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Involvement of sphingomyelinase in insulin-induced phosphatidylinositol 3-kinase activation

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ABSTRACT

Insulin plays a key role in regulating a wide range of cellular functions. Most functions of insulin are believed to occur through activation of phosphatidylinositol-3 kinase (PI-3 kinase) signaling pathways. The present study demonstrates that insulin, C2-ceramide, or phosphocholine (PCho) induced a strong activation of PI-3 kinase activity. This activation was dramatically inhibited by either pretreatment of cells with wortmannin, a widely used PI-3 kinase inhibitor, or constitutive overexpression of a dominant negative p85 subunit of PI-3 kinase (ΔP85). Insulin-induced activation of PI-3 kinase was observed only in the normal human lymphoblast cell line, JY, but not in the sphingomyelinase (SMase)-deficient cell line, MS1418. The PI-3 kinase activation in MS1418 cells could be restored by exposure of the cells to either SMase, C2-ceramide, or PCho. These data provide direct evidence that SMase and its products act as mediators in insulin-induced activation of PI-3 kinase.

Key words: ceramide • Niemann-Pick disease • signal transduction

Insulin plays a central role in a broad range of biological effects, including cell growth; differentiation; transformation; apoptosis; synthesis; and degradation of carbohydrates, lipids, and proteins (1–6). The insulin receptor is a disulfide-linked, heterotetrameric protein complex consisting of two hormone-binding subunits and two signaling β subunits containing tyrosine kinase activity (1, 7). Upon binding insulin, autophosphorylation of the receptor occurs via a transintermolecular reaction between the two β subunits (1, 7). This phosphorylation of tyrosine residues within the kinase domain of the insulin receptor increases catalytic activity, which leads to increased tyrosine phosphorylation of cellular substrates (8). The insulin receptor substrate-1 (IRS-1) is a major substrate for the receptor (8, 9). IRS-1 contains several potential tyrosine phosphorylation sites, at least eight of which are phosphorylated in response to insulin stimulation (9). Some of these sites are within a presumed consensus sequence (YXXM) that dictates association with the SH_2 domain of the regulatory p85 subunit of phosphatidylinositol-3 kinase (PI-3 kinase) (10). The association of IRS-1 with p85 results in the activation of PI-3 kinase (10).

PI-3 kinase is a dimeric enzyme composed of a catalytic subunit (p110) and a regulatory subunit (p85) (11). Although the p85 regulatory subunit has no discernible catalytic activity, it has two SH_2 domains and one SH_3 domain (12). A region between the two SH_2 domains of p85 binds the NH_2-terminus of p110, mediating the constitutive association of the two subunits (12). Binding of p85 to p110 partially activates p110 (13, 14). PI-3 kinase phosphorylates the lipid phosphatidylinositol (PI) on the 3-position of the D-myoinositol ring, yielding phosphatidylinositol-site-3 phosphate (15). Because the enzyme can also use phosphorylated forms of phosphatidylinositol (PI-4-P, PI-4,5-P_2) as substrates, activation of the PI-3 kinase also leads to the generation of PI-3,4-P_2 and PI-3,4,5-P_3 (11, 16, 17). Previous studies suggested that these PI-3 kinase products are potential second messengers (18, 19). Several molecular targets for PI-3,4-P_2 and PI-3,4,5-P_3, which are translocated and activated upon interaction with phosphoinositides, have been identified. The potential of some pleckstrin homology (PH) domains to specifically bind PI-3,4-P_2 and PI-3,4,5-P_3 correlates with *in vivo* data defining the same PH domain-containing proteins as PI-3 kinase effectors (2, 4).

Increasing evidence suggests that PI-3 kinase is responsible for most of the biological effects induced by insulin. Thus, elucidating the mechanism by which insulin activates PI-3 kinase will help us understand the biological effects of insulin. The present study examines the possible role of SMase in insulin-induced PI-3 kinase activation.

MATERIALS AND METHODS

Reagents

C2-ceramide and SMase were from Biomol (Plymouth Meeting, Pa.); insulin and phosphocholine (PCho) were from Sigma (St. Louis, Mo.); wortmannin was from Calbiochem (La Jolla, Calif.); agarose conjugated with monoclonal anti-phosphotyrosine antibody Py20 was from Santa Cruz (Santa Cruz, Calif.); fetal bovine serum (FBS), Eagle's minimal essential medium (MEM), Dulbecco's modified Eagle's minimal essential medium (DMEM) and RPMI1640 were from BioWhittaker (Walkersville, Md.); and [γ-^{32}P]ATP was from DuPont NEN (Boston, Mass.).

