7 homology region also present on other ARF exchange proteins (15–17). GRP1 selectively binds PtdIns(3,4,5)P3 with high affinity through its pleckstrin homology (PH) domain. As expected, GRP1 and its related isoforms respond to insulin by rapid recruitment to cell membranes by a mechanism that requires their PH domains and is blocked by the PI 3-kinase inhibitor wortmannin (17–20). In turn, GTP-binding ARF proteins, the downstream targets of GRP1-like proteins, are known regulators of membrane budding and vesicular trafficking in Golgi, endoplasmic reticulum, and endosomal membranes (21).

GTP-bound ARF proteins appear to regulate membrane budding in concert with their ability to activate phospholipase D (PLD), through the recruitment of coat and adaptor proteins to membranes (21–25). Two isoforms of PLD have been identified, PLD1 and PLD2, that differ in their cellular localizations (26, 27). Recent data suggest that ARF1 can act alone to mediate such events but that the products of PLD activity may facilitate these ARF effects (28, 29). Interestingly, PLD is also activated by protein kinase C (24, 30) as well as Rho-GTPase proteins (24, 26), which have been reported to be modulated by insulin (31, 32). The product of PLD, phosphatidic acid, appears to regulate the production of PtdIns(4,5)P2, which also directly drives recruitment of coat and adaptor proteins to membranes (33–36). Recent results indicate that insulin treatment of adipocytes activates PLD (32, 37), consistent with the hypothesis that stimulation of PI 3-kinase may indeed cause enhanced GTP loading of ARF proteins, which in turn activate the enzyme. However, this result has not yet been confirmed in adipocytes. This is an important issue because the reported role for PLD in vesicle budding and secretion (23, 25) in other...
systems suggests a possible role in insulin-stimulated GLUT4 translocation from intracellular membranes to the cell surface. The aim of the present studies was to clarify the role of PLD in insulin action and GLUT4 regulation. On the positive side, we observed a striking enhancement of GLUT4 translocation upon microinjection of purified PLD into cultured adipocytes treated with a low concentration of insulin. We also observed partial co-localization of PLD1, but not PLD2, with GLUT4 in intracellular membranes of 3T3-L1 adipocytes. However, whereas insulin did stimulate PLD activity in Chinese hamster ovary cells and HeLa cells expressing high levels of insulin receptor, the hormone failed to stimulate PLD activity in 3T3-L1 adipocytes under conditions where phorbol ester was highly effective. Thus, PLD may play in a constitutive manner to promote membrane processes involved in insulin-stimulated GLUT4 translocation.

**EXPERIMENTAL PROCEDURES**

**Materials—**Rabbit polyclonal antibodies against the 20 C-terminal residues of ARF1 was kindly provided by Dr. Stephen Langille. Rabbit polyclonal anti-GLUT4 antibody was raised against the C-terminal 12 amino acids of a monoclonal anti-glutathione S-transferase 3B4 clone (3BC4) was from Genzyme (Cambridge, MA). The rhodamine- or fluorescein-conjugated secondary antibodies were from BIOSOURCE International (Camarillo, CA). Cy2-conjugated goat anti-mouse IgG was from Jackson Immunoresearch (West Grove, PA). Purified PLD preparations from Streptomyces chromofuscus (bacteria), Arachus hypogaea (peanut) and Brussica oleracea var. capitata (cabbage) were from Sigma. The pCDNA3-GLUT4eGF was a gift from Dr. Jeff Pessin. cDNA Constructs—Human PLD1 and PLD2 in the pCGN vector was the kind gift of Dr. Michael Frohman. The deletion mutant of PLD1 (deletion of residues 224–323) was created just before the termination codon of GFP cDNA and GLUT4 cDNA. The first amino acid of GLUT4 starts created just before the termination codon of GFP cDNA and GLUT4 cDNA. Immunofluorescence Microscopy—Serum-starved CHO-T cells were incubated with insulin (100 nM for 30 min) 24 h post-transfection for the indicated times and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) (171 mM NaCl, 10.1 mM Na2HPO4, 3.35 mM KCl, 1.84 mM KH2PO4, pH 7.2). Electroporated adipocytes were prepared as previously described. CHO-T cells were permeabilized with 0.5% Triton X-100 in PBS and 1% fetal bovine serum (buffer A) for 15 min, and 3T3-L1 adipocytes were permeabilized with methanol for 2 min at room temperature and blocked in buffer A. The cells were then incubated for 2 h with the IgG fraction of a polyclonal rabbit anti-HA peptide antibody (2–4 mg/ml) generated in this laboratory or the monoclonal E 3.2.1 glut4 antibody (1:10 dilution of tissue culture supernatant) or with a 1:100 dilution of an anti-ARF1 antibody. The coverslips were washed extensively with buffer A and incubated in a 1:1000 dilution of the appropriate secondary antibody conjugated to fluorescein isothiocyanate or rhodamine. The coverslips were washed again extensively with buffer A, rinsed once with PBS, and mounted on slides with 90% glycerol in PBS + 2.5% 1,4-diazabicyclo(2.2.2)-octane. The stained cells were observed with an Axiovert 35 (Zeiss) microscope equipped with an Olympus OM 4T camera for standard immunofluorescence. Confocal microscopy was performed using a Nikon Diaphot 200 inverted microscope and a MRC1024 processing unit (Bio-Rad). Zoom factors of 1.0–2.0 were used, and the images were analyzed by Lasersharp processing software.

