Overexpression of Catalytic Subunit p110α of Phosphatidylinositol 3-Kinase Increases Glucose Transport Activity with Translocation of Glucose Transporters in 3T3-L1 Adipocytes*

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To elucidate the mechanisms of phosphatidylinositol (PI) 3-kinase involvement in insulin-stimulated glucose transport activity, the epitope-tagged p110α subunit of PI 3-kinase was overexpressed in 3T3-L1 adipocytes using an adenovirus-mediated gene transduction system. Overexpression of p110α was confirmed by immunoblot using anti-tagged epitope antibody. p110α overexpression induced a 2.5-fold increase in PI 3-kinase activity associated with a translocation of GLUT4 glucose transporters from an intracellular to plasma membrane (16). Overexpression of p110α induced an approximate 14-fold increase in basal glucose transport rate, which was also greater than that observed in the stimulated control. No apparent difference was observed in the cellular expression level of either GLUT1 or GLUT4 proteins between control and p110α-overexpressing 3T3-L1 adipocytes. Subcellular fractionation revealed translocation of glucose transporters from intracellular to plasma membranes in basal p110α-overexpressing cells. The translocation of GLUT4 protein to the plasma membrane was further confirmed using a membrane sheet assay. These findings indicate that an increment in PI 3-kinase activity induced by overexpression of p110α of PI 3-kinase stimulates glucose transport activity with translocation of glucose transporters, i.e., mimics the effect of insulin.

One of the major physiological functions of insulin is to stimulate glucose uptake into insulin-sensitive cells, such as adipocytes and myocytes. This effect is primarily due to translocation of GLUT4 glucose transporters from an intracellular compartment to the plasma membrane (1, 2). Binding of insulin to its receptor results in receptor autophosphorylation and activation of the receptor tyrosine kinase, followed by tyrosine phosphorylation of several intermediate proteins including insulin receptor substrate (IRS)1 (3, 4). Tyrosine-phosphorylated IRSs then bind to and thereby regulate Src homology 2 (SH2) domain containing proteins.

Phosphatidylinositol (PI) 3-kinase is one of such signaling molecules (5, 6). It is a heterodimeric enzyme consisting of a regulatory subunit with two SH2 domains and a 110-kDa catalytic subunit (p110α, p110β) (7, 8). Three unique regulatory subunit isoforms (p85α, p85β, p55γ) for PI-3 kinase have been identified (9–12). In addition, an alternatively spliced isoform of p85α (p55α) has been reported recently (13).

Several lines of evidence have indicated that PI 3-kinase activation is important in insulin-stimulated glucose transport. The PI 3-kinase inhibitors, such as wortmannin and LY294002, can block the insulin-stimulated glucose transport and GLUT4 translocation in rat and 3T3-L1 adipocytes (14, 15). Furthermore, inhibition of endogenous PI 3-kinase by microinjection of glutathione S-transferase (GST)-p85α subunit fusion protein (16) or a dominant negative mutant of the p85α regulatory subunit of PI 3-kinase (17) inhibits GLUT4 translocation induced by insulin in 3T3-L1 adipocytes. These findings suggest that PI 3-kinase may be required for insulin-stimulated glucose transport. However, several groups have recently reported that platelet-derived growth factor (PDGF) stimulates PI 3-kinase activity but not glucose transport activity (18, 19), although conflicting results were also reported (20).

We report herein that an increase in PI 3-kinase activity induced by adenovirus-mediated overexpression of the p110α subunit of PI 3-kinase (p110α<sup>p110K</sup>) stimulates glucose uptake with translocation of glucose transporters in 3T3-L1 adipocytes.

EXPERIMENTAL PROCEDURES

Antibodies—As described previously, the antisera which recognized the epitope tagged to exogenous p110α, anti-GLUT1 antisera, and anti-GLUT4 antiserum were raised against synthetic peptides corresponding to residues 510–524 of human GLUT2 (21), residues 478–492 of rabbit GLUT1 (22), and residues 495–509 of rat GLUT4 (23), respectively. The anti-phosphotyrosine monoclonal antibody (4G10) and the antibody against the whole p85α molecule were purchased from UBI. The anti-p85α antibody recognizes all known regulatory subunits of PI 3-kinase including p85α, p55γ, and p55γ, and p85β (13).

Culture—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% CO<sub>2</sub> at 37 °C. Two days after the fibroblasts had reached confluence, differentiation was induced by treating cells with DMEM containing 0.5 mm 3-isobutyl-1-methylxanthine, 4 μg/ml dexamethasone, and 10% fetal bovine serum for 48 h. Cells were refed with DMEM supplemented with 10% fetal bovine serum every other day for the following 4–10 days. More than 90% of the cells expressed the adipocyte phenotype.