Cell culture

JB6 P^+ mouse epidermal cell line, C1 41 (20, 21), stable bovine PI-3 kinase P85 subunit mutant plasmid transfectant (ΔP85 mass1), and vector plasmid SRα transfectant (AP-1 mass1) (5, 6) were cultured in monolayers at 37°C and 5% CO_2 by using Eagle's MEM containing 5% fetal calf serum, 2 mM L-glutamine, and 25 μg of gentamicin per milliliter as described in a previous report (5, 6); EBV-transformed normal human lymphoblast cell lines, JY, or Niemann-Pick disease lymphoblast MS1418 were maintained in the mixture of RPMI1640 and DMEM (1:1, v/v) containing 15% FBS, 2 mM L-glutamine, and 25 μg of gentamicin per milliliter (22, 23).

PI-3 kinase assay

PI-3 kinase activity was assayed as described previously (5, 6, 24). In brief, JB6 cells, C1 41, ΔP85 mass1, or AP-1 mass1 were cultured in monolayers in 100-mm plates. The cells were then incubated in serum-free MEM medium for 3–4 h at 37°C. Insulin (2.5 μg/ml), C2-ceramide, SMase, or PCho was added to cell cultures for PI-3 kinase induction. After 5–10 min at 37°C, the cells were washed once with ice-cold phosphate-buffered saline (PBS) and lysed in 400 μl of lysis buffer (20 mM Tris, pH 8, 137 nM NaCl, 1 mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40, 1 nM dithiothreitol, 0.4 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride) per plate. The lysates were centrifuged and the supernatant fractions were incubated with 40 μl of agarose beads (previously conjugated with the monoclonal antiphosphotyrosine antibody Py20) overnight at 4°C. The beads were washed twice with each of the following buffers: 1) PBS with 1% Nonidet P-40, 1 mM dithiothreitol; 2) 0.1 M Tris (pH 7.6), 0.5 M LiCl, l mM dithiothreitol; and 3) 10 mM Tris (pH 7.6), 0.1 M NaCl, 1 mM dithiothreitol. The beads were incubated for 5 min on ice in 20 μl of buffer 3 and then 20 μl of 0.5 mg/ml phosphatidylinositol (previously sonicated in 50 mM Hepes (pH 7.6), 1 mM EGTA, 1 mM NaH_2PO_4) was added. After 5 min at room temperature, 10 μl of the reaction buffer was added (50 mM $MgCl_2$, 100 mM Hepes (pH 7.6), 250 μM ATP containing 5 μCi of [γ-^{32}P]ATP), and the beads were incubated for an additional 15 min. The reactions were stopped by the addition of 15 μl of 4 N HCl and 130 μl of chloroform:methanol (1:1). After vortexing for 30 s, 30 μl from the phospholipid-containing chloroform phase was spotted onto thin-layer chromatography plates coated with silica gel H containing 1.3% potassium oxalate and 2 mM EDTA applied in H_2O:methanol (3:2). Plates were heated at 110°C for at least 3 h before use. The plates were placed in tanks containing chloroform:methanol:NH_4OH:H_2O (600:470:20:113) for 40–50 min until the solvent reached the top of the plates. The plates were dried at room temperature and autoradiographed. Then the PI-3 phosphate spots were scraped off and counted (5, 6, 24).

RESULTS

Induction of PI-3 kinase activation by insulin, C2-ceramide or PCho in mouse epidermal JB6 cells

SMase can hydrolyze the phosphodiester bond of sphingomyelin yielding ceramide and PCho (22, 23, 25). To determine the possible role of SMase and its products in PI-3 kinase activation, JB6 cells were exposed to either C2-ceramide or PCho. As shown in Figure 1, both C2-ceramide and PCho induced strong activation of PI-3 kinase in JB6 cells. Induction levels of PI-3 kinase activity by C2-ceramide or PCho were similar to those induced by insulin (Fig. 1). The induction of PI-3 kinase activity by C2-ceramide or PCho was blocked by either pretreatment of cells with wortmannin (Figure 2a) or by overexpression of the dominant negative mutant of PI-3 kinase (ΔP85) (Fig. 2b). These data indicated that C2-ceramide and PCho are strong activators of PI-3 kinase. During preparation of this paper, Hanna et al. also reported that ceramide could induce PI-3 kinase in Rat 2 fibroblasts (26).

