p85/p110-type Phosphatidylinositol Kinase Phosphorylates Not Only the D-3, but Also the D-4 Position of the Inositol Ring*

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Activation of p85/p110-type phosphatidylinositol (PI) kinase has been implicated in various cellular activities. This PI kinase phosphorylates the D-4 position with a similar or higher efficiency than the D-3 position when trichloroacetic acid-treated cell membrane is used as a substrate, although it phosphorylates almost exclusively the D-3 position of the inositol ring in phosphoinositides when purified PI is used as a substrate. Furthermore, the lipid kinase activities of p110b for both the D-3 and D-4 positions were completely abolished by introducing kinase-dead point mutations in their lipid kinase domains (ΔKin, respectively). In addition, both PI 3- and PI 4-kinase activities of p51 θ and p110 θ immunoprecipitates were similarly inhibited by either wortmannin or LY294002, specific inhibitors of p110. Insulin induced phosphorylation of not only the D-3 position, but also the D-4 position. Indeed, overexpression of p110 θ in S9F or 3T3-L1 cells induced marked phosphorylation of the D-4 position to a level comparable to or much greater than that of D-3, whereas inhibition of endogenous p85/p110-type PI kinase via overexpression of dominant-negative p85α (Δp85α) in 3T3-L1 adipocytes abolished insulin-induced synthesis of both. Thus, p85/p110-type PI kinase phosphorylates the D-4 position of phosphoinositides more efficiently than the D-3 position in vivo. Thus, p85/p110-type PI kinase may activate downstream targets by increasing not only D-3-phosphorylated (PI-3-P, PI-3,4-P2, and PI-3,4,5-P3) but also D-4-phosphorylated (PI-4-P and PI-4,5-P3) phosphoinositides.

EXPERIMENTAL PROCEDURES

Materials—PI and dexamethasone were purchased from Sigma. 3-Isobutyl-1-methylxanthine and 2-deoxy-β-D-glucose were from Wako Bioproducts and dexamethasone was purchased from Sigma. Fetal bovine serum and RPMI 1640 were purchased from Life Technologies, Inc. Anti-p85 antiserum and anti-phosphotyrosine antibodies were purchased from Upstate Biotechnology, Inc. Anti-hemagglutinin antibodies were purchased from Roche Molecular Biochemicals. Anti-p110α antibodies were raised against synthetic peptides corresponding to residues 1048–1068 of p110α.

Cell Culture—S9F cells were maintained in TC-100 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) at 27 °C. Cells were harvested 2 days post-baculovirus infection. 3T3-L1 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% donor calf serum (Life Technologies, Inc.) in an atmosphere of 10% CO2 at 37 °C. Two days after the fibroblasts had reached confluence, differentiation was induced by treating the cells with DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine, 4 mg/ml dexamethasone, and 10% fetal bovine serum for 48 h. Cells were re-fed with DMEM supplemented with 10% fetal bovine serum every other day. Infection with the indicated adenoviruses was carried out on day 3 post-differentiation induction, and the experiments were conducted on day 5, at which point >90% of the cells expressed the adipocyte phenotype.

Cloning of cDNA—Cloning and construct-reverse transcription-polymerase chain reaction were performed to amplify full-length p110 from human embryonic heart cDNA, based on its reported sequence (9). The entire coding region of p110α cDNA was obtained as described in our previous report (7). The cDNAs coding for the kinase-dead point mutants of p110β (ΔKin) and p110δ (ΔKin), which lack amino acids 917–950 of p110α and amino acids 921–954 of p110δ, were designed as reported previously (10, 11). A portion of human GLUT2 cDNA corresponding to residues 510–524 was ligated to each cDNA to generate catalytic subunits of PI 3-kinase tagged at their C termini.

A variety of growth factors and hormones exert their cellular effects via interactions with specific receptors that possess protein kinase activities. The interaction of most of these ligands with their receptors induces tyrosine kinase activation and phosphorylation of the receptor and/or intracellular substrates. The tyrosine-phosphorylated protein serves as a docking protein for several cytoplasmic substrates with SH2 domains (1–3). p85/p110-type phosphatidylinositol (PI) kinase has been identified through its ability to associate with these tyrosine-phosphorylated substrates (4, 5) and has been thought to be an enzyme that phosphorylates the D-3 position of the inositol ring in phosphoinositides, resulting in formation of PI-3-P, PI-3,4-P2, and PI-3,4,5-P3 (6), based on experiments using purified phosphatidylinositol as a substrate to determine the lipid substrate specificity.

