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Thin Layer Chromatography–Blotting, a Novel Method for the Detection of Phosphoinositides

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Phosphoinositides are believed to be involved in fundamental cellular events such as signal transduction and vesicular trafficking. Aberrant metabolisms of this lipid, caused by mutations in phosphoinositide kinases, phosphatases and lipases are known to be related to variety of human disorders such as diabetes and cancer. While the majority of such information is obtained by analyzing genetic and biochemical properties of phosphoinositide-metabolic enzymes, direct measurement of cellular content of the lipid is hindered by the lack of a simple method that is sensitive enough to measure phosphoinositides present in trace amounts *in vivo*. Here, we describe a novel, thin layer chromatography (TLC)–based method by which cellular phosphoinositides are separated, transferred and detected by specific phosphoinositide-binding domains. This method was applied to follow the generation of minor phosphoinositides, such as PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in response to insulin and to compare PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 levels in several cancer cell lines. The method has potential application not only in investigating the physiological roles of phosphoinositides, but also in diagnosing metabolic disease and cancer by directly assessing phosphoinositide levels in samples obtained from patients.

Key words: cancer, insulin, phosphoinositide, phosphoinositide binding domain, thin layer chromatography (TLC)–blotting.

Phosphoinositides are minor components of less than one per cent in cellular membranes that play critical roles in a wide range of physiological events such as cell proliferation, survival, motility and intercellular membrane trafficking (*1–3*). Aberrant activities of phosphoinositide-metabolic enzymes are known to cause a variety of diseases including cancer, diabetes, myopathy and Lowe syndrome (*4–6*). Nevertheless, direct evidence for alternations of phosphoinositide contents has rarely been presented. To date, the most common method to measure cellular contents of phosphoinositide is radioisotope labeling, in which cellular lipids are metabolically labeled and separated by high performace chromatography (*7*). Recently, mass spectrometry has been utilized to measure phosphoinositides (*8*). Both of these methods, while possessing large advantages, cannot be easily adapted for high throughput analysis. Thus, a new method is needed that is convenient and user-friendy. Here, we developed a TLC blot assay to detect phosphoinositides in lipid samples extracted from culture cells. The lipids were separated by TLC, transferred onto PVDF membrane and overlaid by phosphoinositide-binding domains. Several domains are reported to recognize a specific phosphoinositide strongly and selectively: the PH domains of PLC δ1, TAPP1, FAPP1, GRP1 recognize PtdIns(4,5)P_2, PtdIns(3,4)P_2, PtdIns(4)P and PtsIns (3,4,5)P_3, respectively, and the tandem version of Hrs FYVE domain specifically binds to PtsIns(3)P, which was used broadly as a marker of intracellular localization of PtsIns(3)P (*9–15*). Here, we applied this property to detect individual phosphoinositides.

This method allows highly sensitive detection of minor phosphoinositides such as PtsIns(3,4,5)P_3 by simple procedures without radiolabeling. It is potentially a powerful tool for assessing the correlation between phosphoinositide metabolism and various diseases.

MATERIALS AND METHODS

Materials—LY294002 and Wortmannin were purchased from Wako. Rabbit anti-GST polyclonal antibody was purchased from Santa Cruz Biotechnology. Anti–rabbit alkaline phosphatase conjugate was purchased from Promega Corp. Phosphatidylinositol (PtdIns) was purchased from Sigma. PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 were purchased from Cell Signals Inc.

Cell Culture—B16, B16F1, B16F10 and U87MG cells were grown at 37°C in Dulbecco's Modified Eagle medium (Nissui) containing 10% fetal bovine serum. Chinese hamster ovary cells overexpressing human Insulin receptors (CHO-IR cells) were cultured in Ham's F12 medium (Gibco) containing 10% fetal bovine serum (FBS). A172 and T98G cells were cultured in RPMI1640 medium (Gibco) containing 10% FBS.

Protein Purification—E. coli (JM109) cells transformed with the expression vectors were grown at 37°C in 700 ml of Luria-Bertani broth for 3 h. Expression of GST-tagged proteins was induced by addition of IPTG and β-mercaptoethanol each to a final concentration of 1 mM, and the

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bacteria were grown at 25°C for 3 h. Bacteria were harvested by centrifugation at 10,000 × *g* for 10 min, and the pellet was lysed by resuspension in 10 ml of lysis buffer containing 40 mM Tris-HCl (pH 7.4), 200 mM NaCl, 5 mM EDTA and 1% Triton X-100 and then sonicated at 4°C for 5 min. The solution was ultracentrifuged at 100,000 × *g* for 30 min, and the supernatant was incubated for 2 h at 4°C with 0.1 ml of glutathione-Sepharose beads (Amersham Biosciences) equilibrated in lysis buffer. The beads were washed three times with 10 ml of wash buffer containing 40mM Tris-HCl ph 7.4, 500 mM NaCl, 5 mM EDTA and 1% Triton X-100. GST-tagged proteins were eluted by addition of solution containing 50 mM glutathione and 50 mM Tris-HCl (pH 7.4). Purified proteins were stocked at –80°C.