Insulin induces PI-3 kinase activation in normal JY lymphoblasts, but not in SMase-deficient MS1418 lymphoblasts

To investigate the role of SMase in insulin-induced activation of PI-3 kinase, we exposed both EBV-transformed normal lymphoblasts (JY) and SMase-deficient lymphoblasts (MS1418) to insulin for PI-3 kinase induction. The results show that insulin-induced PI-3 kinase activation was observed only in normal JY cells but not in SMase-deficient MS1418 cells (Fig. 3). These results suggest that SMase is involved in PI-3 kinase activation by insulin.

Restoration of PI-3 kinase activation by C2-ceramide, Pcho, or SMase in SMase-deficient MS1418 cells

To further demonstrate a role for SMase in PI-3 kinase activation, we treated MS1418 cells with C2-ceramide, PCho, or SMase to examine whether the deficiency of PI-3 kinase activation in MS1418 cells could be restored. Addition of exogenous C2-ceramide directly into the cell culture medium caused activation of PI-3 kinase in both JY and MS1418 cells (Fig. 4a). The time course study indicated that exposure of MS1418 cells to SMase, PCho, or C2-ceramide induced a very strong activation of PI-3 kinase within 5 min (Fig. 4b) and the activity returned toward the basal level by 15 min after exposure (Fig. 4b). The results from a concentration response study (Fig. 4c) are consistent with the above findings that treatment of cells with SMase can restore PI-3 kinase activation in MS1418 cells. These experiments provide evidence that the lack of insulin-induced PI-3 kinase activation in MS1418 cells is due to a deficiency of SMase.

DISCUSSION

Our present studies demonstrate that SMase is involved in insulin-induced activation of PI-3 kinase in mouse epidermal JB6 cells, human lymphoblast (JY), and MS1418 cells. This conclusion is based on the following experimental observations: 1) insulin, C2-ceramide, or phosphocholine (PCho) can stimulate PI-3 kinase activity in JB6 cells; 2) this insulin-induced activation of PI-3 kinase is observed in the normal human lymphoblast cell line, JY, but not in a SMase-deficient cell line, MS1418; and 3) the deficiency of PI-3 kinase activation in MS1418 cells could be restored by exposure of the cells to SMase, C2-ceramide, or PCho.

Several extracellular stimulations can result in activation of SMase. This result, in turn, causes hydrolysis of sphingomyelin, a phospholipid largely confined to the outer leaflet of cellular membranes, and stimulates the generation of ceramide and PCho (23–25). These activating agents include ultraviolet (UV) or ionizing irradiation, heat shock, nerve-growth factor, TNF-α, endotoxin, interferon-γ, interleukin-1, Fas, and CD28 (27, 28). Also, a number of direct targets for ceramide action have been identified, including ceramide-activated protein kinase, ceramide-activated protein phosphatase, and protein kinase Cζ. Growing evidence indicates an important role of SMase and its products, ceramide, and PCho, as secondary messengers in the regulation of cell growth and differentiation, cell–cell contact, and oncogenesis, depending on cell type (29–32). Indeed, synthetic ceramide analogs and SMase have been shown to mimic the vitamin D3 in inducing monocytic differentiation in HL60 cells (27, 28). TNF-α has been reported to induce apoptosis and rapid sphingomyelin hydrolysis to ceramide in a number of cell models, including U937 monocytic cells, HL60 cells, and L929 fibrosarcoma cells (33). Further, synthetic ceramide analogs and SMase can mimic the action of TNF-α in the initiation of

apoptosis (33). Ultraviolet C and X-ray irradiation can lead to an increase in ceramide production in exposed cells, and exogenous ceramide may induce activation of both Erks and JNKs (22, 23, 33). Our previous studies also demonstrated that UV-induced JNKs activation depends on SMase (23). In the present study, we found that SMase, ceramide, and PCho induce strong activation of PI-3 kinase in JB6 cells. In the lymphoblast cell system, insulin-induced PI-3 kinase activation is observed only in a normal human lymphoblast (JY) but not in a SMase-deficient cell line (MS1418). Moreover, exposure of cells to SMase, ceramide, or PCho leads to significant activation of PI-3 kinase in both JY cells and MS1418 cells. Exogenous ceramide, PCho, or SMase could restore PI-3 kinase activation in SMase-deficient cells, which suggests that the lack of response of MS1418 cells to insulin in terms of PI-3 kinase activation is due to its deficiency of SMase. Thus, we conclude that SMase and its products are involved in insulin-induced PI-3 kinase activation.