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‡ The abbreviations used are: IRS, insulin receptor substrate; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; GST, glutathione S-transferase; PDGF, platelet-derived growth factor; DMEM, Dulbecco’s modified Eagle’s medium; pfu, plaque-forming units; PM, plasma membrane; LDM, low density microsome.

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Cloning and Construct—Reverse transcription-polymerase chain reaction was performed to amplify cDNA of p110\textsubscript{a}PI3K using the bovine brain RNA as a substrate and oligonucleotides based on its reported sequence (7) as primers, yielding cDNA of p110\textsubscript{a}PI3K covering the entire coding region. A portion of human GLUT2 cDNA corresponding to residues 510–524 was ligated to p110\textsubscript{a}PI3K cDNA to encode p110\textsubscript{a}PI3K. Adex1CAp110, a 3-kinase tagged with the epitope tag at its C terminus, was constructed.

Gene Transduction—The recombinant adenoviruses Adex1CAlacZ (24) and Adex1Cdlp110\textsubscript{a}, which encode Escherichia coli lacZ and the epitope-tagged bovine p110\textsubscript{a}PI3K, respectively, were constructed by homologous recombination between the expression cosmid cassette and the parental virus genome as described previously (25, 26). 3T3-L1 adipocytes were incubated with DMEM containing the adenoviruses for 1 h at 37 °C, and the growth medium was then added. Experiments were performed 3 to 4 days after the infection. When the adenovirus Adex1CAlacZ was applied at a multiplicity of infection of 200–300 pfu/cell, lacZ gene expression was observed in more than 90% of 3T3-L1 adipocytes on postinfection day 3 (data not shown). Infection with Adex1CAp110\textsubscript{a} or Adex1CAp110\textsubscript{a} resulted in no apparent differences in extent of differentiation into adipocytes, numbers of differentiated adipocytes, or morphological features in 3T3-L1 adipocytes, as compared with untreated cells on postinfection day 3. In addition, 3T3-L1 adipocytes infected with Adex1CAlacZ exhibited no significant differences in glucose transport activity as compared with noninfected cells on postinfection day 3 (data not shown). Therefore, in the present study, recombinant adenoviruses were applied at a multiplicity of infection of approximately 200–300 pfu/cell, and 3T3-L1 adipocytes infected with Adex1CAlacZ virus were used as a control.

Immunoblotting—3T3-L1 adipocytes were lysed and boiled in Laemmli buffer containing 10 mM dithiothreitol and subjected to SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose filters. The filters were incubated with antisera, subsequently with 125I-protein A. The blots were exposed to x-ray film. The dried thin layer chromatography plates were visualized for the radioactivities (B). Results shown in C and D were quantitated ones using an image analyzer. The values are means ± S.D. for triplicate measurements in a representative experiment. Three other separate experiments yielded similar results.

Preparation of Plasma Membrane Sheets and Immunofluorescence—3T3-L1 fibroblasts plated on sterile glass coverslips were induced to differentiate into adipocytes. Plasma membrane sheets were prepared by sonication as described (28). Adherent plasma membranes were fixed in 2% paraformaldehyde and processed for indirect immunofluorescence using anti-GLUT4 antisemur (1:100 dilution) followed by rhodamine-conjugated secondary antibodies.

RESULTS

Overexpression of p110\textsubscript{a}PI3K was achieved utilizing an adenovirus-mediated gene transduction system in 3T3-L1 adipocytes, as demonstrated by immunoblotting with the antibody against the tagged epitope (Fig. 1A). Control (LacZ-expressing) and p110\textsubscript{a}PI3K-overexpressing 3T3-L1 (p110\textsubscript{a}PI3K) adipocytes were incubated with or without insulin for 15 min, and the PI 3-kinase activity was measured. In control cells, insulin stimulated the coimmunoprecipitation of PI 3-kinase activity with tyrosine-phosphorylated protein (maximum approximately 35-fold) (Fig. 1, B and C) and that with the regulatory subunits of PI 3-kinase (maximum approximately 2-fold) (Fig. 1D) in a dose-dependent manner. Similar results were obtained in parental 3T3-L1 adipocytes. Overexpression of PI3K-L1 induced only a very modest elevation of PI 3-kinase activity in precipitates with the anti-phosphotyrosine antibody in the basal condition (Fig. 1C). PI 3-kinase activity was greatly increased in precipitates with the anti-phosphotyrosine antibody or anti-p85\textsubscript{a} antibody. Immunocomplexes were precipitated with protein A-Sepharose (Pharmacia Biotech Inc.). PI 3-kinase activity was assayed in the immunoprecipitates as reported previously (12). The results were quantitated using an image analyzer BAS2000 (Fujix).