**Measurement of PLD Activity—**Cells were grown in 6-well tissue culture plates, serum-starved overnight, and labeled with 50 μCi/well [3H]myristic acid for 1–4 h. After the indicated periods of stimulation with 100 nM insulin, 1-butanol was added to a final concentration of 1.7% for 15 min, and the samples were placed on ice, rinsed with cold PBS, and the lipids extracted by the method of Bligh and Dyer (38). The dried samples were resuspended in chloroform/ methanol (2:1), and the phospholipids were resolved on oxalate-treated Silica Gel 60 TLC plates (Whatman) by developing with chloroform/methanol/acetic acid (65:40: 2). The plates were air-dried, treated with ENHANCE (NEN Life Science Products), and exposed to a Kodak X-Omat AR film. Following autoradiography, phosphatidylbutanol (PtdBuOH) and phosphatidylcholine (PtdCho) spots were cut from the chromatography plates, and the radioactivity was monitored in a Beckman LS9000 β counter.

**Microinjection and Assay of PLD Effects—**Adipocytes differentiated for 7 days were replated on acid-etched glass coverslips using trypsin and incubated in DMEM containing 25 mM glucose and 10% fetal bovine serum. Before experiments, adipocytes differentiated for 11–15 days were incubated 2 h in serum-free medium and injected with 2 mg/ml glutathione S-transferase (GST as a marker for cellular injection) and purified PLD diluted in 100 mM KCl, 5 mM NaH2PO4, pH 7.2. Adipocytes were microinjected using an Eppendorf injection apparatus with an injection pressure of 50–100 hectopascals and an injection time of 0.2–0.5 s. The injection volume is 1–10% of the cell volume under these conditions. Following microinjection, adipocytes were incubated with or without insulin for 30 min and fixed in 100% methanol (6 min, −20°C). Coverslips were blocked for 10 min in PBS containing 1% (v/v) fetal bovine serum. Coverslips were incubated overnight at 4°C with PBS containing 1% fetal bovine serum and the anti-GLUT4 antibody (1:500). The next day coverslips were washed in PBS (2 × 10 min) and blocked for 30 min in PBS containing 5% non-immune goat serum. Coverslips were incubated 1 h with a rhodamine-conjugated goat anti-rabbit IgG to determine the subcellular distribution of GLUT4. Cells were washed twice with PBS, rinsed in distilled water, and mounted on glass slides in mounting medium (100 μM Tris, pH 8.5, 10% polyvinyl alcohol, 20% glycerol, and 0.1% p-phenylenediamine). Injected adipocytes were examined using fluorescence microscopy (Zeiss Axioskop, × 630 magnification) and scored for the presence of GLUT4 immunoreactivity at the plasma membrane. Adipocytes judged as having substantial cell surface GLUT4 immunoreactivity had a narrow band of intense fluorescence encircling the cell.