Previous studies have shown that PI-3 kinase and its products Ptdins (3, 4) P2, and Ptdins (3, 4, 5) P3 are important regulators of cell mitogenic signaling, cell survival, cytoskeletal remodeling, metabolic control, and vesicular trafficking (2–4). The introduction of the N-terminal SH_2 domain of the P85 subunit of PI-3 kinase into cells abrogates insulin- or IGF-1-stimulated DNA synthesis and prevents insulin stimulation of c-Fos protein expression (34, 35). The microinjection of a dominant-negative $p21^{ras}$ mutant or anti-ras antibody inhibits insulin-induced DNA synthesis (35). A constitutively activated mutant P110 induced transcription from the *fos* promoter, whereas co-expression of dominant negative Ras blocked this response (34). Other studies have shown that Ptdins (3, 4) P2 and Ptdins (3, 4, 5) P3 are elevated in cells transformed by v-abl, v-src, and polyoma middle T and that decreased levels of these lipids correlate with impaired cell transformation by mutated forms of these oncogenes (36, 37, 38). The presence of insulin-like growth factor I receptor (IGF-IR) was reported to be an obligatory requirement for the establishment and maintenance of the tumor phenotype (39–42). Cells derived from mouse embryos with a targeted disruption of the IGF-IR gene (R^- cell) cannot be transformed by SV40 T antigen or by an activated and overexpressed Ha-ras, even by a combination of both. In contrast, either one of them transforms very efficiently the corresponding wild-type cells or other 3T3-like cells (43). If a plasmid expressing a wild-type human IGF-I receptor cDNA is stably transfected into R^- cells, the cells can be transformed by SV40 T antigen. This finding indicates that the defect in transformability is specifically caused by the lack of IGF-IR (42). Substantial evidence shows that PI-3 kinase is a critical component of signaling pathways used by the cell surface receptors for a variety of mammalian growth factors or other stimulators (36, 16, 18, 44), especially IR and IGF-IR. Recently, insulin was shown to activate the Ras-Raf-MAP kinase pathway by interacting and activating its receptors (13). Hu et al. suggested that activation of this Ras/MAP kinase pathway is critical for the effect of insulin on mitogenesis and c-fos expression (13). Others have shown that neither insulin nor phorbol ester regulation of phosphoenolpyruvate carboxykinase gene expression requires activation of the Ras/MAP kinase pathway (45). In contrast, PI-3 kinase is required in this event (45). Our previous studies demonstrated that PI-3 kinase is required for TPA- or EGF-induced AP-1 transactivation and cell transformation (5, 6). The important role of PI-3 kinase in development was supported by using transgenic and gene knockout mice (46). Mice homozygous for a p110α deletion mutant in the p85 binding site, as well as animals homozygous for a p110β without a functional catalytic domain, are developmentally retarded and die between E9.5 and E10.5 (46). Consistent with this result, complete disruption of p85α severely impairs embryonic development and most of these animals

do not survive to birth (46). Thus, investigation of mechanisms that activate PI-3 kinase may provide new understanding of the development of some human diseases. Type C Niemann-Pick (NP-C) disease is an autosomal recessive lipidosis distinguished by a unique error in cellular trafficking of exogenous cholesterol (47). NP-C is usually present in late childhood and patients die within their second decade (47). SMase levels were found to be low in cells from NP-C patients (47), although not to the extensive degree that occurs in type A and B Niemann-Pick disease (48). In these latter diseases, cells deficient in SMase form the primary metabolic lesion that leads to substantial accumulation of sphingomyelin (48). Unesterified cholesterol, sphingomyelin, phospholipids, and glycolipids are stored in excess in the liver and spleen (47, 48). Cultured fibroblasts show a unique disorder of cellular cholesterol processing, in which delayed homeostatic responses to exogenous cholesterol loading are associated with cholesterol accumulation in lysosomes (48). Accumulation of unesterified cholesterol is known to be accompanied by accumulation of sphingomyelin (48). In NP-C, a parallel loss of regulatory responses in both uptake and esterification of low-density lipoprotein-induced cholesterol occurs in cultured fibroblasts (47). Because PI-3 kinase plays an important role in mediating metabolism of lipids, we speculate that deficiency of PI-3 kinase activation in response to insulin may at least be part of the molecular mechanism for abnormal metabolism of lipids in patients with Niemann-Pick disease. This hypothesis is supported by the findings that SMase enhances low-density lipoprotein uptake in macrophages (49).