FIG. 1. Overexpression of p110\textsubscript{a}PI3K in 3T3-L1 adipocytes determined by immunoblot analysis (A–D). A, total cellular proteins were prepared from control (lane 1) and p110\textsubscript{a}PI3K-overexpressing 3T3-L1 (lane 2) adipocytes, subjected to SDS-polyacrylamide gel electrophoresis, and immunoblotted with anti-tagged epitope antiserum. B–D, lysates were prepared from control (open circles) and p110\textsubscript{a}PI3K-overexpressing 3T3-L1 adipocytes (closed circles) after incubation with the indicated concentrations of insulin and were immunoprecipitated with anti-phosphotyrosine (B and C) or anti-regulatory subunits of PI 3-kinase (D). PI 3-kinase activity was then assayed in the immunoprecipitates. The dried thin layer chromatography plates were visualized for the radioactivities (B). Results shown in C and D were quantitated ones using an image analyzer. The values are means ± S.D. for triplicate measurements in a representative experiment. Three other separate experiments yielded similar results.

2-Deoxy-[\textsuperscript{3}H]glucose uptake in response to a 15-min incubation with 10–10 to 10–5 M insulin was measured in control and p110\textsubscript{a}PI3K adipocytes. Insulin dose-dependently stimulated 2-deoxyglucose uptake in control 3T3-L1 adipocytes (Fig. 2), and the maximally stimulated values were similar (approximately 12-fold) in control and parental 3T3-L1 adipocytes. Overexpression of p110\textsubscript{a}PI3K induced a marked increase (approximately 14-fold) in the basal glucose transport rate. Insulin further stimulated the glucose uptake in p110\textsubscript{a}PI3K-L1 in a dose-dependent manner (maximum approximately 1.4-fold) (Fig. 2).
To begin addressing the mechanism whereby overexpression of p110α<sup>PI3K</sup> stimulated hexose transport activity, cellular expression levels of the two glucose transporter isoforms (GLUT1 and GLUT4), known to be expressed in 3T3-L1 adipocytes, were determined. As shown in Fig. 3, A and B, no apparent differences in cellular expression levels of GLUT1 and GLUT4 were observed between control and p110α-L1 adipocytes.

We next tested whether overexpression of p110α<sup>PI3K</sup> affected the subcellular distribution of glucose transporters by immunoblot analysis of the plasma membrane (PM) fraction and the intracellular low density microsome (LDM) fraction (Fig. 3, C and D). Insulin caused 4- and 6-fold increases in the amounts of plasma membrane GLUT1 and GLUT4, respectively, in control adipocytes. Corresponding decreases in GLUT1 and GLUT4 were observed in the LDM fraction of these cells. Strikingly, p110α<sup>PI3K</sup> overexpression induced translocation of GLUT1 and GLUT4 from the LDM fraction to the PM fraction, in a fashion similar to the insulin effect in control cells. Insulin addition to p110α-L1 adipocytes had no apparent effects on the subcellular distributions of either GLUT1 or GLUT4.

We further confirmed the translocation of GLUT4 protein to the plasma membrane using the membrane sheet assay method. Fig. 4 demonstrates cell surface GLUT4 expression in control and p110α-L1 adipocytes treated in the absence or presence of insulin (100 nM) for 15 min. In control cells, very little GLUT4 staining was observed on the plasma membrane, whereas insulin treatment increased surface GLUT4 staining substantially (Fig. 4). On the other hand, cell surface GLUT4 expression in p110α-L1 adipocytes was already intense in the basal state, and insulin appeared to have virtually no further effects, consistent with the results obtained by the subcellular fractionation method. These findings demonstrate that overexpression of p110α<sup>PI3K</sup> exerts stimulatory effects on the translocation of glucose transporters from intracellular to plasma membranes.

DISCUSSION

In this study, overexpression of p110α<sup>PI3K</sup>, using an adenovirus-mediated gene transduction system, induced translocation of glucose transporters from intracellular low density microsomes to the plasma membrane and, thus, increased the glucose transport rate without changing the total amount of glucose transporter protein. Recently, several findings on the relationship between PI 3-kinase activity and glucose transport in 3T3-L1 adipocytes have been reported. PDGF caused no significant stimulation of glucose transport activity in 3T3-L1 adipocytes, despite increasing PI 3-kinase activity to a level approaching that elicited by insulin (18, 19). We obtained similar results in PDGF-treated 3T3-L1 adipocytes (data not shown). In addition, introduction of a thio-phosphotyrosine peptide into permeabilized 3T3-L1 adipocytes stimulated PI 3-kinase to the same extent as insulin, while having little stimulatory effect on glucose transport activity (29). These authors concluded that another signaling pathway, in addition to the activation of PI 3-kinase, might be required for the stimulation of GLUT4 translocation and glucose transport activity.