**RESULTS**

Preliminary experiments were conducted to test whether PLD activity might be required for insulin stimulation of glucose transport in 3T3-L1 adipocytes. Primary cultures were known to drive the generation of adenosine phosphatidic acid from phosphatidylcholine during catalysis by PLD, thus inhibiting the formation of phosphatidic acid itself (39). Secondary or tertiary alkaloids do not appreciably participate in this reaction. This divergence in activity between primary versus secondary or tertiary alkaloids has prompted experiments demonstrating
Phospholipase D in GLUT4 Translocation

Fig. 1. Microinjection of PLD into 3T3-L1 adipocytes potentiates insulin action on GLUT4 translocation. A, 3T3-L1 adipocytes were incubated in serum-free medium for 2 h and microinjected with the indicated concentrations of PLD purified from S. chromofuscus. Sixty minutes later adipocytes were incubated with media alone (open diamonds) or 0.5 nM insulin (open squares) for 30 min, fixed in methanol, and immunostained with anti-GLUT4 antibody. The percentages of microinjected adipocytes exhibiting GLUT4 at the plasma membrane were determined. B, 3T3-L1 adipocytes were microinjected with 2 units/ml PLD purified from either cabbage, peanuts, or S. chromofuscus. One hour after injection adipocytes were incubated for 30 min with media alone, 1 nM insulin, or a maximally effective concentration of insulin (10 nM). The percentage of microinjected adipocytes with plasma membrane staining for GLUT4 was determined. The means ± S.D. are shown.

selective blockade of PLD-mediated processes from the former (40–44). Thus, in the present studies, 3T3-L1 adipocytes were incubated with 0.75 or 1.25% 1-butanol or 2-butanol in the presence or absence of insulin prior to assay of deoxyglucose transport (not shown). Both alcohols were inhibitory, but the primary alcohol was much more effective in attenuating deoxyglucose uptake at the higher concentration than was 2-butanol (almost complete inhibition versus about 50% inhibition). Although not conclusive, these preliminary results were encouraging and consistent with the hypothesis that PLD activity may be involved in insulin-regulated glucose transport.

A second approach taken to determine whether PLD activity may regulate GLUT4 glucose transporter translocation involved microinjection of single 3T3-L1 adipocytes with various preparations of purified enzyme followed by microscopic assessment of GLUT4 localization (45). Microinjection of up to 10 units/ml purified PLD (S. chromofuscus) had little effect on the subcellular distribution of GLUT4 in unstimulated cells (Fig. 1A). However, microinjection of PLD caused a concentration-dependent increase in cell surface GLUT4 in 3T3-L1 adipocytes incubated with 0.5 nM insulin. This low concentration of insulin had little or no effect alone but acted synergistically with injected PLD. The percentage of injected cells with GLUT4 at the cell surface was maximal at an injected concentration of 2 units/ml PLD. Since the injected volume is approximately 10% of the cell volume, we estimate the intracellular concentration of PLD in the injected adipocytes at 0.2 units/ml. To address the possibility that the increase in cell surface GLUT4 following injection of PLD might be due to a co-purifying bacterial contaminant, we also injected PLD purified from peanut or cabbage. Injection of cabbage or peanut PLD into 3T3-L1 adipocytes significantly enhanced the percentage of cells with GLUT4 at the plasma membrane (Fig. 1B) relative to un.injected cells stimulated with 1 nM insulin. The effect of injected peanut or cabbage PLD on GLUT4 translocation in the presence of the low concentration of insulin was approximately half that of a maximally effective concentration of insulin (10 nM insulin) in these experiments. Injection of PLD at 2 units/ml did not enhance GLUT4 translocation in response to 10 nM insulin, consistent with the concept that insulin and PLD are stimulating GLUT4 translocation through a similar mechanism.

Experiments were also conducted to examine the cellular localization of PLD isoforms with respect to GLUT4. Preliminary studies were conducted by transiently expressing Myc epitope-tagged PLD1 or HA epitope-tagged PLD2 in CHO-T cells followed by immunofluorescence microscopy using anti-Myc or anti-HA antibodies to localize these proteins. As previously reported (46), PLD1 localized to intracellular vesicular structures that were often concentrated in the perinuclear region of these cells (Fig. 2). Insulin treatment did not disrupt the association of PLD1 with intracellular membranes but appeared to cause the dispersion of PLD1-associated membranes away from the perinuclear region. Interestingly, deletion of the
region of the PLD1 cDNA encoding residues 224–323 yielded an expressed protein that failed to associate with intracellular membranes and instead localized in the cytoplasm of CHO-T cells (Fig. 2). Residues 224–323 within PLD1 have been noted to exhibit weak but significant similarity to pleckstrin homology domains, which are known to direct the membrane targeting of many proteins. Expressed HA-tagged PLD2 also displayed a cytoplasmic disposition but also localized to plasma membranes that could be visualized when the focal plane was adjusted to the top surface of cells (Fig. 2). Insulin had no detectable effect on the disposition of expressed HA-tagged PLD2 in these cells.

