Roles of Phosphatidylinositol 3-Kinase and Ras on Insulin-stimulated Glucose Transport in 3T3-L1 Adipocytes

アデノウィルスベクターによる遺伝子導入系を用いた3T3-L1脂肪細胞におけるインスリン刺激による糖取り込み促進作用の機構の解析

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ABSTRACT

Exogeneous proteins involved in signal transduction were overexpressed in 3T3-L1 adipocytes using an adenovirus-mediated gene transduction system. First, overexpression of a dominant negative p85α regulatory subunit of PI 3-kinase (Δp85α) or of a dominant negative Ras (N17Ras) blocked each signaling pathway. Functional expression of Δp85α and N17Ras was confirmed by marked inhibition of insulin-stimulated PI 3-kinase activity and mitogen-activated protein kinase activity, respectively. N17Ras expression did not affect glucose transport activity, whereas Δp85α expression inhibited insulin-stimulated glucose transport with impairment of GLUT4 translocation. Thus, the Ras signaling pathway does not play a major part in insulin-stimulated glucose transport, but PI 3-kinase activation, via phosphotyrosyl proteins and heterodimeric PI 3-kinase, is required for translocation of GLUT4 glucose transporter.

Next, the epitope-tagged p110α subunit of PI 3-kinase was overexpressed in 3T3-L1 adipocytes. p110α overexpression induced an increase in PI 3-kinase activity associated with its regulatory subunits in the basal state, an increase exceeding that of the maximally insulin-stimulated control cells, and also induced an increase in the basal glucose transport rate, which was greater than that observed in the stimulated control. Subcellular fractionation revealed translocation of glucose transporters from the intracellular membrane to the plasma membrane in basal p110α-overexpressing cells even in the basal state, which was further confirmed using a membrane sheet assay. These findings indicate that an increment in PI 3-kinase activity stimulates glucose transport activity with translocation of glucose transporters, i. e., mimics the effect of insulin.

Thus, I demonstrate here that the activation of PI 3-kinase is required and sufficient for translocation of GLUT4 glucose transporter, resulting in activation of glucose transport.

INTRODUCTION

The biological actions of insulin are important for regulation of growth, differentiation, and metabolism. One of the most important metabolic actions of insulin is to stimulate glucose uptake into insulin-sensitive cells, such as adipocytes and myocytes. The ability of insulin to recruit glucose transporters (primarily GLUT4) to the cell surface in a dose-dependent and reversible manner is thought to account for the majority, if not all, of the increase in glucose transport seen with insulin stimulation (1, 2). The signaling mechanisms involved in insulin-stimulated glucose transport and GLUT4 translocation are not well understood, although tremendous progress has been made in understanding the molecular mechanisms of insulin signaling. 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, which is responsible for phosphorylating PI, PI-4-P, and PI-4,5-P_2 , 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) and an alternatively spliced isoform of p85α (p55α) for PI-3 kinase have been identified (9-13).

The exact function of PI 3-kinase in mammalian cells is not known, although PI 3-kinase has been implicated in the regulation of various cellular activities (5, 6), including proliferation, differentiation,

membrane ruffling, and prevention of apoptosis. In addition, 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 activation is required for insulin-stimulated glucose transport. However, since the glucose transport activity or PI 3-kinase activity could not be estimated in 3T3-L1 adipocytes in which the dominant negative proteins were microinjected, the effect of specific blocking of the signaling pathway from insulin receptor to PI 3-kinase on glucose transport activity, which may be determined by both translocation of glucose transporters and their intrinsic activity, remains unclear.

On the other hand, it was also reported that several growth factors stimulated Ras (p21-ras)-mediated activation of PI 3-kinase in PC12 and COS cells, possibly due to direct binding between Ras-GTP and the catalytic subunit of PI 3-kinase (18), suggesting the possibility that insulin-stimulated glucose transport is partially mediated by the Ras signaling pathway in adipocytes. On the contrary, it was also reported that Ras lies downstream of PI 3-kinase in several signaling pathways (19-22), although it is not known whether PI 3-kinase-dependent Ras activation is involved in insulin-stimulated glucose transport in adipocytes.

In addition, several groups have recently reported that platelet derived growth factor (PDGF) stimulates PI 3-kinase activity but not

glucose transport activity (23, 24), suggesting that another signaling pathway, in addition to the activation of PI 3-kinase, is required for the stimulation of GLUT4 translocation and glucose transport activity. However, conflicting results were also reported (25). The signal from the mutated PDGF receptor which activates PI 3-kinase cascade alone could induced the translocation of co-expressed epitope-tagged GLUT4 glucose transporter. Thus, controversy persists as to whether PI 3-kinase activation is sufficient for insulin-stimulated glucose transport.