However, the results of the present study indicate that PI 3-kinase activation alone stimulates translocation of GLUTs and, thus, activates glucose transport activity. One interpretation of these findings is that activation of PI 3-kinase by insulin is qualitatively different from that by either PDGF or the thio-phosphotyrosine peptide, including the possibility that insulin activates PI 3-kinase in different subcellular locations than do the peptide and PDGF. A previous study (30) showed that activated PI 3-kinase has the same intracellular location as tyrosine-phosphorylated IRS1 in insulin-stimulated adipocytes, whereas the thio-phosphotyrosine peptide appears to inhibit the association of PI 3-kinase activity with IRS1. The tyrosyl-phosphorylated IRS1-p85 complex formed in response to insulin was demonstrated to be localized in a very low density vesicle subpopulation. These vesicles could be distinguished from vesicles containing the insulin receptor which was endocytosed from the plasma membrane (30). On the other hand, tyrosyl-phosphorylated PDGF receptors, the p85 subunit of PI 3-kinase, and activated PI 3-kinase are all found in isolated clathrin-coated vesicles after PDGF stimulation in 3T3-L1 cells, indicating that both receptor and activated PI 3-kinase enter the endocytic pathway (31). These data suggest that the subcellular redistribution of PI 3-kinase activity in response to PDGF is different from that induced by insulin. Increased PI 3-kinase activity was observed in every LDM subpopulation, including a very low density vesicle subpopulation, when p110α-L1 adipocytes were homogenized and frac-
Overexpression of PI3K and Glucose Transport

One of the advantages of transient expression using an adenovirus-mediated gene transduction system is that long-term effects of gene transduction are much smaller than those in cells stably expressing the gene product. Furthermore, transient expression is not hampered by the problem of cell selection. Long-term selection of stable cell lines can lead to the establishment of specific cell lines which express many other genes necessary for survival during the selection period. We achieved expression of the intended protein in almost all 3T3-L1 adipocytes, while avoiding the selection process, using the adenovirus-mediated gene transduction system.

The downstream components of the pathway from PI 3-kinase through glucose transport have yet to be elucidated. Recently, several downstream targets of PI 3-kinase have been identified, such as Akt (34) and the small G-protein Rac (35). However, Rac does not couple PI 3-kinase to insulin-stimulated glucose transport in 3T3-L1 adipocytes (36). Other as yet unknown pathway(s) may be involved in insulin-stimulated glucose transport. p110-α adipocytes may serve as a model for studying the steps downstream from PI 3-kinase.

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REFERENCES

1. Birnbaum, M. J. (1992) Int Rev. Cytol. 137, 239–297
2. James, D. E., and Piper, R. (1993) J. Cell. Biochem. 50, 307–612
3. White, M. F., and Kahn, C. R. (1994) J. Biol. Chem. 269, 1–4
4. Keller, S. R., and Lienhard, G. E. (1994) Trends Cell Biol. 4, 115–119
5. Panayotou, G., and Waterfield, M. D. (1993) BioEssays 15, 171–177
6. Kapeller, R., and Cantley, L. C. (1994) BioEssays 16, 565–576
7. Iles, I., Otsu, M., Volini, S., Fry, M. J., Gout, I., Hirano, H., Yazaki, Y., and Oka, Y., and Saito, A. (1995) J. Biol. Chem. 270, 8527–8530
8. Nakamura, T., Vlahos, C. J., Cheatham, L., Wang, L., Blumenstein, B., and Williams, L. T. (1993) Cell 65, 75–82
9. Skolnik, E. Y., Margolis, B., Mohammad, M., Lowenstein, E., and Schlessinger, J. (1991) Cell 65, 83–90
10. Otsu, M., Iles, I., Gout, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Totty, N., Hsuan, S. A., Courtneidge, S. A., Parker, C. J., and Waterfield, M. D. (1992) Cell 70, 419–429
11. Hu, P., Mondino, A., Skolnik, E. Y., and Shleissinger, J. (1993) Mol. Cell. Biol. 13, 7677–7688
12. Escobedo, J. A., Navankaattusatts, S., Kavanagh, W. M., Milfray, D., Vliet, J., and Williams, L. T. (1995) Mol. Cell. Biol. 15, 6635–6646