p110β Is Up-regulated during Differentiation of 3T3-L1 Cells and Contributes to the Highly Insulin-responsive Glucose Transport Activity*

Received for publication, December 23, 1999, and in revised form, March 4, 2000
Published, JBC Papers in Press, March 16, 2000, DOI 10.1074/jbc.M9103911199

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Activation of p85/p110 type phosphatidylinositol kinase is essential for aspects of insulin-induced glucose metabolism, including translocation of GLUT4 to the cell surface and glycogen synthesis. The enzyme exists as a heterodimer containing a regulatory subunit (e.g. p85α) and one of two widely distributed isoforms of the p110 catalytic subunit: p110α or p110β. In the present study, we compared the two isoforms in the regulation of insulin action. During differentiation of 3T3-L1 cells into adipocytes, p110α was up-regulated ~10-fold, whereas expression of p110α was unaltered. The effects of the increased p110 expression were further assessed by expressing epitope tagged p110β and p110α in 3T3-L1 cells using adenovirus transduction systems, respectively. In vitro, the basal lipid kinase activity of p110β was lower than that of p110α. When p110α and p110β were overexpressed in 3T3-L1 adipocytes, exposing cells to insulin induced each of the subunits to form complexes with p85α and tyrosine-phosphorylated IRS-1 with similar efficiency. However, whereas the kinase activity of p110β, either endogenous or exogenous, was markedly enhanced by insulin stimulation, only very small increases of the activity of p110α were observed. Interestingly, overexpression of p110β increased insulin-induced glucose uptake by 3T3-L1 cells without significantly affecting basal glucose transport, whereas overexpression of p110α increased both basal and insulin-stimulated glucose uptake. Finally, microinjection of anti-p110β neutralizing antibody into 3T3-L1 adipocytes abolished insulin-induced translocation of GLUT4 to the cell surface almost completely, whereas anti-p110α neutralizing antibody did only slightly. Together, these findings suggest that p110β plays a crucial role in cellular activities evoked acutely by insulin.

Activation of phosphatidylinositol (PI) 3-kinase has been implicated in the regulation of various cellular activities, including proliferation (1, 2), differentiation (3), membrane ruffling (4, 5), and prevention of apoptosis (6–12). It is now clear that at least four types of PI 3-kinases exist, including mammalian homologs of Saccharomyces cerevisiae VPS34 (13), a G-protein-activated form termed p110γ (14), and p170 having C-terminal sequences similar to the phosphoinositide-binding C2 domain (15). The best known form of PI 3-kinase is p85/p110, which exists as a heterodimer consisting of a regulatory and a catalytic subunit. The regulatory subunit contains two Src homology 2 domains and functions as an adaptor protein transmitting the signal from a tyrosine-phosphorylated protein to the catalytic p110 subunit (16). To date, five isoforms of the regulatory subunit and three isoforms of the catalytic subunit (p110α, p110β, and p110δ) have been identified. The regulatory subunits include two 85-kDa proteins (p85α and p85β) (17), two 55-kDa proteins (p55α and p55γ) (18–20), and one 50-kDa protein (p50α) (21, 22). The respective tissue distributions of the five isoforms differ, as do the levels of their insulin-induced activation of the associated catalytic subunits (21–23). This suggests that the different regulatory subunit isoforms function within specific signal transduction pathways induced by various growth factors and hormones. On the other hand, although some reports suggest different roles for p110α and p110β, far less is known about functional differences of the catalytic subunits.

Insulin-induced activation of PI 3-kinase in 3T3-L1 adipocytes is itself sufficient to elicit translocation of the GLUT4 glucose transporter to the cell surface with a resultant increase in glucose uptake (24–26). In addition, we have observed that expression of 110β is markedly increased in 3T3-L1 cells during their differentiation into adipocytes. The aim of the present study was to better understand the respective roles of the p110α and p110β subunits in the regulation of phosphoinositide metabolism by insulin. This was accomplished by examining the effects of p110 catalytic subunits overexpressed in 3T3-L1 adipocytes using an adenovirus transduction system and by microinjecting neutralizing anti-p110α and anti-p110β antibodies into the cells.