Although it has been suggested that PI 3-kinase activation is important for insulin-stimulated glucose transport, the signaling pathway from insulin receptor to glucose transport activity in insulin-responsive cells, such as adipocytes and myocytes, remains unclear. This is partly due to the difficulty of gene transduction into such highly differentiated cells. In the present study, the functional expression of exogeneous proteins in 3T3-L1 adipocytes was achieved using an adenovirus-mediated gene transduction system. First, to elucidate the signaling pathway involved in insulin-stimulated PI 3-kinase activation as well as involvement of Ras-PI3K cross-talk and of the Ras-MAPK cascade, I blocked each signaling pathway via the induction of the dominant negative p85α regulatory subunit of PI 3-kinase (Δp85α) or of dominant negative Ras (N17Ras) in 3T3-L1 adipocytes. This system enables us to study the relationship among PI 3-kinase activity, MAP kinase activity, and glucose transport activity in 3T3-L1 adipocytes, which was not elucidated by using the microinjection technique. In addition, to further investigate the role of PI 3-kinase in insulin-induced glucose uptake, the p110α subunit of PI 3-kinase (p110α^{PI3K}) is overexpressed in 3T3-L1 adipocytes. I report here that an increase in PI 3-kinase activity induced by overexpression of p110α PI 3-kinase stimulates glucose uptake with translocation of glucose transporters in 3T3-L1 adipocytes.

EXPERIMENTAL PROCEDURES

Antibodies

The antiserum which recognized the epitope tagged to exogenous p110α, anti-GLUT1 antiserum, and anti-GLUT4 antiserum were raised against synthetic peptides corresponding to residues 510-524 of human GLUT2 (26), residues 478-492 of rabbit GLUT1 (27), residues 495-509 of rat GLUT4 (28), 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, p55α, p55γ, and p85β (13). The monoclonal anti-Ras antibody was also purchased from Transduction Laboratories.

Cell Culture

3T3-L1 fibroblasts were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% donor calf serum (GIBCO) in an atmosphere of 10% CO_2 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-methyl-xanthine, 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 (14).

Cloning and Construct

The cDNA fragment encoding amino acid residues 508-733 of mouse p85α was generated by the reverse transcription (RT)-polymerase chain reaction (PCR) based on the reported sequence (9) using mRNA obtained from MIN6, a mouse insulinoma cell line (29). Using the amplified fragment as a probe, a full-length p85α cDNA was isolated by screening

a cDNA library from MIN6 (30). This coding region of p85α was subcloned into the pBluescript vector (Stratagene) and its full nucleotide sequence was determined using an automatic sequencer (Applied Biosystems Inc.). Compared with the reported sequence (9), the codon CCC for 46Pro was replaced by CAG for 46Gln in the cloned p85α cDNA, presumably due to a polymorphism among the mouse strains, since 46Gln is conserved among several species including human and bovine (11).

To construct a dominant negative mutant of p85α that cannot bind to the p110 catalytic subunit, PCR was performed using p85α cDNA as a template to amplify a fragment containing nucleotides 994-1990 of p85α, nucleotide sequences for the hemagglutinin tag, and the NaeI site at its 3'-end. The PstI-DraI fragment (nucleotides 1033-2092) of p85α was replaced by the PstI-NaeI fragment of the PCR product, resulting in replacement of the binding site (amino acid residues 479-512) of p85α to the p110 catalytic subunit (31) with an HA tag sequence (YPYDVPDYA). Dominant negative Ras cDNA containing an asparagine substitution at position 17 (N17Ras) was kindly provided by Dr. Y. Takai.

RT-PCR was also performed to amplify cDNA of $p110\alpha^{PI3K}$ using the bovine brain RNA as a substrate and oligonucleotides based on its reported sequence (7) as primers. The amplified fragments were subcloned, and fragments without mutations were selected and ligated, yielding cDNA of $p110\alpha^{PI3K}$ covering the entire coding region. A portion of human GLUT2 cDNA corresponding to residues 510-524 was ligated to $p110\alpha^{PI3K}$ cDNA to encode p110α subunit of PI 3-kinase tagged with the epitope at its C-terminus.