Lipid Extraction—Cells cultured in 15-cm dishes were washed with PBS and scraped loose. The cell pellet was resuspended in 750 μl of chloroform/methanol/1 N HCl (40:80:1 v/v/v). After sonicating, 250 μl of chloroform and 450 μl of 1 N HCl were added, and the mixture was vortexed vigorously. After centrifugation, the organic phase was collected and washed with aqueous phase of 500 μl of $CHCl_3$/MeOH/1 N HCl (10:10:4 v/v/v), and organic phase was washed with 500 μl of MeOH/0.1 M EDTA (1:0.9 v/v). The lower phase was collected and evaporated under N_2 gas.

To determine the total cellular phosphate of phospholipids, extracted phospholipids were oxidized with 250 μl of perchloric acid at 180°C. After cooling, 750 μl of H_2O, 250 μl 2.5% hexaammonium heptamolybdate and 500 μl 10% ascorbic acid were added, and the mixture was incubated at room temperature until the color was developed. Absorption measured at 795 nm.

Thin Layer Chromatography—TLC plates (Merck, Silica gel-60) were developed with a solvent system containing 1.2% potassium oxalate in MeOH/water (2:3 v/v). Before spotting, plates were heated for 15 min at 110°C. The phosphoinositides sample was dissolved in 10 μl of $CHCl_3$/MeOH (2:1 v/v) and spotted on the TLC plate. The TLC plate was then developed in $CHCl_3$/acetone/MeOH/AcOH/water (80:30:26:24:14 by volume). After drying the TLC plate, phosphoinositide was stained with Dittmer-Lester reagent (*16*).

TLC Blotting—The TLC plate was soaked in a transfer solution containing 0.2% $CaCl_2$/MeOH/2-propanol (20:7:40 v/v/v) for 30 s. PVDF and PTFE (polytetrafluoroethylene) membranes were placed on the TLC plate and then pressed at 0.07 Pa at 180°C in TLC thermal blotter (ATTO). After drying, the PVDF membrane was immersed in methanol for 1 min, then soaked in the blocking solution containing the 3% non-fat milk and 1% BSA for at least 30 min at room temperature. The membrane was then reacted with GST-domain in 0.05% Tween 20-PBS at a final concentration of 1 μg/ml for 1 h. The membrane was washed three times with 0.05% Tween 20-PBS, and anti–GST rabbit antibody diluted 1:2,000 with 0.05% Tween 20-PBS was added, and incubated for 30 min. The membrane was washed three times, and reacted with alkaline phosphatase–conjugated anti–rabbit IgG antibody diluted 1:7,500 with 0.05% Tween 20-PBS for