In order to evaluate the relative subcellular localizations of PLD1 and GLUT4, a fusion construct was engineered containing GLUT4 and green fluorescent protein (GLUT4eGFP) as described previously (47). Preliminary experiments confirmed the co-localization of GLUT4eGFP with endogenous GLUT4 in 3T3-L1 adipocytes (not shown). As observed in CHO-T cell (Figs. 2 and 3A), expressed Myc-tagged PLD1 localized to intracellular membranes in 3T3-L1 adipocytes as did GLUT4eGFP (Fig. 3). Analysis of the intracellular sites where these proteins localized by confocal microscopy revealed partial co-localization of PLD1 and GLUT4eGFP under the conditions of these experiments (Fig. 3, right panels). Taken together, the data in Figs. 2 and 3 reveal that PLD1 is a component of at least some of the intracellular membranes that sequester GLUT4 in 3T3-L1 adipocytes.

The results described above indicating that PLD potentiates the action of insulin on GLUT4 translocation (Fig. 1) and is present at membrane sites that also contain GLUT4 (Fig. 3) raise the question whether PLD may be a target of insulin signaling. Extensive investigations have shown that PLD is potently stimulated by many hormones and other external stimuli (24), and some reports have claimed similar effects of insulin (37, 48). In the present studies, the effects of insulin receptor signaling and the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) on PLD activity in intact cells were assessed in three different model systems as follows: CHO-T and HeLa cells expressing high levels of insulin receptors and 3T3-L1 adipocytes. In these experiments, PLD activity in intact cells incubated with primary alcohol is estimated based on the production of alcoholic phosphatidic acid. Fig. 4 shows that in the CHO-T cell model insulin elicited a rapid stimulation of PLD activity that appeared to be maximal after only 5 min of treatment (Fig. 4A). Both insulin and PMA caused severalfold stimulations of PLD activity (Fig. 4B). However, only the insulin-stimulated PLD activity could be inhibited by the PI 3-kinase inhibitor wortmannin (Fig. 4B). These results...
indicating that insulin but not PMA regulates PLD activity through a PI 3-kinase-dependent pathway in CHO-T cells expressing human insulin receptors.

One mechanism that might account for the results presented in Fig. 4 that has been raised in previous studies involves stimulation of PLD activity through activated ARF proteins (48, 49). GTP loading of ARF1 in intact cells is sensitive to the toxin brefeldin A (50), which has been reported to block insulin-activated PLD activity (49). However, in our experiments in CHO-T cells, this does not appear to be the case. Fig. 5 shows that incubation of CHO-T cells with brefeldin A is indeed able to disrupt ARF1 function, as evidenced by the disruption of Golgi morphology visualized by immunofluorescence microscopy using anti-ARF antibody. B, number of counts in PtdBuOH normalized for the amount of counts in PtdCho. The bars are means of quadruplicate determinations, and the error bars are the S.D. Similar results were obtained in three other experiments.

In these cells PMA was also an effective stimulator of the enzyme, whereas expression of tyrosine kinase-deficient insulin receptors was without effect, as expected.