Gene Transduction

The recombinant adenoviruses Adex1CAlacZ (32), Adex1CAΔp85α, Adex1CAN17Ras, and Adex1CAp110α, which encode Escherichia coli lacZ, Δp85α, N17Ras, and the epitope-tagged bovine p110α^{PI3K}, respectively, were constructed by homologous recombination between the expression cosmid cassette and the parental virus genome as described previously (33, 34). 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 (m.o.i.) of 200-300 pfu/cell, lacZ gene expression was observed in more than 90% of 3T3-L1 adipocytes on post-infection day 3 (data not shown). Infection with Adex1CAlacZ Adex1CAΔp85α, Adex1CAN17Ras, or Adex1CAp110α 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 post-infection day 3. In addition, 3T3-L1 adipocytes infected with Adex1CAlacZ exhibited no significant differences in glucose transport activity as compared with non-infected cells on post-infection day 3 (data not shown). Therefore, in the present study, recombinant adenoviruses were applied at an m.o.i. of approximately 200-300 pfu/cell and 3T3-L1 adipocytes infected with Adex1CAlacZ virus were used as a control.

Immunoblotting

To determine the expression of exogenous p110α^{PI3K}, and cellular expression levels of glucose transporter isoforms, 3T3-L1 adipocytes in a 12-well tissue culture dish were lysed and boiled in Laemmli buffer containing 10 mM dithiothreitol and subjected to SDS-polyacrylamide (7.5% for p85α and p110α of PI 3-kinase, 10% for glucose transporters, and 15% for Ras) gel electrophoresis and transferred onto nitrocellulose

filters. The immunoblots were visualized using anti-rabbit or anti-mouse immunoglobulin G coupled to horseradish peroxidase and the enhanced chemiluminescence detection kit (Amersham).

Mitogen-activated protein (MAP) kinase assay

Adipocytes were serum-deprived for 3 h prior to treatment with insulin for 5 min. The MAP kinase assay was performed as previously described by Waga et al. (35). The cells were washed, frozen at -70 °C and lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 2 mM dithiothreitol, and 100 mM NaCl), followed by partial purification using Q-Sepharose (Pharmacia). The MAP kinase activity was determined using a BIOTRAK MAP kinase enzyme assay system (Amersham).

PI 3-Kinase Assay

The cells were serum-starved, as described above, and solubilized in ice-cold lysis buffer containing 20 mM Tris pH 7.5, 137 mM NaCl, 1 mM $CaCl_2$, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and 100 μM sodium orthovanadate. Lysates were extracted by centrifugation at 15,000 x g for 10 min and incubated with anti-phosphotyrosine antibody or anti-p85α antibody. Immunocomplexes were precipitated with protein A-Sepharose (Pharmacia Biotech). PI 3-kinase activity was assayed in the immunoprecipitates as previously reported (12). PI 3-kinase activities were detemined in 0.1 ml of the reaction mixture consisting of 40 mM Tris, pH7.4, 0.5 mM $MgCl_2$, and 0.1 mM (1 μCi)[γ-^{32}P]ATP. The reaction was carried out at RT for 15 min and stopped by the addition of 20 μl of 8M HCl and 160 μl of $CHCl_3$/methanol (1:1). The organic phase was spotted on a silica gel plate (Merck, Silica Gel 60). The plate was developed in $CHCl_3$/methanol/25% NH_4OH/H_2O

(120:96:16:23.2). The dried plate was visualised and the results were quantitated using an image analyzer BAS2000 (Fujix).

Glucose Transport Assay

3T3-L1 adipocytes in a 12-well culture dish were serum-starved for 3 h in DMEM containing 0.2% bovine serum albumin, and glucose-free incubation was performed for 45 min in Krebs-Ringer phosphate (KRP) buffer (137 mM NaCl, 4.7 mM KCl, 10 mM sodium phosphate [pH 7.4], 0.5 mM $MgCl_2$, 1 mM $CaCl_2$) (14). Cells were then incubated with or without various concentrations of insulin for 15 min and the uptake of 2-deoxy-D-glucose was measured as previously described (27). Near confluent monolayers of 3T3-L1 cells in 12-well plates were incubated with 0.1 mM of 2-deoxy-D-[1,2-^{3}H]glucose (ICN) for 4 min at 37 ^{o}C. The reaction was terminated by the addition of ice-cold KRP buffer containing 0.3 mM phloretin. The cells were washed and solubilized with 0.1 % Triton X-100 and the radioactivity was measured.