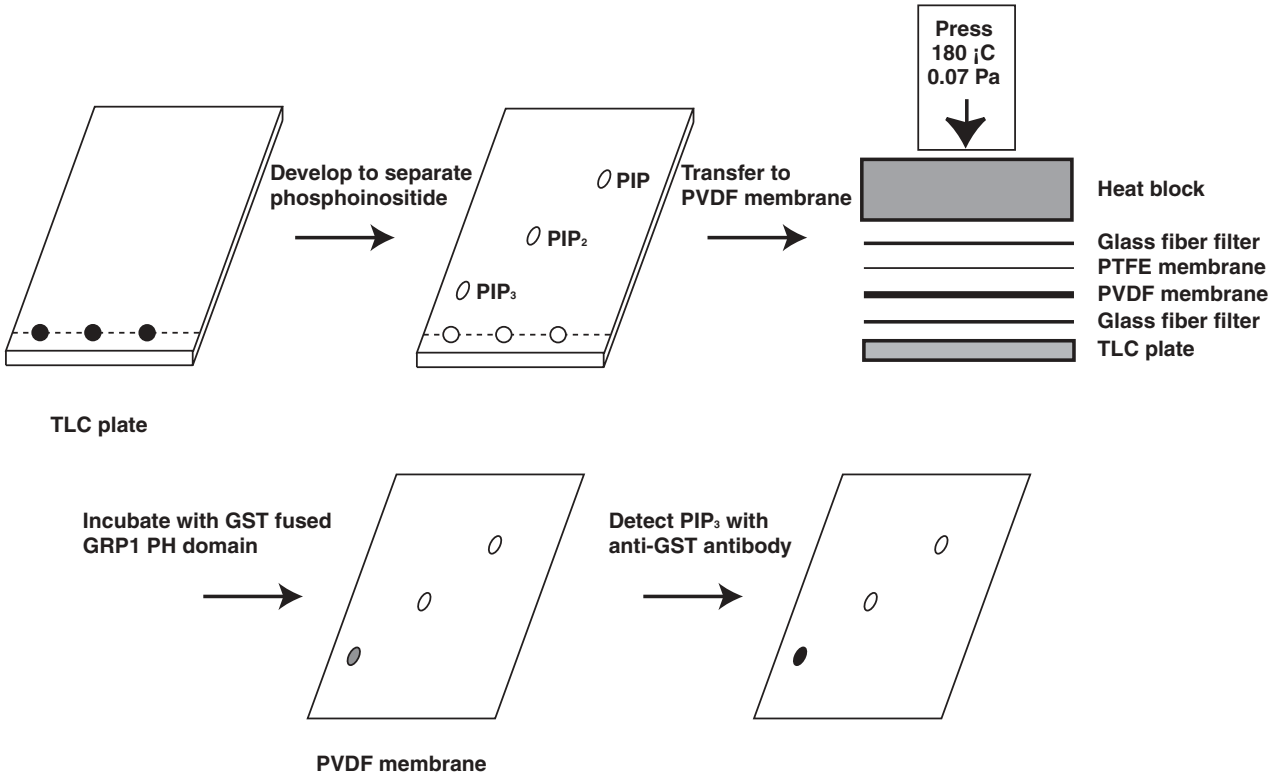

Fig. 1. **Schematic diagram of TLC blotting method.** Phosphoinositides extracted from cells were spotted onto a TLC plate, developed and transferred to a PVDF membrane at 0.07 Pa at 180°C. The membrane was incubated with blocking solution including 5% non-fat milk and 1% BSA. Each phosphoinositide was detected by use of the corresponding GST-fused phosphoinositde-binding domain.