* This work was supported in part by Grant-in-aid for Scientific Research 09470214 (to T. A.) from the Ministry of Education, Science, and Culture of Japan and by a grant for diabetes research (to T. A.) from Sankyo Co. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: PI, phosphatidylinositol; IRS, insulin receptor substrate; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; Ab, antibody; PAGE, polyacrylamide gel electrophoresis; M.O.I., multiplicity of infection.

This paper is available on line at http://www.jbc.org

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MATERIALS AND METHODS
PI, PI 4-phosphate, PI 4,5-bisphosphate, and dexamethasone were purchased from Sigma; 3-isobutyl-1-methylxanthine and 2-deoxy-D-glucose were from WAKO (Osaka, Japan); the ECL detection system was from Amersham Pharmacia Biotech; [γ-32P]ATP and 2-deoxy-D-[3H]glucose were from NEN Life Science Products. All other reagents from commercial sources were of analytical grade.

Antibodies—Phosphotyrosine-specific antibody (Ab) 4G10 and anti-p85 antiserum were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Monoclonal anti-GLUT4 Ab was from Genzyme (Cambridge, MA). The anti-p110α and anti-p110β Abs were raised in rabbits against synthetic peptides corresponding to residues 1048–1068 of the p110α protein and to residues 1039–1070 of the p110β protein, respectively. These Abs were affinity-purified on Affi-Gel-10 (Bio-Rad) columns. These corresponding peptides had been coupled. They were then extensively dialyzed against phosphate-buffered saline (PBS). Abs raised against IRS-1 and the C-terminal GLUT2 tag were prepared as described previously (24, 27).

Cell Culture—3T3-L1 fibroblasts to be transduced using recombinant adenovirus were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% donor calf serum (Life Technologies, Inc.). After microinjection, 3T3-L1 adipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Life Technologies, Inc.) and then incubated in serum-free DMEM for 3 h. Microinjection of the adenovirus were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% donor calf serum (Life Technologies, Inc.).

PI 3-Kinase Assay—PI 3-kinase assays were carried out using recombinant adenovirus such that similar levels of p110α or p110β proteins were expressed (Fig. 2A, top panel). Recombinant adenoviruses were obtained by homologous recombination of those cosmids and the parental virus genome as described previously (24).

Immunoprecipitation and Western Blotting—Cells were lysed in PBS containing 1% Triton, 0.35 mg/ml phenylmethylsulfonyl fluoride, and 100 μM sodium vanadate, after which the lysates were centrifuged for 10 min at 15,000 χ g at 4 °C to remove insoluble materials. For immunoprecipitation, the supernatants were incubated with the indicated Abs, which were protein A-Sepharose and anti-phosphotyrosine antibody (Ab) 4G10 and anti-p85. After washing with ice-cold PBS, the cell lysates were cleaved of insoluble materials by centrifugation (15,000 × g, 4 °C, 10 min) and immunoprecipitated with the indicated Ab and protein A-Sepharose. PI 3-kinase activities in the immunoprecipitates were measured as described previously (24) using PI as a substrates. The results were quantitated with Fuji BAS2000 (Tokyo, Japan).

Glucose Transport Assay—3T3-L1 adipocytes plated in 24-well culture dishes were serum-starved for 3 h in DMEM containing 0.2% bovine serum albumin and then incubated with or without selected concentrations of insulin for 5 min, washed with ice-cold PBS, and lysed with PBS containing 1% Nonidet P-40, 0.35 mg/ml phenylmethylsulfonyl fluoride, and 100 μM sodium vanadate. Cell lysates were cleared of insoluble material as centrifugation (15,000 × g, 4 °C, 10 min) and immunoprecipitated with the indicated Ab and protein A-Sepharose. PI 3-kinase activities in the immunoprecipitates were measured as described previously (24) using PI as a substrates. The results were quantitated with Fuji BAS2000 (Tokyo, Japan).