However, our findings that the lipid products of p85/p110-type PI kinase in vivo differ markedly from those produced by a conventional in vitro method used to determine the substrate specificity of PI kinase are of great interest. The results of this study show that p110α and p110β phosphorylate the D-4 position of phosphoinositides more efficiently than the D-3 position in vivo. Thus, p85/p110-type PI kinase may activate downstream targets by increasing not only D-3-phosphorylated (PI-3-P, PI-3,4-P2, and PI-3,4,5-P3) but also D-4-phosphorylated (PI-4-P and PI-4,5-P3) phosphoinositides.

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The abbreviations used are: PI, phosphatidylinositol; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography.
Gene Transduction—The entire coding regions of p110α, p110β, and their kinase-dead point mutants, all of which have an epitope GLUT2 tag at their C termini, were cloned into transfer vectors (either pBacPAK5 or pBacPAK9). The entire coding region of LacZ from *Escherichia coli* was also cloned into pBacPAK9. Recombinant baculoviruses were produced by homologous recombination between the recombinant transfer vectors and their parental virus genome according to the manufacturer’s instructions (CLONTECH). To obtain recombinant adenoviruses, the expression cosmid cassette pAdexCawi was ligated with each of the cDNAs coding for either LacZ from *E. coli* or epitope-tagged p110α, followed by homologous recombination between the recombinant cosmid cassette and its parental virus genome, as described previously (12). Recombinant adenovirus expressing Δ85α was prepared as described previously (12).

Immunoprecipitation and Western Blotting—Cells were lysed in PBS containing 1% Triton, 0.35 mg/ml phenylmethylsulfonyl fluoride, and 100 mM sodium vanadate. Cell lysates were centrifuged at 15,000 × g for 10 min at 4 °C to remove insoluble materials. The supernatants were incubated with the indicated antibodies, followed by the addition of protein A-Sepharose (Amersham Pharmacia Biotech). Alternatively, in some experiments, lysates were immunoprecipitated with anti-hemagglutinin antibodies, followed by the addition of protein G-Sepharose (Amersham Pharmacia Biotech). The immune complexes were collected by centrifugation, washed with PBS containing 1% Triton X-100, boiled in Laemmli’s buffer containing 100 mM dithiothreitol, 2% sodium dodecyl sulfate, and subjected to SDS-polyacrylamide gel electrophoresis. Immunoblotting was performed with the ECL system according to the manufacturer’s instructions. In some experiments, the band intensities were quantified with a Model GS-525 molecular imager (Bio-Rad).

**In Vitro Generation of 32P-Labeled Phosphoinositides (PI 3-Kinase Assay)—**Sf9 cells infected with baculovirus were lysed with PBS containing 1% Nonidet P-40 and 0.55 mg/ml phenylmethylsulfonyl fluoride and then immunoprecipitated with anti-C-terminus GLUT2 tag antibodies and protein A-Sepharose. 3T3-L1 adipocytes (in 24-well culture dishes) were serum-starved for 3 h in DMEM containing 0.2% bovine serum albumin. The cells were incubated with 10^−6 M insulin for 5 min; washed with ice-cold PBS; and then lysed with PBS containing 1% Nonidet P-40, 0.35 mg/ml phenylmethylsulfonyl fluoride, and 100 mM sodium vanadate. Cell lysates were precleared of insoluble materials by centrifugation (15,000 × g, 4 °C, 10 min) and were subjected to immunoprecipitation with anti-phosphotyrosine antibodies and protein A-Sepharose. The PI 3-kinase activity in the immunoprecipitates was measured as described previously (7). When indicated, the membrane fraction of Sf9 cells, prepared as described previously (13), was used as the assay system instead of purified PI from a commercial source. The reaction products were deacylated and analyzed by HPLC. In some experiments, wortmannin or LY294002 was added to the reaction mixture of the in vitro kinase assay, and the inhibitory effects on 32P-labeled phosphoinositide synthesis were investigated.

To exclude the possibility that D-3-phosphorylated phosphoinositides may activate some unknown PI 4-kinase in the membrane fraction, resulting in the synthesis of D-4-phosphorylated phosphoinositides, purified D-3-phosphorylated phosphoinositides were prepared by incubating purified PI, PI-4-P, or PI-4,5-P_{2}, with nonradioactive ATP and p110α immunoprecipitates in *vitro*. Phosphoinositides labeled with unlabeled phosphate groups on their D-3 positions (PI-3-P, PI-3,4-P_{2}, and PI-3,4,5-P_{3}) were separated by TLC, followed by chloroform extraction. Each of them was dried and mixed with Sf9 cell membrane fraction in the presence of [γ-32P]ATP.