Subcellular Fractionation

Fractionation of subcellular membranes from 3T3-L1 adipocytes was done essentially as described (36). Cells were scraped vigorously and were homogenized in 20 mM Hepes, 1 mM EDTA, 255 mM sucrose, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4 (termed HES buffer in this study) in a Potter-Elvehjem glass-Teflon type homogenizer at 4 ^{o}C. The homogenate was centrifuged at 19,000 g for 20 min, yielding a pellet designated as a high-density microsomal fraction (HDM). The supernatant from this spin was centrifuged at 180,000 g for 75 min, yielding a pellet designated as low density microsomes (LDM). The pellet obtained from the initial spin was layered onto 1.12 M sucrose in HES buffer and centrifuged at 100,000 g in a Beckman SW-41 rotor for 60 min. This yielded a white fluffy band at the interface [plasma membrane fraction (PM)] and a viscous brown pellet

[mitochondria/nuclei fraction]. Aliquots of subcellular membrane fractions containing equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted as described above.

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 (37). Cells were cultured on glass coverslips. After treatments with or without insulin, coverslips were washed in phosphate-buffered saline (PBS), followed by a 10-s treatment with 0.5 mg/ml polylysine in PBS. The cells were swollen by three 5-s incubation in a hypotonic buffer (1/3x buffer A), transferred to buffer A (70 mM KCl, 30 mM Hepes, 5 mM Mg Cl_2, 3 mM EGTA, pH 7.5), and immedeately broken open by placing under an ultrasonic microprobe (Kontes Co.). Adherent plasma membranes were fixed in 2% paraformaldehyde and processed for indirect immunofluorescence using anti-GLUT4 antiserum (1:100 dilution) followed by rhodamine-conjugated secondary antibodies.

RESULTS

1. Overexpression of Δp85α or N17Ras in 3T3-L1 Adipocytes

Expression of Δp85α or N17Ras in 3T3-L1 Adipocytes

Overexpression of Δp85α and N17Ras was achieved utilizing an adenovirus-mediated gene transduction system in 3T3-L1 adipocytes. Immunoblotting with antibodies against the regulatory subunits of PI 3-kinase (Fig. 1A) or Ras (Fig. 1B) revealed a large amount of exogeneously expressed Δp85α or N17Ras with the degradated products in 3T3-L1 adipocytes.

Effect of Δp85α or N17Ras Expression on PI 3-kinase activity in 3T3-L1 Adipocytes

To further confirm the functional expression of Δp85α, PI 3-kinase activity in the immunoprecipitate with tyrosine-phosphorylated protein was assayed. Control (lacZ-expressing), Δp85α-overexpressing, and N17Ras-expressing 3T3-L1 adipocytes were incubated with several concentrations of insulin for 15 min and the PI 3-kinase activity was measured. In control cells, insulin stimulated coimmunoprecipitation of PI 3-kinase activity with tyrosine-phosphorylated protein (maximum approximately 35-fold) (open circles in Fig. 2A). Essentially the same results were obtained in parental 3T3-L1 adipocytes (closed squares in Fig. 2A). Basal PI 3-kinase activity associated with tyrosine-phosphorylated protein was not changed by overexpression of N17Ras and insulin also stimulated coimmunoprecipitation of PI 3-kinase activity with tyrosine-phosphorylated protein in N17Ras-expressing 3T3-L1 adipocytes to an extent similar to that observed in control cells (closed circles in Fig. 2A). On the other hand, Δp85α-overexpression inhibited PI 3-kinase activity (by approximately 40%) in precipitates with the anti-phosphotyrosine antibody in the basal state. Furthermore, stimulation of

Fig. 1

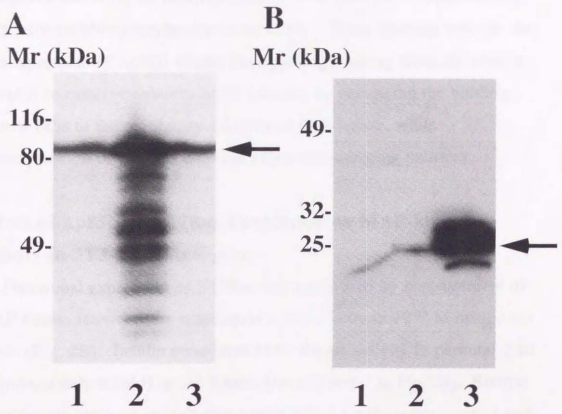

Fig. 1. Overexpression of Δp85α and N17Ras in 3T3-L1 adipocytes determined by immunoblot analysis with the antibodies against the regulatory subunits of PI 3-kinase (A) and Ras (B). Total cellular proteins were prepared from control (lane 1), Δp85α-expressing (lane 2), and N17Ras-expressing (lane 3) 3T3-L1 adipocytes, subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted.