In conclusion, we used several approaches to study the role of SMase in insulin-induced PI-3 kinase activation. PI-3 kinase activation can be observed in JB6 cells treated with insulin, ceramide, or PCho. Insulin induces PI-3 kinase activation in normal lymphoblasts, but not in SMase-deficient lymphoblasts. Exposure of SMase-deficient cells to SMase, ceramide, or PCho can restore PI-3 kinase activation. These results demonstrate that SMase plays a role in insulin-induced PI-3 kinase activation. These data reveal that the lack of PI-3 kinase activation by insulin may be a possible mechanism for abnormal lipid metabolism in Niemann-Pick disease. Further investigations will focus on the effects of SMase, ceremide, or PCho on uptake and esterification of low-density lipoprotein-derived cholesterol in animals and cultured cells.

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Fig. 1

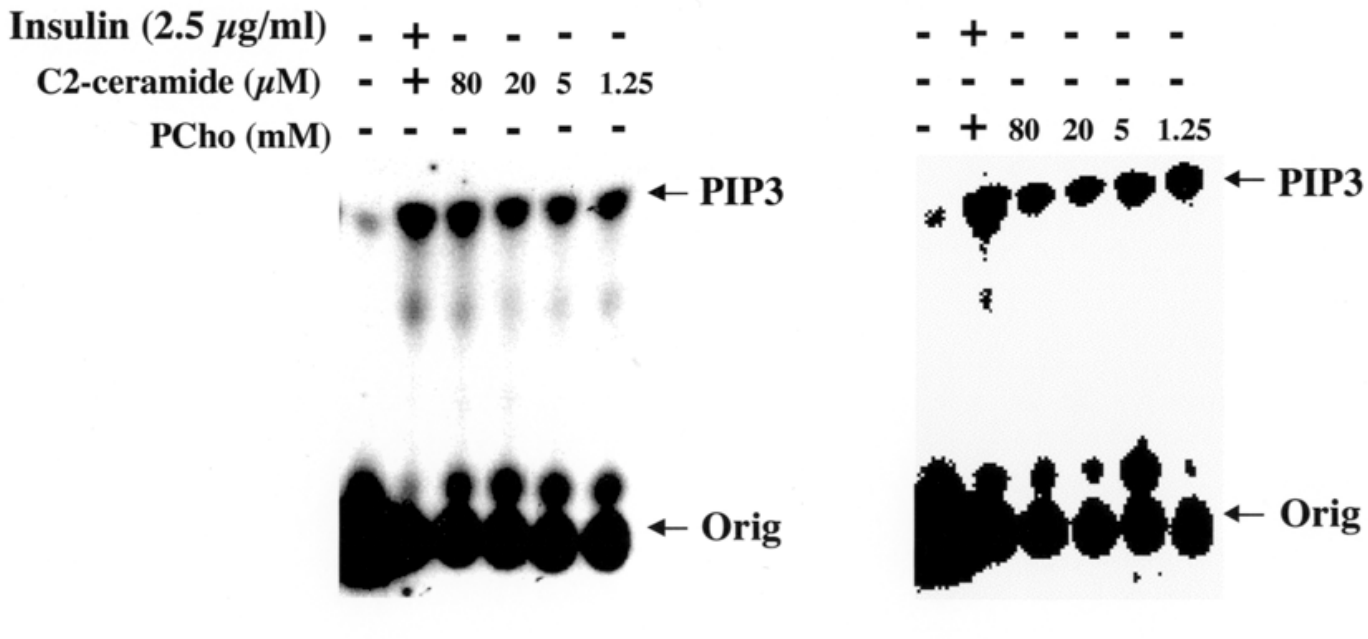

Figure 1. Induction of PI-3 kinase activity by insulin, C2-ceramide, or PCho in JB6 cells. JB6 cells were exposed to insulin (2.5 μg/ml) for 10 min, or different doses of C2-ceramide or PCho for 5 min at 37°C in 5% CO_2. The cells were harvested, and PI-3 kinase activity (PIP3) was measured as described in Materials and Methods.