A quite different profile of PLD regulation was observed in 3T3-L1 adipocytes, which express endogenous GLUT4 and serve as a unique cell culture model for insulin action on glucose transport in fat cells. In 3T3-L1 adipocytes, PMA was a strong activator of PLD (Fig. 7), consistent with its effects in CHO-T and HeLa cells (Figs. 4 and 5). In contrast, insulin was without effect on PLD activity in intact 3T3-L1 adipocytes, as determined by PtdBuOH formation in total cell extracts (Fig. 7). In order to enhance the sensitivity of the assay in these cells, we took advantage of the fact that the product of PLD activity PtdBuOH is membrane-bound. Thus, one can analyze this reaction product in different membrane fractions derived from cells that had been incubated in the presence of the alcohol. We performed such analyses in plasma membranes (not shown), high density microsomes, and low density microsomes purified from 3T3-L1 adipocytes treated without or with insulin or PMA, with results similar to those obtained with total cell extracts, i.e. no effect of insulin (Fig. 7). PMA treatment of 3T3-L1 adipocytes stimulated PLD activity in all of these membrane fractions. These data indicate that insulin has little or no effect on PLD activity in 3T3-L1 adipocytes compared with that observed for PMA under these experimental conditions.

**FIG. 4.** Activation of PLD in CHO-T cells in response to insulin. A, time course of stimulation. CHO-T cells were incubated in the absence of serum overnight and labeled with [3H]myristic acid for 1 h. At time 0, cells were stimulated with 10^{-7} M insulin, and 1-butanol was added at the indicated times for 15 min, and the amount of PtdBuOH produced was determined as described under “Experimental Procedures.” B, sensitivity of stimulation of PLD to wortmannin. CHO-T cells were serum-starved overnight, and wortmannin was added at a final concentration of 100 nM 10 min before stimulation with 100 nM insulin or 1 μM PMA. After 5 min, 1-butanol was added, and the reaction was terminated after 15 min. The upper panel shows the autoradiograph of the labeled PtdBuOH. The bar graph shows the means of the intensities of the spots as determined by the “NIH image” analysis program, and the error bars are the S.D. (n = 2). Similar results were obtained in three other experiments.

**FIG. 5.** Lack of effect of brefeldin A on activation of PLD in CHO-T cells by insulin. CHO-T cells were labeled with [3H]myristic acid for 1 h. 100 μM brefeldin A was added to half of the cultures, and 100 nM insulin was added 10 min later. After 5 min, 1-butanol was added, and the reactions were terminated after a further 15 min of incubation as described under “Experimental Procedures.” A, brefeldin A-treated and control cells stained with an anti-ARF1 antibody. B, number of counts in PtdBuOH normalized for the amount of counts in PtdCho. The bars are means of quadruplicate determinations, and the error bars are the S.D. Similar results were obtained in three other experiments.

**DISCUSSION**

The potential role of PLD activity in membrane trafficking pathways has been highlighted over the past several years in several model systems of constitutive as well as regulated (22, 23, 25) secretion. In permeabilized cells, nascent secretory vesicle budding from the trans-Golgi is enhanced by PLD as well as the small GTPase protein ARF1, an activator of PLD activity (42, 51). This effect of PLD is mediated through its product phosphatidic acid generated through hydrolysis of phosphati-
HeLa cells were grown in 6-well tissue culture plates and transfected with 0.75 μg of pCMV5 encoding the insulin receptor (IR), the kinase-inactive mutant of the insulin receptor (IR*), or pCMV5 containing no insert. Cells were serum-starved overnight and labeled with [3H]myristic acid for 4 h. PLD assays were then performed on the transfectants as described under “Experimental Procedures.” Following autoradiography, areas containing PtdBuOH and PtdCho were cut from the chromatography plates and counted in a β-counter. A, autoradiograph of a chromatography plate. B, PLD activation expressed as the ratio of PtdBuOH/PtdCho. The bars are the means ± S.D., n = 3. Similar results were obtained in two other experiments.

diphosphatidylcholine. Similarly, regulated secretory activity in adrenal cells is associated with the translocation of PLD bound to intracellular chromatin granules to the cell surface membrane (52). ARF-regulated PLD activity also localizes to secretory vesicles and mobilizes to the plasma membrane in response to stimulation of neutrophils with N-formylmethionyl-leucyl-phenylalanine, an agonist that induces inflammation. PLD also appears to be a key element in the process of phagocytosis (40), which likely involves movement of intracellular membranes to the plasma membrane of the extending pseudopods (53). Taken together, these and other studies have established a potential link between PLD and membrane trafficking processes, prompting the present evaluation of PLD involvement in membrane movements of GLUT4 in response to insulin.