RESULTS

Altered Expression of p110α and p110β during Differentiation of 3T3-L1—Differentiation of 3T3-L1 cells into adipocytes was induced as described under “Materials and Methods,” and expression of p110α and p110β, along with that of C/EBPα and GLUT4 as differentiation markers, was measured by immunoblotting using their specific Abs, respectively. To avoid influences from cell division after induction, each sample was normalized by protein contents. As shown in Fig. 1, C/EBPα was detected from Day 2, showed maximum expression on Day 4 and continued to express at a high level (Fig. 1A). GLUT4 could be detected from Day 2, and its expression level continued to increase (Fig. 1B). Expression level of p110α was unchanged during the differentiation (Fig. 1C). On the other hand, although the expression of p110β was under detectable level before induction, it could be detected from Day 2 and continued to increase significantly during differentiation, of which the time course was very similar to that of GLUT4 expression (Fig. 1D).

p110α and p110β Overexpressed in 3T3-L1 Adipocytes—p110α or p110β was overexpressed in 3T3-L1 adipocytes by using recombinant adenovirus such that similar levels of p110α or p110β proteins were expressed (Fig. 2A, top panel). From the optical density of the blots, the degree to which p110α and p110β were overexpressed was calculated to be approximately 8-fold over their endogenous expression levels in 3T3-L1 cells (Fig. 2A, middle and bottom panels). Overexpressed p110α and p110β were not recognized by the anti-p110α and anti-p110β Abs, respectively (Fig. 2A, middle and bottom panels), indicating that these antibodies are highly specific for their corresponding isoforms.

The association of the overexpressed p110α and p110β isoforms with the p85α regulatory subunit was shown by the presence of p85α in the respective immunoprecipitates (Fig. 2B). p110α and p110β exhibited a very similar binding ability with the regulatory subunit, p85α, in 3T3-L1 cells. In addition, it was also revealed that IRS-1, which was tyrosine-phosphorylated and bound to p85α in the presence of insulin, complexed with p110α or p110β with equal efficiency (Fig. 2C). Thus, it appears that p110α and p110β are not different in regard to their molecular association with the regulatory subunit or with IRS-1.

PI 3-Kinase Activities of p110α and p110β at the Basal Level and Their Responses to Insulin Stimulation—To compare PI 3-kinase activities of p110α and p110β, either catalytic subunit was overexpressed in 3T3-L1 cells and immunoprecipitated with anti-GLUT2 tag Ab (Fig. 3A). Without insulin stimulation, overexpressed p110α exhibited 34-fold higher PI 3-kinase activity was determined by scintillation counting. Microinjection of Anti-p110 Abs into 3T3-L1 Adipocytes—3T3-L1 adipocytes were trypsinized, reseeded onto acid-washed glass cover slips, and then incubated in serum-free DMEM for 3 h. Microinjection of the affinity-purified anti-p110 Abs or control rabbit IgG at a final concentration of 2 mg/ml in PBS was carried out using a semiautomated Eppendorf microinjection system with an injection pressure of 100 hectopascals and an injection time of 0.8 s. The injection volume is approximately 10% of the cell volume under these conditions. In each experiment, 200–250 cells were microinjected with each anti-p110α, anti-p110β Ab, or control rabbit IgG.
activity as compared with overexpressed p110α, despite these catalytic subunits having similar affinity for p85α, as shown in Fig. 2B.

The concentration response curves for endogenous p110α and p110β were next investigated. As shown in Fig. 3B, insulin-stimulated lipid kinase activity in the p110β immunoprecipitate was increased as much as 3.4-fold over basal activity in the absence of insulin. In contrast, insulin stimulation increased the kinase activity in the p110α immunoprecipitate by only about 60%. When the two catalytic subunits were then overexpressed using the adenovirus expression system, the kinase activity in the p110β immunoprecipitates from the cells overexpressing p110β were insulin-dependently increased as much as 19-fold (Fig. 3C), whereas the kinase activity in the p110α immunoprecipitate from the cells overexpressing p110α was unaffected. To avoid any effect caused by using different Abs, anti-p110α and anti-p110β, for immunoprecipitation, overexpressed p110α or p110β were immunoprecipitated by anti-GLUT2 tag Ab (Fig. 3D). Essentially the same results were obtained, clearly indicating that PI 3-kinase activity catalyzed by p110β is highly insulin-sensitive, whereas the activity catalyzed by p110α is not.
The aim of the present study was to better understand regulation of the PI kinase activities associated with two isoforms of its catalytic subunit, p110α and p110β, and to assess how regulatory differences might influence the cellular effects of insulin. In fact, during differentiation of 3T3-L1 cells into adipocytes, in which they acquire insulin-induced glucose uptake, expression of p110α is unchanged, whereas that of p110β is that used for anti-p110α Ab (data not shown).