Fig. 2

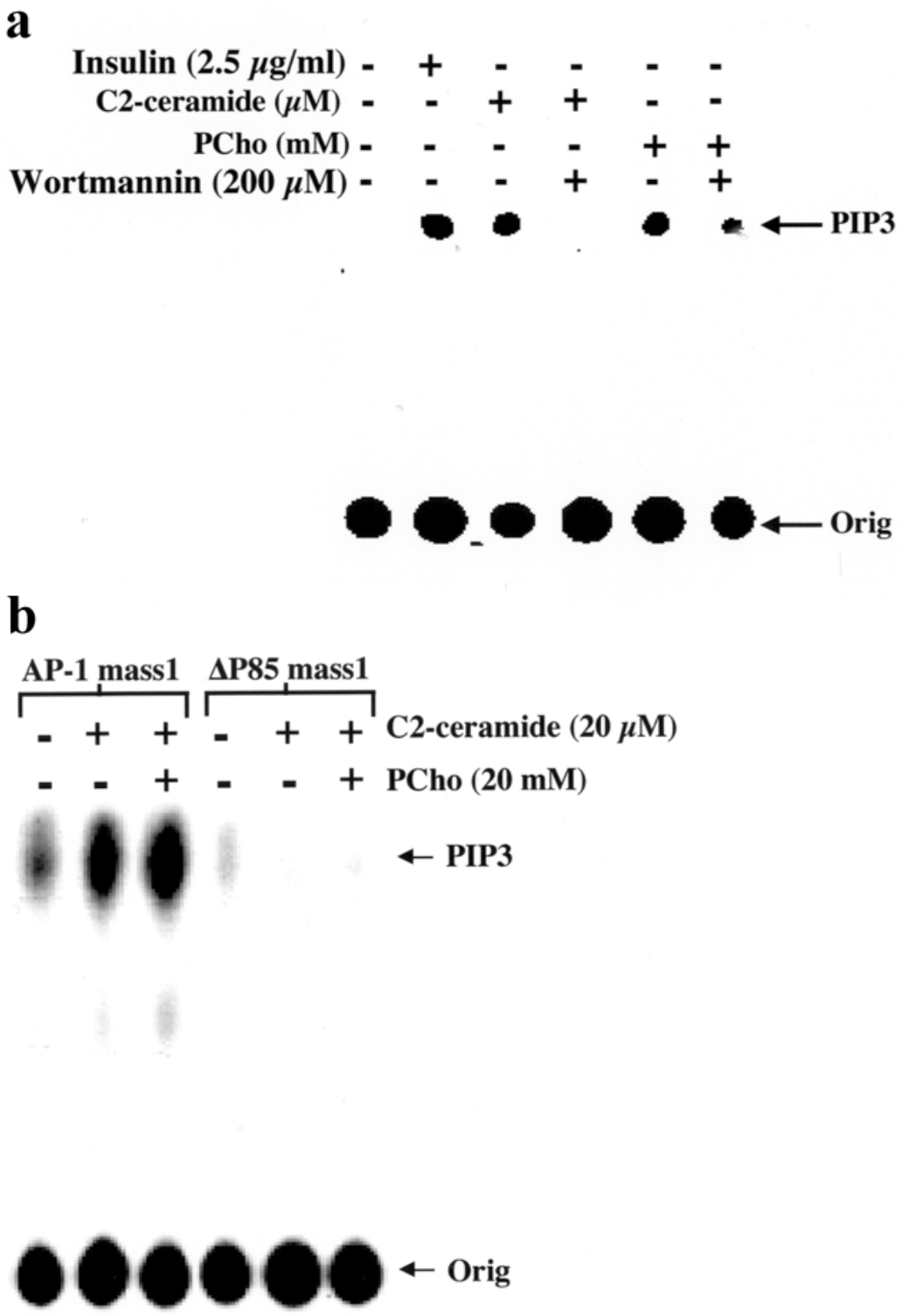

Figure 2. Blocking of C2-ceramide- or PCho-induced PI-3 kinase activity by either pretreatment of cells with wortmannin or overexpression of dominant negative PI-3 kinase mutant (ΔP85 mass1). **(a)** JB6 C1 41 cells were pretreated with or without wortmannin (200 μM) for 30 min. Then the cells were exposed to either insulin (2.5 μg/ml) for 10 min, or C2-ceramide (20 μM) or PCho (20 mM) for 5 min at 37°C in 5% CO_2. **(b)** Cells (ΔP85 mass1 or AP-1 mass1) were or were not treated with C2-ceramide (20 μM) or PCho (20 mM) for 5 min at 37°C in 5% CO_2. The cells were then harvested, and PI-3 kinase activity (PIP3) was measured as described in Materials and Methods.