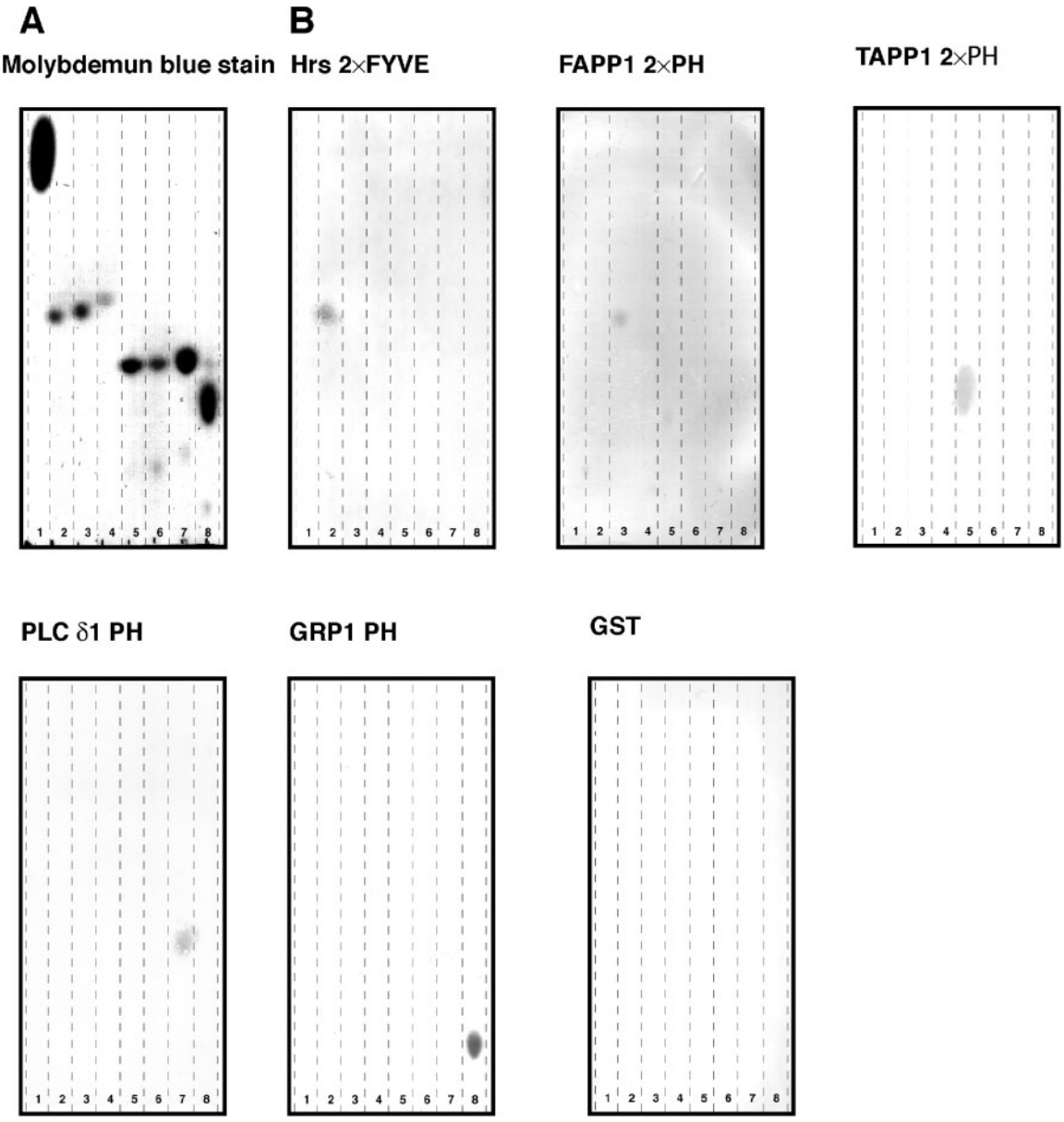

Fig. 2. **TLC blots of phosphoinositides.** Phosphoinositides (5 μg) were separated by TLC in a solvent system of $CHCl_3$/MeOH/Acetone/acetic acid/H_2O (80:30:26:24:14 by volume). TLC plates were stained with Ditter and Lester reagent. Phosphoinositides (0.2 μg) were developed as in (A) and transferred to PVDF membranes. Membranes were incubated with the indicated GST-fusion proteins, and the products were detected with alkaline phosphatase–conjugated GST antibody. Lane 1, PtdIns; Lane 2, PtdIns(3)P; Lane 3, PtdIns(4)P; Lane 4, PtdIns(5)P; Lane 5, PtdIns(3,4)P_2; Lane 6, PtdIns(3,5)P_2; Lane 7, PtdIns(4,5)P_2; and Lane 8, PtdIns(3,4,5)P_3.

30 min. The membrane was washed and stained with NBT and BCIP system.

RESULTS

Separation and Quantification of Phosphoinositides by TLC Blotting—The TLC blotting method developed by Ishikawa and Taki (*16*, *17*) for the detection of sphingoglycolipidis shown schematically in Fig. 1. We utilized this method to separate and quantify phosphoinositides from cells. First, we confirmed that phosphoinositides were separated according to the number of phosphates by TLC (Fig. 2A). Phosphoinositides on the TLC plate were then transferred to PVDF membranes. We found that the transfer efficiency was approximately 60% by comparing PtdIns(3,4,5)P_3 transferred to the membrane with the same sample directly spotted on the membrane (data not

Table 1. **The Phosphoinositides and their corresponding binding domains.**

Phosphoinositide	Domain
PtdIns(3)P	Hrs 2×FYVE
PtdIns(4)P	FAPP1 2×PH
PtdIns(3,4)P_2	TAPP1 2×PH
PtdIns(4,5)P_2	PLC δ1 PH
PtdIns(3,4,5)P_3	GRP1 PH

shown). To detect phosphoinositides, we used the phosphoinositide-binding domains as shown in Table 1, which successfully recognized PtdIns(3)P, PtdIns(4)P, PtdIns(3,4)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Fig. 2B). Phosphoinositide amounts were calculated from the data obtained by spotting authentic samples of each phosphoinositide on the TLC.

We used this method to measure cellular amounts of phosphoinositides. Their concentrations were calculated from standard curves generated from control lipids, whose linearity was evidenced by the relationship between the amount of the control lipid used and density of each spot (Fig. 3A). Because phosphoinositides are metabolized quickly by kinases, phosphatases or phospholipases, the