The studies presented here provide two lines of evidence that PLD may be involved at some stage of the membrane recycling pathway traversed by the GLUT4 glucose transporter. First, partial co-localization of expressed, epitope-tagged PLD1 with GLUT4 fused to green fluorescent protein is evident in both 3T3-L1 adipocytes and CHO-T cells (Fig. 3). The GLUT4-green fluorescent protein fusion construct, like endogenous GLUT4, localizes to both a predominant perinuclear disposition and to small vesicular structures in the periphery of the cells (Figs. 2 and 3).

The partial co-localization of PLD1 with GLUT4 includes both these populations of GLUT4-containing membranes. This overall distribution of PLD1 in intracellular membranes is not unlike that reported in other cell types (46). Our results on the cellular localization of PLD2 to cytoplasm and plasma membrane also appear to agree with previous studies (46), making it unlikely that present at significant levels in GLUT4-containing membranes. An interesting and novel finding in our experiments is the apparent requirement of the PH domain for PLD1 association with intracellular membranes (Fig. 2). Thus, deletion of this domain conferred a cytosolic disposition to the enzyme. These findings are consistent with extensive published data showing that PH domains from a large number of proteins bind membrane components such as phosphoinositides (54–57). Juxtaposed to the PH domain on PLD1 is a region denoted as a PX domain, similar to regions on several proteins that are involved in membrane trafficking (26). The PX domain may thus also be involved in membrane localization. It will be interesting to test this hypothesis and to determine the membrane component or components that associate with the PLD1 PH domain.

A second set of data suggesting that PLD may be involved in GLUT4 translocation to the cell surface membrane in response to insulin derives from direct effects of microinjected enzyme in 3T3-L1 adipocytes (Fig. 1). The results indicating that increasing intracellular PLD activity has little effect on GLUT4 localization in either the absence or presence of maximal concentrations of insulin suggests that there is little or no toxic effect caused by this manipulation. Importantly, the stimulatory effect of a submaximal concentration of insulin is potentiated by microinjection of PLD. Reinforcing the reproducibility of this response is the observation that PLD enzyme preparations from multiple sources have the same stimulatory effect on GLUT4 translocation when added with submaximal concentrations of insulin (Fig. 1). These data indicate that one or more events triggered by insulin signaling are required for GLUT4 translocation to become sensitive to PLD action. It has been suggested that insulin signaling to GLUT4 may require two regulatory pathways, only one of which requires PI 3-kinase. In experiments not illustrated we found that wortmannin blocks GLUT4 translocation that is stimulated when PLD is microinjected into cells that are then exposed to low concentrations of
insulin. Taken together, the results indicate that PI 3-kinase activation by insulin is required for PLD to potentiate its action on GLUT4 translocation.

The data and considerations described above also raise the hypothesis that PLD may be a target of insulin action and that insulin-stimulated PLD activity may constitute a necessary signaling pathway for GLUT4 regulation. PLD activity is regulated by a remarkable number of agonists in a vast array of cell types (24). Considerable data support the activation of PLD through the actions of protein kinase C as well as small G proteins such as ARF proteins, Rho, Rac, and Ral (24–26). Recent results have implicated signaling pathways initiated by receptor tyrosine kinases in the activation of PLD (24), including the insulin receptor tyrosine kinase. However, many of these latter results have been obtained in cells expressing very high levels of insulin receptors (48). Similar data in fat cells have been reported by one group (37) but not by others. Our data strongly indicate that insulin receptor signaling can activate PLD activity in cells artificially expressing high levels of insulin receptors. Thus, in both CHO-T cells stably expressing human insulin receptors and HeLa cells transiently expressing human insulin receptors PLD activation is observed (Figs. 5 and 6). However, in contrast to a report on fibroblasts expressing high levels of human insulin receptors (49), in our studies the stimulation of PLD by insulin was blocked by wortmannin (Fig. 4) but not brefeldin A (Fig. 5). The results of the present work suggest that in CHO-T cells insulin activation of the enzyme occurs by a mechanism that requires PI 3-kinase but is independent of brefeldin A-sensitive ARF exchange factors.