Fig. 3

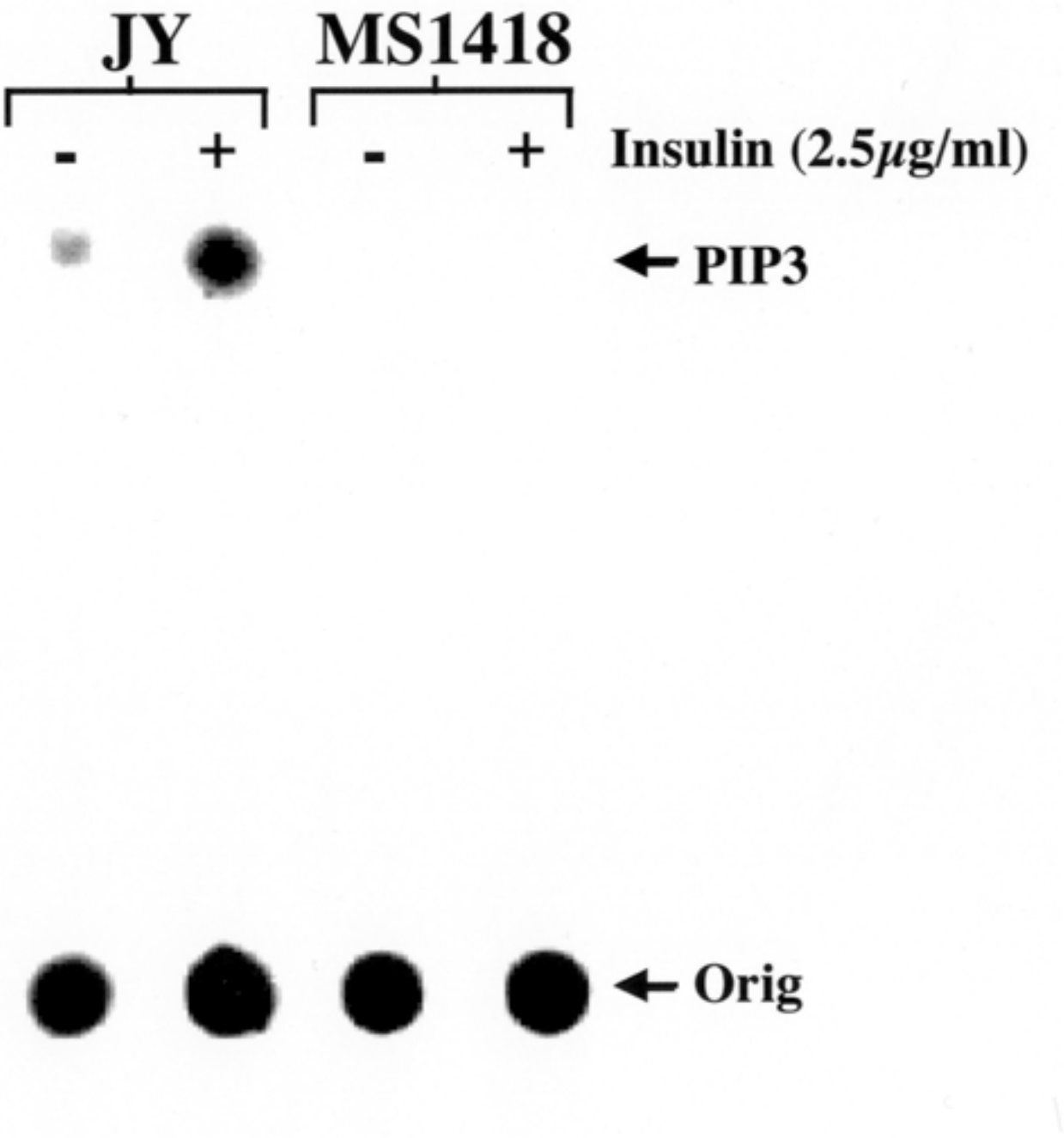

Figure 3. Induction of PI-3 kinase by insulin in normal lymphoblasts, but not in SMase-deficient MS1418 lymphoblasts. Lymphoblasts (JY or MS1418) were or were not treated with insulin for 10 min at 37°C in 5% CO_2. The cells were then harvested and PI-3 kinase activity (PIP3) was measured as described in Materials and Methods.