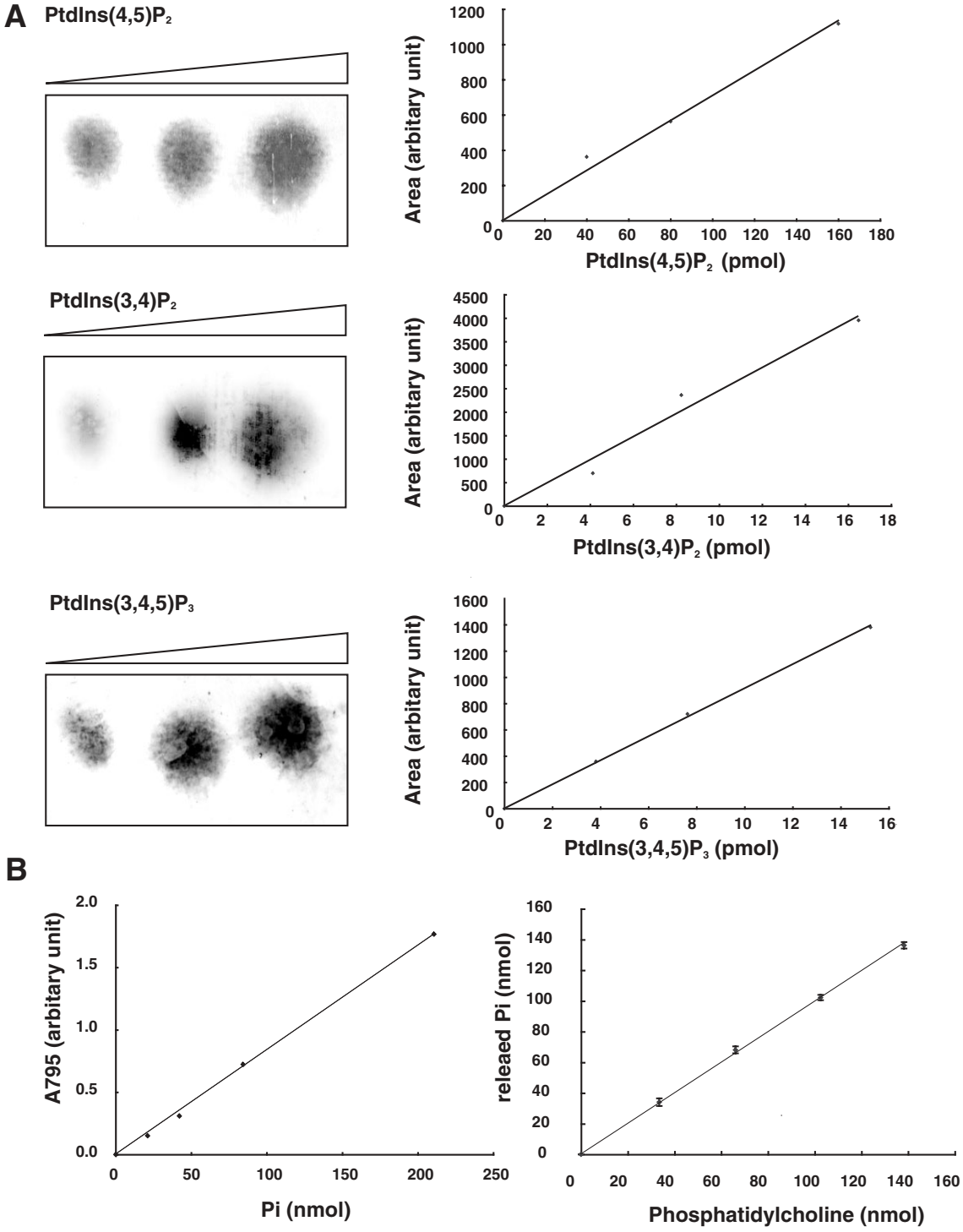

Fig. 3. **Standard curves of phosphoinostides on TLC blots.** Standard curves of PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIsnP(3,4,5)P_3. Phosphoinositides were detected by TLC blotting and the stained areas were quantified by use of ImageJ software. Standard curve of inorganic phosphate (P_i) (left) and the curve of Pi released from PtdCho used as a standard (right).