**In Vivo Generation of 32P-Labeled Phosphoinositides—**3T3-L1 adipocytes and infected with an adenovirus containing p110α, p110β, or control LacZ DNA were phosphate-starved overnight in phosphate-free DMEM and then labeled with [32P]orthophosphate (0.1 mCi/ml) for 2 h. The radioactivity was detected with an on-line radiochemical detector.

**HPLC Analysis of Phosphoinositides—**The extracted lipid was deacylated and subjected to anion-exchange HPLC using a Partisphere strong anion-exchange column (Whatman) as described previously (14). The radioactivity was detected with an on-line radiochemical detector. Deacylated [γ32P]Pi-4-P and [γ32P]Pi-4,5-P_{2} were used as internal standard.

**RESULTS AND DISCUSSION**

p85/p110-type PI kinase has been identified through its ability to associate with many tyrosine-phosphorylated substrates and has been considered to phosphorylate the D-3 position of the inositol ring in phosphoinositides (6, 16–18). However, of great interest are our findings that the lipid products of p85/p110-type PI kinase in *vivo* differ markedly from those produced by a conventional *in vitro* method used to determine the substrate specificity of each PI kinase.

First, recombinant baculoviruses that express either p110α or p110β with a tagged epitope at their COOH terminus were prepared. Sf9 cells were infected with either p110α or p110β recombinant virus or control LacZ virus, and similar expression levels were obtained among them (Fig. 1A). Immunoprecipitates with antibodies against the tag were subjected to *in vitro* lipid kinase assays using purified PI as substrates, followed by HPLC analysis (Fig. 1, B and C). The major product was PI-3-P with p110α immunoprecipitates, although small amounts of PI-4-P and PI-3,4-P_{2} were also detected. Based on the amounts of PI-3-P, PI-4-P, and PI-3,4-P_{2} produced, the *in vitro* lipid kinase activity of p110α immunoprecipitates at the D-4 position was estimated to be 4% of that at the D-3 position. On the contrary, immunoprecipitates of p110β, another isoform of the catalytic subunit, produced <1% of the PI-3-P produced by p110α immunoprecipitates, and no generation of PI-4-P or PI-3,4-P_{2} was detected. These results indicate that the catalytic subunits of p85/p110-type PI kinases, p110α and p110β, are indeed PI kinases for the D-3 position.

Quite different results were obtained when trichloroacetic acid-treated cell membranes were used as substrates for p110α or p110β, instead of purified PI. Treating the cell membranes with 10% trichloroacetic acid apparently denatures the protein and converts cellular PI to immunoprecipitable PI. The mass of PI-3-P, PI-4-P, and PI-3,4-P_{2} was detected. These results indicate that the catalytic subunits of p85/p110-type PI kinases, p110α and p110β, are indeed PI kinases for the D-3 position.
immunoblotted with anti-C-terminal GLUT2 tag antibodies. Anti-C-terminal GLUT2 tag immunoprecipitates from Sf9 cells overexpressing LacZ were then separated by SDS-polyacrylamide gel electrophoresis and the washed immunoprecipitates were quantified with an on-line radiochemical detector. Three other separate experiments yielded similar results.

In vitro generation of phosphoinositides from purified PI by p110α and p110β expressed in Sf9 cells. Sf9 cells were infected with recombinant baculoviruses containing control LacZ (Cont.), p110α, or p110β (B). These cells were lysed and immunoprecipitated with anti-C-terminal GLUT2 tag antibodies. The washed immunoprecipitates were then separated by SDS-polyacrylamide gel electrophoresis and immunoprecipitates from Sf9 cells overexpressing LacZ (B, upper panel), p110α (middle panel), or p110β (lower panel) were subjected to PI kinase assay. Purified PI and [γ-32P]ATP were mixed with the immunoprecipitates as described under “Experimental Procedures.” The 32P-labeled phosphoinositides generated were separated by HPLC (B), yielding three peaks corresponding to PI-3-P, PI-4-P, and PI-3,4-P2, which were quantified with an on-line radiochemical detector (C). Three other separate experiments yielded similar results.

![Graph A](image1.png)

![Graph B](image2.png)

| Transfection | LacZ | p110α | p110β |
|--------------|------|-------|-------|
| PI-3-P       | 0    | 2360  | 15    |
| PI-4-P       | 0    | 95    | 0     |
| PI-3,4-P2    | 0    | 35    | 0     |

The increases in the amounts of PI-3,4-P2, PI-4,5-P2, and PI-3,4,5-P3 were comparable to the increases in PI-4-P and PI-4,5-P2, which were comparable to the increase in PI-3-P induced by p110α overexpression and even greater than the increases in PI-3,4-P2 and PI-3,4,5-P3, which suggests that p110α overexpression induces accumulation of not only D-3-phosphorylated, but also D-4-phosphorylated phosphoinositides in vivo. Essentially the same results were obtained with the overexpression of both p110 and its regulatory subunit, p85 (data not shown). p110β overexpression induced accumulation of D-4-phosphorylated phosphoinositides at a much higher level than that of D-3 in Sf9 cells, which suggests that p110β may function as a PI 4-kinase predominantly in intact cells. Further study should be addressed to determine the mechanism that causes the difference in substrate specificity between p110α and p110β.