A striking feature of the present work is the lack of effect of insulin on PLD activity in 3T3-L1 adipocytes, which do respond to insulin with enhanced GLUT4 translocation (Fig. 1). This failure of insulin to activate the enzyme in adipocytes occurs under conditions where our assay readily detects PLD activation by phorbol ester (Fig. 7). Furthermore, we enhanced the sensitivity of our assay conditions by isolating specific membrane fractions from cells that were incubated with or without insulin prior to monitoring the phosphatidylbutanol turnover product of PLD in these membrane fractions. These data strongly indicate that PLD is not significantly stimulated by insulin in these cells under conditions where GLUT4 regulation occurs. This conclusion is also consistent with the findings (58) and view of Exton (24), who recently cataloged the many agents that reproducibly regulate PLD without including insulin.

In the absence of an effect of insulin on PLD in normal insulin-sensitive cell types that respond to the hormone with increased GLUT4 translocation, how might PLD potentiate the action of hormone on GLUT4? One possibility is that phosphatidic acid produced by PLD in intracellular membranes that sequester GLUT4 acts to enhance the budding of vesicles destined for transport to the cell surface. Recent work has indicated that budding of Golgi vesicles in a cell-free system requires ARF1 protein but not phosphatidic acid (29). These results suggested that PLD activity may not be absolutely required for Golgi membrane transport. However, acidic phospholipids such as phosphatidic acid were found to enhance ARF1-mediated membrane budding in this system (28, 59). Thus, it seems plausible that phosphatidic acid produced by microinjected PLD may enhance a mechanism stimulated by insulin signaling that serves to cause GLUT4-containing vesicles to bud. Although we have as yet no direct evidence to support this hypothesis, it should be a useful one for devising future experiments.

Acknowledgments—We thank Dr. M. Frohman for providing PLD cDNA constructs. We acknowledge the excellent assistance of Jane Erickson in preparation of the manuscript.

Note Added in Proof—During the review process for this manuscript, a report (SciarrA, V. A., Rudge, S. A., Prestwich, G. D., Frohman, M. A., Englebrecht, J., and Morris, A. J. (1999) EMBO J. 18, 5911–5921) appeared showing PtdIns(4,5)P2 binding to PLD2 does not require its PH domain and that PtdIns(4,5)P2 binding was not required for membrane association of PLD2. These data are consistent with our present results indicating the PLD PH domain is a membrane targeting region and raises the question of its endogenous ligand.