Effect of Microinjecting p110 Abs on Insulin-induced Translocation of GLUT4 to the Cell Surface—To analyze the isoform-specific functions of endogenously expressed p110α and p110β, inhibitory anti-p110α or anti-p110β Ab was microinjected into 3T3-L1 adipocytes, followed by membrane sheet assay. Microinjection of control rabbit IgG had no effect on insulin-induced translocation of the GLUT4 transporter to the cell surface (Fig. 5, A and B). Microinjection of either anti-p110α or anti-p110β Ab into cells had no effect on GLUT4 localization in the basal state (data not shown). Microinjection of anti-p110α Ab attenuated insulin-evoked GLUT4 translocation by approximately 25% (Fig. 5C; also quantified in Fig. 5E). In contrast, microinjection of anti-p110β Ab inhibited insulin-evoked GLUT4 translocation to the cell surface by approximately 94% (Fig. 5D; also quantified in Fig. 5E).

**DISCUSSION**

p85/p110 type PI kinase has been implicated in a wide range of cellular activities, including control of proliferation (1, 2), cytoskeletal organization (4, 5), prevention of apoptosis (6–12), neurite outgrowth (3), vesicular trafficking (31), and insulin-induced translocation of the glucose transporter to the cell surface (1, 32). Most of these findings have been derived from experiments in which lipid kinase activity was diminished by antagonists such as wortmannin and LY294002 or by microinjection or overexpression of a dominant negative p85α mutant that binds to tyrosine phosphorylated proteins but lacks a p110 binding site. More recent studies entailing overexpression of constitutively active p110α or GLUT2-tagged p110α have shown that elevation of this lipid kinase activity is itself sufficient to induce some of these cellular activities, including translocation of GLUT4 glucose transporter and activation of an intracellular signaling pathway known to mediate proliferation (24–26).

FIG. 5. **Effect of microinjecting anti-p110 Abs on GLUT4 translocation.** Images from membrane sheet assay samples showing the distribution of GLUT4 on the surfaces of 3T3-L1 adipocytes microinjected with control IgG (A and B), anti-p110α Ab (C), or anti-p110β Ab (D). Once microinjected, the cells were incubated without (A) or with (B–D) 10^{-7} M insulin for 15 min. 100 images were taken for each sample. Two representative images from each sample are shown in A–D. Average fluorescence intensities and their S.E. are shown in E.
markedly up-regulated (Fig. 1). On the basis of in vitro PI 3-kinase assays of p110α and p110β expressed in 3T3-L1 adipocytes, basal p110α activity is substantially lower than that of p110α (Fig. 3A). However, p110β appears to be highly insulin-sensitive, whereas p110α was activated only slightly by insulin (Fig. 3, B–D). Although the two isoforms bind to p85α and to IRS-1 with similar efficiency (Fig. 2, B and C), by these bindings, p110β may change its conformation more dramatically than does p110α. Such a difference may be attributable to isoform-specific binding of p110 to unknown molecules, although further investigations are required. It should be noted, however, that each isoform-specific anti-p110 Abs markedly inhibited PI kinase activity of p110α or p110β (30). Nonetheless, because the lipid kinase activity of p110 incubated with each concentration of insulin should be similarly inhibited, it still seems reasonable to conclude that the p110β isoform is a highly insulin-sensitive form of the catalytic subunit, whereas the p110α isoform is a rather insulin-insensitive form.