Finally, to demonstrate that endogenous p85/p110 produces not only D-3-phosphorylated, but also D-4-phosphorylated phosphoinositides, and to examine the effect of insulin on the level of phosphorylation of phosphoinositides, either p110α or dominant-negative p85α was overexpressed in 3T3-L1 adipocytes, and the cellular level of each phosphoinositide in the absence or presence of insulin was investigated. p110α was overexpressed in 3T3-L1 adipocytes using an adenovirus expression system (Fig. 4A, upper panel) at a level approximately five times that expressed endogenously in 3T3-L1 cells (lower panel). The cellular ATP level was essentially unaltered by p110 overexpression (data not shown). The amount of overexpressed p110α with the adenovirus expression system is much smaller than that with the baculovirus expression system. Thus, increases in the amounts of PI-3,4-P3, PI-4,5-P2, and PI-3,4,5-P3 accumulated in 3T3-L1 adipocytes (Fig. 4B) were smaller than those in Sf9 cells (Fig. 3), but such increases were still obvious. Interestingly, the increase in PI-4-P in 3T3-L1 adipocytes overexpressing p110α was much larger than that in 3T3-L1, although they were almost comparable in Sf9 cells overexpressing p110α. The reason for these differences according to cell type remains unclear, but the conformation of p110 may differ somewhat by an unknown modification, such as serine/threonine phosphorylation, according to whether p110 is expressed in Sf9 cells or in 3T3-L1 adipocytes.

Insulin showed no additive effect on the amount of each phosphoinositide in p110α-overexpressing cells, which also
3,4,5-P<sub>3</sub> decreased to nearly undetectable levels and that of the cell surface was observed (7). In addition, very clear results with anti-C-terminal GLUT2 tag antibodies. A, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunodetection with anti-C-terminal GLUT2 tag antibodies. B, the washed immunoprecipitates were subjected to PI kinase assay. The membrane fraction from uninfected Sf9 cells was also prepared. After 10% trichloroacetic acid treatment, membrane fractions were incubated with immunoprecipitates together with [γ-<sup>32</sup>P]ATP. The [γ-<sup>32</sup>P]ATP in the presence of various concentrations of wortmannin or LY294002. The amount of glycerophosphoinositides was analyzed by HPLC. PI 3-kinase activity (squares) represents the amount of PI-3-P and PI-4,5-P<sub>2</sub> and PI-4-kinase activity (triangles) represents the amount of PI-4-P and PI-3,4-P<sub>2</sub>. Lysates were prepared from Sf9 cells overexpressing p110<sub>a</sub> (open symbols) or p110<sub>b</sub> (closed symbols). PI kinase assay was performed in the presence of wortmannin (C) or LY294002 (D). Three other separate experiments yielded similar results.

agrees well with our previous report that no additive effect of insulin and p110<sub>a</sub> overexpression on GLUT4 translocation to the cell surface was observed (7). In addition, very clear results were obtained in the experiment using recombinant baculoviruses containing p110<sub>a</sub>, p110<sub>b</sub>, ΔKinα, or ΔKinβ. These cells were lysed, and lysates were immunoprecipitated with anti-C-terminal GLUT2 tag antibodies. A, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunodetection with anti-C-terminal GLUT2 tag antibodies. B, the washed immunoprecipitates were subjected to PI kinase assay. The membrane fraction from uninfected Sf9 cells was also prepared. After 10% trichloroacetic acid treatment, membrane fractions were incubated with immunoprecipitates together with [γ-<sup>32</sup>P]ATP, as described under "Experimental Procedures." D-3-Phosphorylated Phosphoinositide indicates PI-3-P, PI-3,4-P<sub>2</sub>, PI-3,4,5-P<sub>3</sub>, or a mixture of these three, all of which are prepared by incubating purified PI, PI-4-P, or PI-4,5-P<sub>2</sub> with [γ-<sup>32</sup>P]ATP in the presence of various concentrations of wortmannin or LY294002. The amount of glycerophosphoinositides was analyzed by HPLC. PI 3-kinase activity (squares) represents the amount of PI-3-P and PI-4,5-P<sub>2</sub>, and PI-4-kinase activity (triangles) represents the amount of PI-4-P and PI-3,4-P<sub>2</sub>. Lysates were prepared from Sf9 cells overexpressing p110<sub>a</sub> (open symbols) or p110<sub>b</sub> (closed symbols). PI kinase assay was performed in the presence of wortmannin (C) or LY294002 (D). Three other separate experiments yielded similar results.