REFERENCES

1. Czech, M. P., and Verdera, S. (1999) J. Biol. Chem. 274, 1865–1868
2. Pessin, J. E., Thurmond, D. C., Elmenhorst, J. C., Coker, K. J., and Okada, S. (1999) J. Biol. Chem. 274, 2593–2596
3. Charron, M. J., Katz, E. B., and Olson, A. L. (1999) J. Biol. Chem. 274, 3253–3256
4. Yang, J., and Holman, G. D. (1993) J. Biol. Chem. 268, 4600–4603
5. Okada, T., Kawanou, Y., Sakakihara, T., Hazeki, O., and Ui, M. (1994) J. Biol. Chem. 269, 3569–3573
6. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Koh, C. R. (1994) Mol. Cell. Biol. 14, 4902–4911
7. Sharma, P. M., Egoza, K., Hsuan, J. L., Huvar, I., Boss, G. R., and Olefsky, J. M. (1998) J. Biol. Chem. 273, 18528–18537
8. White, M., and Kahn, C. R. (1994) J. Biol. Chem. 269, 1–4
9. Corvera, S., and Czech, M. P. (1998) Trends Cell Biol. 8, 442–446
10. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) J. Biol. Chem. 271, 31372–31377
11. Wang, Q., Somwar, R., Bilan, P. J., Liu, Z., Jin, J., Woodgett, J. R., and Klip, A. (1999) Mol. Cell. Biol. 19, 4008–4018
12. Coffer, P. J., Jin, J., and Woodgett, J. R. (1998) Biochem. J. 335, 1–13
13. Bandyopadhyay, G., Standaert, M. L., Zhao, L., Yu, B. Avignon, A., Galloway, L. J., Karlam, P., Mosecz, J., and Farese, R. V. (1997) J. Biol. Chem. 272, 2551–2558
14. Kotani, K., Ogawa, W., Matsumoto, M., Kitamura, T., Sakae, H., Hino, Y., Miyake, K., Sano, W., Akotu, K., Ohno, S., and Kasuga, M. (1998) Mol. Cell. Biol. 18, 6971–6982
15. Klarlund, J. K., Guilherme, A., Holik, J. K., Virbasius, J. V., Chawla, A., and Czech, M. P. (1997) Science 273, 1927–1930
16. Klarlund, J. K., Rameh, L., Canley, L. C., Buxton, J. M., Holik, J. K., Sakelis, C., Patiki, V., Corvera, S., and Czech, M. P. (1998) J. Biol. Chem. 273, 1589–1602
17. Langille, S. E., Patiki, V., Klarlund, J. K., Buxton, J. M., Holik, J. K., Chawla, A., Corvera, S., and Czech, M. P. (1998) J. Biol. Chem. 273, 27099–27104
18. Venkateswarlu, K., Oatey, B. P., Tavare, J. M., and Cullen, P. J. (1998) Curie. Biol. 8, 463–466
19. Ashery, U., Koch, H., Scheuss, V., Brose, N., and Retting, J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1094–1099
20. Venkateswarlu, K., Gunn-Moore, F., Oatey, B. P., Tavare, J. M., and Cullen, P. J. (1999) Biochem. J. 335, 139–146
21. Moss, J., and Vaughan, M. (1998) J. Biol. Chem. 273, 21431–21434
22. Moss, J., and Vaughan, M. (1998) Mol. Cell. Biol. 183, 153–157
23. Jones, D., Morgan, C., and Cockcroft, S. (1999) Biochem. Biophys. Acta 1439, 229–244
24. Exton, J. H. (1999) Biochim. Biophys. Acta 1439, 121–133
25. Chavrier, P., and Goud, E. (1999) Curie. Opt. Cell Biol. 11, 466–475
26. Frohman, M. A., Sung, T. C., and Morris, A. J. (1999) Biochem. Biophys. Acta 1439, 175–186
27. Meier, K. E., Gibbs, T. C., Knoepp, S. M., and Ella, K. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13676–13680
28. Tolias, K. F., and Canley, L. C. (1999) Chem. Phys. Lipids 98, 69–77
29. Standaert, M. L., Avignon, A., Yamada, K., Bandyopadhyay, G., and Farese, R. V. (1996) Biochem. J. 313, 1039–1046
30. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Physiol. Pharmacol. 37, 911–917
31. Morris, A. J., Frohman, M. A., and Englebrecht, J. (1997) Anal. Biochem. 252, 239–249
46. 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) *Curr. Biol.* **7**, 191–201
47. Min, J., Okada, S., Kanzaki, M., Elmendorf, J. S., Coker, K. J., Ceresa, B. P., Syu, L. J., Noda, Y., Saltiel, A. R., and Pessin, J. E. (1999) *Mol. Cell* **3**, 751–760
48. Shome, K., Vasudevan, C., and Romero, G. (1997) *Curr. Biol.* **7**, 387–396
49. Rizzo, M. A., Shome, K., Vasudevan, C., Stolz, D. B., Sung, T. C., Frohman, M. A., Watkins, S. C., and Romero G. (1999) *J. Biol. Chem.* **274**, 1131–1139
50. Chardin, P., and McCormick, F. (1999) *Cell* **97**, 153–155
51. Chen, Y. G., and Shields, D. (1996) *J. Biol. Chem.* **271**, 5297–5300
52. Caumont, A. S., Galas, M. C., Vitale, N., Aunis, D., and Bader, M. F. (1998) *J. Biol. Chem.* **273**, 1373–1379
53. Cox, D., Tseng, C. C., Bjekic, G., and Greenberg, S. (1999) *J. Biol. Chem.* **274**, 1240–1247
54. Bottomley, M. J., Salim, K., and Panayotou, G. (1998) *Biochim. Biophys. Acta* **1436**, 165–183
55. Rebecchi, M. J., and Scarlata, S. (1998) *Annu. Rev. Biophys. Biomol. Struct.* **27**, 503–528
56. Katan, M., and Allen, V. L. (1999) *FEBS Lett.* **452**, 36–40
57. Irvine, R. (1998) *Curr. Biol.* **8**, R557–R559
58. Augert, G., and Exton, J. H. (1988) *J. Biol. Chem.* **263**, 3600–3609
59. Spang, A., Matsuoka, K., Hamamoto, S., Schekman, R., and Orci, L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 11199–11204