In addition, we can speculate the reason why the insulin-induced response of endogenous p110β was lower than that obtained from the experiment using overexpressed p110β. In the plain or Lac-Z expressing 3T3-L1 cells, p85α is present as a heterodimer with endogenous p110α or p110β. Because IRS-1 possesses four potential binding sites for the Src homology 2 domain of p85α and thus can bind to two molecules of p85α, a detectable amount of p110α was co-immunoprecipitated by the anti-p110β antibody in the insulin-stimulated condition, although this amount was small (data not shown). This co-immunoprecipitated “low-insulin responsive” p110β is likely to reduce the insulin responsiveness of the anti-p110β immunoprecipitate as compared with the control 3T3-L1 cells. In case of p110β-overexpressing 3T3-L1 cells, most p85α is occupied by overexpressed p110β, and the amount of p110α bound with p85α is much smaller than in control cells, and thus the effect of co-immunoprecipitated p110α becomes much smaller.

Earlier reports have suggested that different p110 catalytic subunit isoforms have distinct functions. For example, the p110α subunit is involved in platelet-derived growth factor- and epidermal growth factor-mediated mitogenic responses, but not in responses mediated by bombesin or lysophosphatidic acid (33). In contrast, p110β is apparently necessary for insulin- and lysophosphatidic acid-mediated mitogenic responses but not platelet-derived growth factor-mediated responses (34). Moreover, in response to insulin stimulation, p110β but not p110α associates with the GLUT4 glucose transporter compartment (35). It should be noted that the previous studies showing that overexpressed p110α induces GLUT4 translocation may not directly indicate the importance of p110α itself, because these membrane-targeted or constitutively active forms of p110α are considered to function in all of the membrane fractions but are not limited to the membrane of a specific compartment. Such an ectopic expression of p110α, which is always active even without insulin stimulation, might have brought insulin-independent phosphoinositide production at a place where phosphoinositide is critical to cause GLUT4 translocation. Because p110β seems to require insulin stimulation to be an active form, overexpression of p110β without insulin treatment might not cause translocation of GLUT4. Thus, it seems very likely that both subcellular localization of the enzyme and kinetics of the kinase activity are key factors in determining the specific functions of the various PI kinase isoforms.

Taking into consideration that both the subcellular localization and kinetics of p110 may influence cellular function, we tried to determine which endogenous catalytic subunit, p110α or p110β, is involved in insulin-induced GLUT4 translocation, by microinjecting isoform-specific anti-p110 to neutralize activities of each p110 (Fig. 5). The neutralizing antibodies used in this study were highly isoform-specific and very strongly inhibited the lipid kinase activity of the corresponding isoform. The obtained data clearly suggested that p110β plays a major role in transmitting the signals to translocate GLUT4. On the other hand, the contribution of p110α to insulin-induced GLUT4 translocation is likely to be relatively small.

To date, some evidence has been obtained suggesting that high levels of basal PI 3-kinase activity associated with p110α may be important for prevention of apoptosis. On the other hand, acute cellular activities rapidly evoked by growth factors or hormones may require a large increase in PI kinase activity against a background of low basal activity. In this way, maintenance of the appropriate levels of basal and stimulated PI kinase activities may be governed by the ratio of the expressions of the p110α and p110β isoforms. We therefore speculate that PI kinase activity derived from p110α functions mainly in carrying out such “housekeeping” activities as prevention of apoptosis and sustaining basal glucose uptake, whereas p110β plays a crucial role in carrying out cellular responses to insulin and possibly other hormones as well.

This is the first report differentiating the two isoforms of the PI kinase catalytic subunit as regards regulation of basal and insulin-stimulated lipid kinase activity and glucose transport. The respective roles played by p110α and p110β may be applicable to the signal transduction cascades activated not only by insulin but also by other growth factors. In addition, we have recently reported that p85/p110 type PI kinase phosphorylates both the D-3 and the D-4 position of the inositol ring in vivo (36). In this report, it was shown that p110β exhibits a higher ratio of PI 4-kinase activity/PI 3-kinase activity than p110α. Additional studies will be required before a full understanding of the mechanisms underlying the localization and regulation of p110α activities under basal and stimulated conditions can be obtained.

Acknowledgments— We thank Drs. I. Saito and Y. Kanegae for helpful advice and generous gifts of the recombinant Adex1CAlacZ, the expression cosmid cassette, and the parental adenovirus DNA terminal protein complex.

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