FIG. 2. In vitro generation of phosphoinositides from cell membranes by p110<sub>a</sub>, p110<sub>b</sub>, and their kinase-dead point mutants expressed in Sf9 cells (A and B) and the effect of PI 3-kinase inhibitors on lipid kinase activities (C and D). A and B, Sf9 cells were infected with recombinant baculoviruses containing p110<sub>a</sub>, p110<sub>b</sub>, ΔKinα, or ΔKinβ. These cells were lysed, and lysates were immunoprecipitated with anti-C-terminal GLUT2 tag antibodies. A, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunodetection with anti-C-terminal GLUT2 tag antibodies. B, the washed immunoprecipitates were subjected to PI kinase assay. The membrane fraction from uninfected Sf9 cells was also prepared. After 10% trichloroacetic acid treatment, membrane fractions were incubated with immunoprecipitates together with [γ-<sup>32</sup>P]ATP, as described under "Experimental Procedures." D-3-Phosphorylated Phosphoinositide indicates PI-3-P, PI-3,4-P<sub>2</sub>, PI-3,4,5-P<sub>3</sub>, or a mixture of these three, all of which are prepared by incubating purified PI, PI-4-P, or PI-4,5-P<sub>2</sub> with [γ-<sup>32</sup>P]ATP in the presence of various concentrations of wortmannin or LY294002. The amount of glycerophosphoinositides was analyzed by HPLC. PI 3-kinase activity (squares) represents the amount of PI-3-P and PI-4,5-P<sub>2</sub>, and PI-4-kinase activity (triangles) represents the amount of PI-4-P and PI-3,4-P<sub>2</sub>. Lysates were prepared from Sf9 cells overexpressing p110<sub>a</sub> (open symbols) or p110<sub>b</sub> (closed symbols). PI kinase assay was performed in the presence of wortmannin (C) or LY294002 (D). Three other separate experiments yielded similar results.
isoforms of protein kinase C (21). In addition, recently, the substrate specificity of PI 4-P-5-kinases, which has been reported based on in vitro assay, was also corrected (15). Thus, it is understandable that results obtained from the artificial in vitro assay do not reflect the actual intracellular situation.

The other possible reason is that many investigators employed stimulation with epidermal growth factor, nerve growth factor, or platelet-derived growth factor to analyze the function of p85/p110-type PI kinase (22–24). Stimulation with these growth factors does activate p85/p110-type PI kinase, but phospholipase C, which hydrolyzes PI-4,5-P2 (25), is also activated. Thus, as reported previously, epidermal growth factor, nerve growth factor, or platelet-derived growth factor does not increase the amount of PI-4,5-P2, despite the possibly generation by activated p85/p110-type PI kinase of PI-4,5-P2 probably via PI-4-P production. Because of this complicated alteration in the amount of D-4-phosphorylated phosphoinositide, it seems that earlier studies did not pay attention to the effect of p85/p110-type PI kinase on the amount of D-4-phosphorylated phosphoinositide. In contrast, insulin stimulation does not lead to phos-
phospholipase Cγ activation (26). Thus, the generation of D-4-phosphorylated phosphoinositides (PI-4-P and PI-4,5-P2) by p85/p110-type PI kinase became as apparent as that of D-3-phosphorylated phosphoinositides (PI-3-P, PI-3,4-P2, and PI-3,4,5-P3). However, even in the case of insulin stimulation, since the cell has significant basal PI 4-kinase activity, if cells are prelabeled with 32P for too long a period (12 h or longer), the effect of insulin for a very short time would be difficult to detect. This is the reason we labeled the cells for 2 h. In the living body, cells are always stimulated by many factors in the serum. It is thus reasonable to consider the PI 4-kinase activity of p85/p110 to be important for the regulation of D-4-phosphorylated phosphoinositides since p85/p110 is likely to be the only enzyme that phosphorylates the D-4 position in response to insulin.

The importance of D-4-phosphorylated phosphoinositides has been established by several investigators. PI-4,5-P2 regulates the function of several actin-binding proteins (27–31) and raises the possibility that the substrate specificity of various lipid kinases and phosphatases, when incubated with purified phosphoinositide in an in vitro assay, might be different from the true, i.e., cellular, substrate specificity.

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