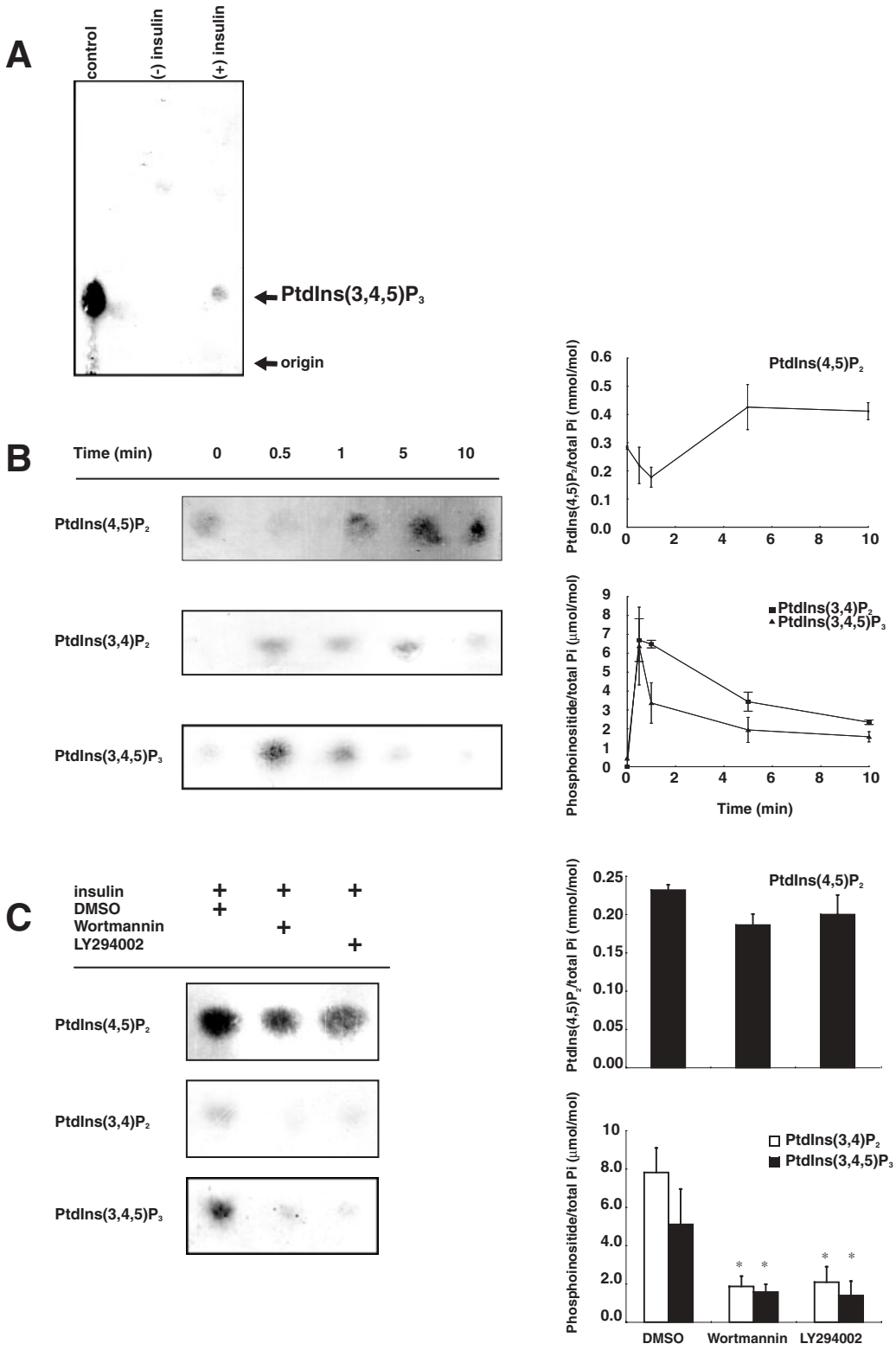

Fig. 4. **Changes in phosphoinositide levels in response to insulin.** CHO-IR cells cultured on 15-cm dishes were serum-starved overnight and stimulated with 100 nM insulin for 30 s. Lipids were extracted and separated by TLC, and then endogenous PtdIns(3,4,5)P_3 was visualized as described in "MATERIALS AND METHODS." Changes of PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 levels in response to insulin as detected by the GST- PLC δ1 PH, TAPP1 2×PH and GRP1 PH domains, respectively. Values were normalized to the amount of total phospholipid P_i. Data shown are the mean ± SEM of three independent measurements. Inhibition of PtsIns(3,4)P_2 and PtsIns (3,4,5)P_3 formation by Wortmannin and LY294002 in culture cells. Before stimulation with 100 nM insulin for 30 s, cells were incubated with 100 nM Wortmannin or 25 μM LY294002 for 30 min. Data are the means ± SEM of three independent determinations $^*p < 0.01$.

calculated values were normalized by the amount of total phospholipids, such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, as determined from the amount of inorganic phosphate (P_i) released after the digestion with perchloric acid (Fig. 3B).

Changes in Phosphoinositides after Insulin Stimulation—This new quantitative analysis of phosphoinositides was applied to monitor the changes in cellular amounts of this lipid in response to insulin treatment (*18*). CHO-IR cells, which express insulin receptors, were stimulated with 100 nM insulin for various times, and then cellular phospholipids were extracted. An aliquot (1/10 volume) of the extracted phospholipids was used to measure inorganic phosphate, and the remainder was used for the TLC-blotting. As shown in Fig. 4A, production of PtdIns(3,4,5)P_3 in response to insulin was detected which started to increase just after the insulin treatment and reached a maximum at 30 s (10-fold increase over the level at 0 s), decreased rapidly. Production of PtdIns(3,4)P_2 also increased up to 30 s and then decreased at 5 min. In contrast PtdIns(4,5)P_2 decreased to about 60% of control and recovered to the basal level by 10 min (Fig. 4B). We next examined the effects of Wortmannin and LY294002, two well-established PtdIns 3-kinase inhibitors, on the levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in insulin-stimulated cells (*19*, *20*). As shown in Fig. 4C, these inhibitors blocked production of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 but not PtdIns(4,5)P_2.