Fig. 4

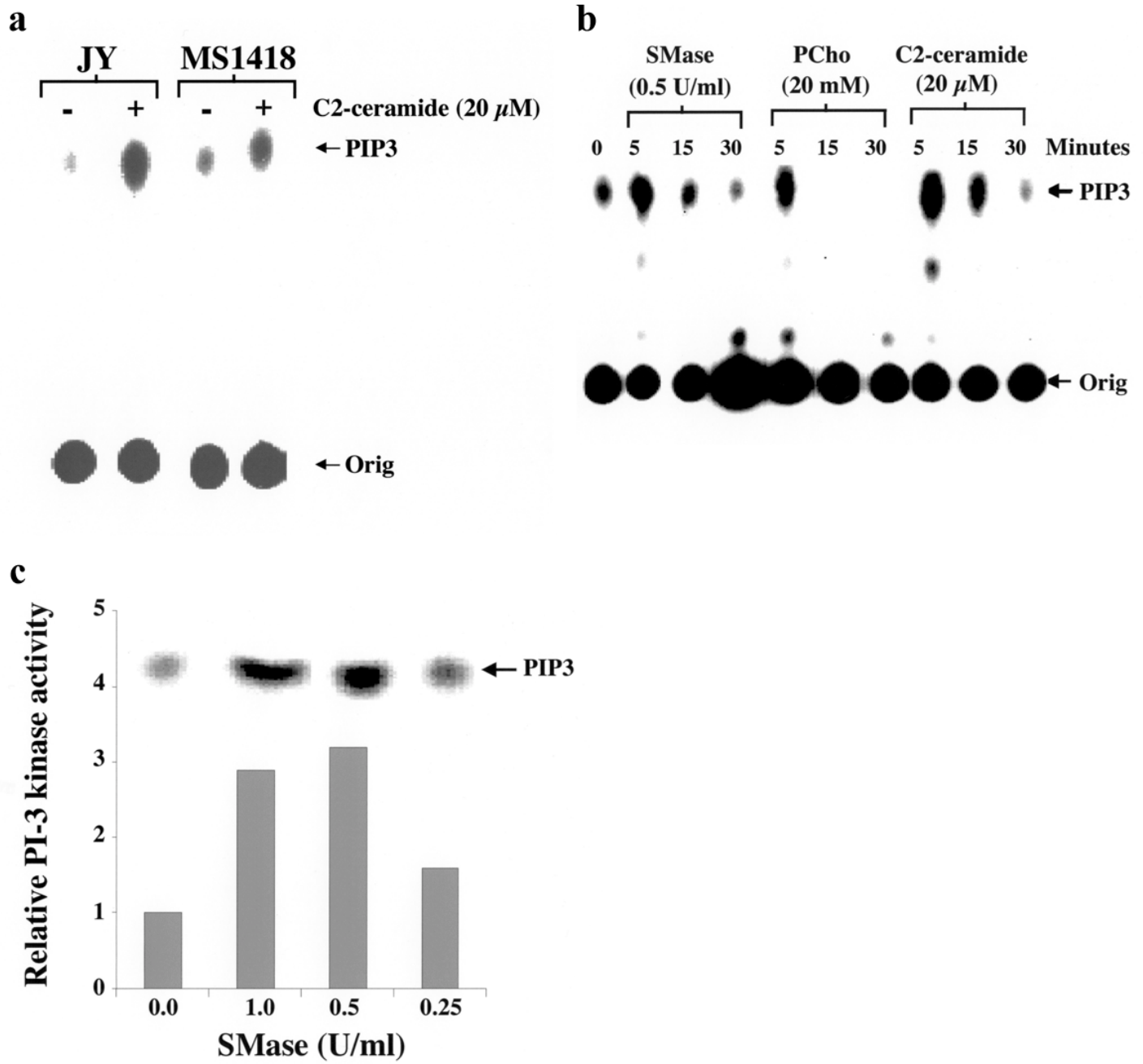

Figure 4. Restoration of PI-3 kinase activation in MS1418 cells by C2-ceramide, PCho or SMase. **(a)** Lymphoblasts (JY or MS1418) were or were not treated with C2-ceramide (20 μM) for 5 min at 37°C in 5% CO_2. **(b)** MS1418 cells were exposed to SMase (0.5 U/ml), PCho (20 mM) or C2-ceramide (20 μM) for the time as indicated. **(c)** MS1418 cells were exposed to different concentrations of SMase for 5 min. The cells were then harvested and PI-3 kinase activity (PIP3) was measured as described in Materials and Methods.