Determination of PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 Concentrations in a Variety of Cancer Cells—It has been reported that some cancer cells lack PTEN, and the absence of this tumor suppressor is thought to increase the PtdIns(3,4,5)P_3 levels, leading to the formation of cancer (*21–24*). We thus used the TLC blotting to measure the levels of PtdIns(3,4,5)P_3 and PtdIns(4,5,)P_2 in cancer cells. Mouse B16 melanoma cell and its derivative malignant cell lines were used (*25*), as well as A172, T98G and U87MG human glioblastoma cell lines, which show loss of PTEN expression or function. We extracted phospholipids from cells cultured in 15-cm dishes and measured levels of PtdIns(3,4,5)P_3 by TLC blotting. B16F10 cells had two times higher levels of PtdIns(3,4,5)P_3 than those of B16 and B16F1 cells (Fig. 5A). In contrast, PtdIns(4,5)P_2 levels in B16F10 cells were one-third lower than those in B16 cells. Phosphorylation of Akt on Thr308 and Ser473 was highly elevated, indicating that the kinase was activated downstream of PtdIns(3,4,5)P_3 in the metastastic B16F10 cell line. Such phosphorylation was not observed in the parent non-metastatic cell line, B16 (Fig. 6). Expression of p110α was also increased in B16F10 cells, although PTEN expression was not changed. These results suggest that PtdIns 3-kinase signals are up-regulated in B16F10 cells. On the other hand, in the glioblastoma cell lines, Akt was highly phosphorylated in U87MG cells, and p110α expression was increased in T98G cells (Fig. 6). Despite the fact that all these glioblastoma cell lines retain

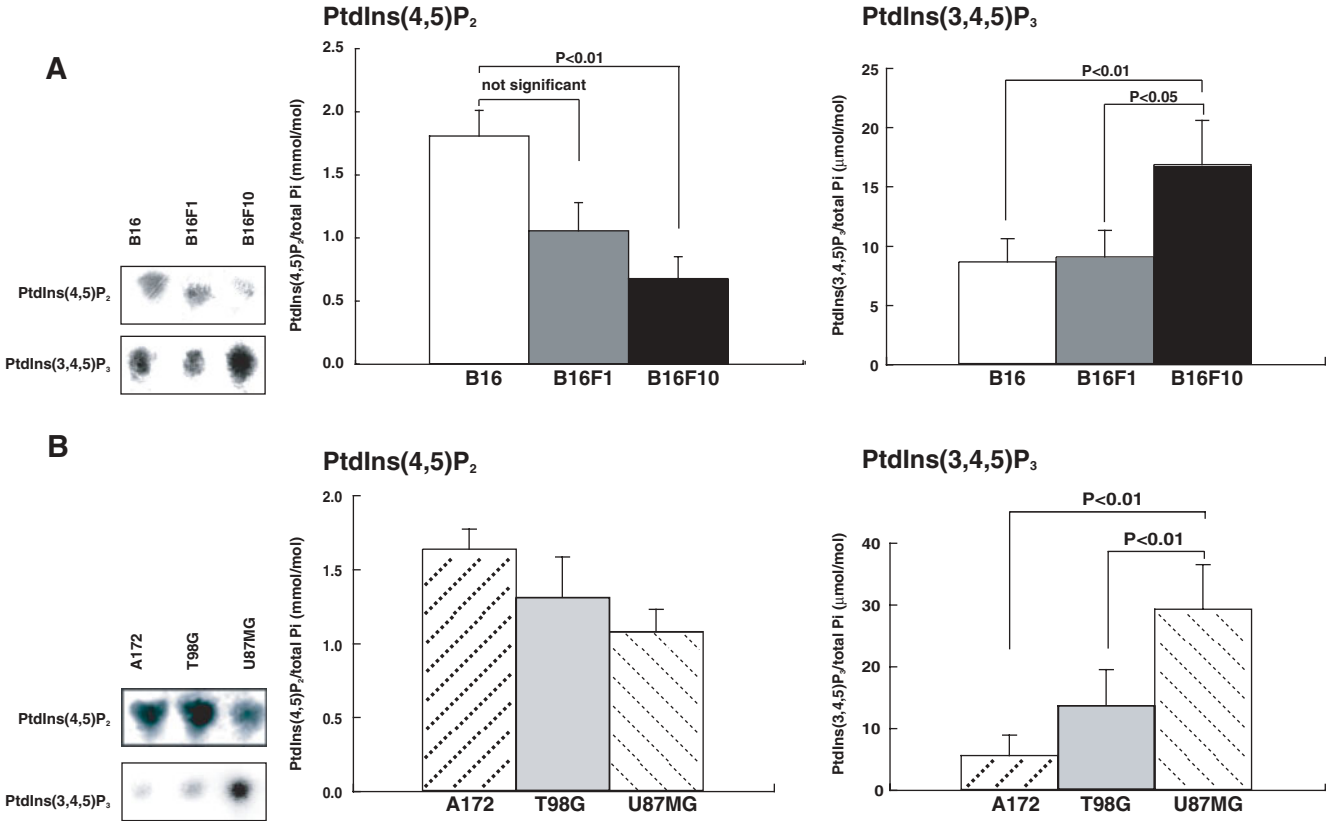

Fig. 5. **PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 concentrations in a variety of cancer cells.** PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 levels were quantified in (A) mouse malignant melanoma cell lines (B16 (white bar), B16F1 (gray bar) and B16F10 (black bar)) or (B) human PTEN–deficient cell lines [A172 (bar with diagonal dotted lines from bottom left to top right), T98G (gray bar) and U87MG (bar with diagonal dotted lines from top left to bottom right)]. Data are the means ± SEM of three independent determinations.

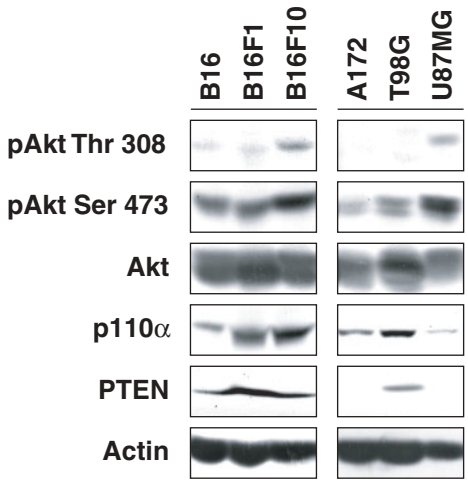

Fig. 6. **Expression of Akt, PTEN and PtsIns 3-kinase p110α.** Levels of Akt, PTEN, p110α and actin were examined by Western blotting in various cancer cell lines. Akt phosphorylation was determined with antibodies specific for Akt phosphorylated at Ser473 or Thr308.

dysfunctional PTEN, only U87MG cells showed a high level of PtdIns(3,4,5)P_3 (Fig. 5B). Interestingly, the level of PtdIns(3,4,5)P_3 in A172 cells was quite low, even though PTEN was absent. Akt activation was not observed in A172 cells, indicating that Akt activation was correlated with PtdIns(3,4,5)P_3 levels (*26*).

DISCUSSION

To measure the endogenous phosphoinositides contents, we adapted a TLC-blotting method with sufficiently high sensitivity to detect changes in phosphoinositide contents, especially those of minor phosphoinositides such as PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. On this count, the sensitivity of this new detection method is comparable to that of radiolabeling methods. Additionally, our results about the changes in phosphoinositides in response to insulin (Fig. 4B) are consistent with those from experiments based on radiolabeling methods (*7*). We also measured the absolute concentrations of phosphoinositides in several tumor cell lines. In B16 cell lines, the PtdIns(3,4,5)P_3 level with the progression of malignancy increased (Fig. 5A), but the PtdIns(4,5)P_2 level is decreased (Fig. 5A), suggesting the properties of B16F10 such as higher metastatic capability and activated proliferation are due to the elevated PtdIns(3,4,5)P_3 level. Interestingly, not all PTEN-deficient cell lines show increased levels of PtdIns(3,4,5)P_3 (Fig. 5B). Furthermore, this difference is not correlated with the expression level of p110α (catalytic subunit of PtdIns 3-kinase) (Fig. 6), suggesting that the cellular amount of PtdIns(3,4,5)P_3 is not correlated with the expression levels of metabolic enzymes. This indicates the importance of direct measurement of phosphoinositides.

With the development of molecular biology, metabolic enzymes have been extensively studied mainly with respect to their expression levels and catalytic mutations. These enzymes play critical roles in the cell, and dysfunction of these enzymes causes a variety of severe diseases (*4*). However, little quantitative information is available on phosphoinositides, which should be the critical output for the regulation of various cellular events. Several reasons for this technological retardation are conceivable: (i) phosphoinositides are present in very small amounts in the cell, (ii) conventional methods using radioisotopes are complicated and are not suitable for high-throughput assays (*7*). Here we have established a novel TLC blot assay that overcomes these difficulties. This provides researchers with a convenient tool for detection of phosphoinositides, and may be applied more broadly to the diagnosis and treatment of diseases that are caused by abnormal phosphoinositide metabolism.

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