PI 3-kinase activity by insulin was markedly inhibited and even in maximally stimulated state, only an approximately 2-fold increase above the basal value was observed (open squares in Fig. 2A), i. e., Δp85α expression inhibited the maximal insulin-stimulated PI 3-kinase activity with tyrosine-phosphorylated protein to 6%. These findings indicate that overexpression of Δp85α blocks the signaling pathway from the insulin receptor to catalytic subunits of PI 3-kinase by occupying the binding sites of IRSs to the regulatory subunits of PI 3-kinase, while overexpression of N17Ras does not affect this signaling pathway.

Effect of Δp85α or N17Ras Expression on MAP-kinase activity in 3T3-L1 Adipocytes

Functional expression of N17Ras was confirmed by measurement of MAP kinase activity after stimulation with or without 10^{-6} M insulin for 5 min (Fig. 2B). Insulin stimulated MAP kinase activity in parental cells (approximately 8-fold) in cell lysates (lanes 1 and 2 in Fig 2B). Similar results were obtained in lacZ-expressing 3T3-L1 adipocytes (lanes 3 and 4 in Fig 2B) and in Δp85α-overexpressing 3T3-L1 adipocytes (lanes 5 and 6 in Fig 2B). In marked contrast, only a slight elevation (1.2-fold) of MAP kinase activity was observed in lysates from N17Ras-expressing 3T3-L1 adipocytes (lanes 7 and 8 in Fig 2B). Thus, N17Ras expression exerted a dominant inhibitory effect on the insulin-stimulated Ras-MAP kinase cascade.

Effect of Δp85α or N17Ras Expression on Glucose Transport activity in 3T3-L1 Adipocytes

2-Deoxy-[^{3}H] glucose uptake in response to a 15-min incubation with several concentrations of insulin was measured in control, Δp85α-expressing, and N17Ras-expressing 3T3-L1 adipocytes. Insulin

Fig. 2

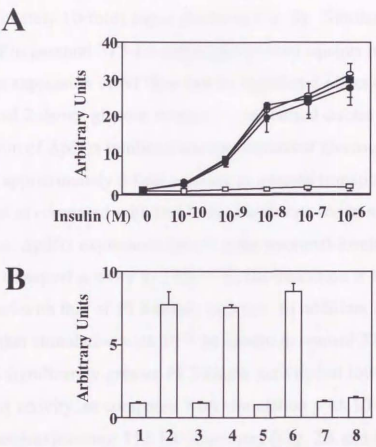

Fig. 2. Functional expression of Δp85α and N17Ras in 3T3-L1 adipocytes confirmed by PI 3-kinase activity assay (A), and of MAP kinase activity assay (B). A, lysates were prepared from parental (closed squares), lacZ-expressing (open circles), Δp85α-overexpressing (open squares), and N17Ras-expressing (closed circles) 3T3-L1 adipocytes after incubation with indicated concentrations of insulin for 15 min, and were immunoprecipitated with anti-phosphotyrosine antibody. PI 3-kinase activity was then assayed in the immunoprecipitates. Results were quantitated using an image analyzer. B, lysates were prepared from parental (lanes 1 and 2), lacZ-expressing (lanes 3 and 4), Δp85α-overexpressing (lanes 5 and 6), and N17Ras-expressing (lanes 7 and 8) 3T3-L1 adipocytes after incubation with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) 10^{-6}M insulin for 5 min, and partially purified using Q-Sepharose, followed by determination of the MAP kinase activity. The values are means ± S.D. for triplicate measurements in a representative experiment. Three other separate experiments yielded similar results.

stimulated 2-deoxy-glucose uptake in control 3T3-L1 adipocytes (approximately 10-fold) (open circles in Fig. 3). Similar results were obtained in parental 3T3-L1 adipocytes (closed squares in Fig. 3). Whereas expression of N17Ras had no significant effect on the insulin-stimulated 2-deoxy-glucose transport rate (closed circles in Fig. 3), expression of Δp85α inhibited insulin-stimulated glucose transport and only an approximately 3-fold increase in glucose transport activity was observed as compared with that in the basal state (open squares in Fig. 3). Thus, Δp85α expression inhibited the maximal insulin-stimulated glucose transport activity to 27%, i. e., the inhibition is less remarkable compared with that of PI 3-kinase activity. In addition, it is interesting to note that stimulation with 10^{-9} M insulin in control 3T3-L1 adipocytes induced significantly greater PI 3-kinase activity but lower glucose transport activity, as compared with stimulation with 10^{-6} M insulin in Δp85α-overexpressing 3T3-L1 adipocytes (Fig. 2A and Fig. 3). Thus, a discrepancy was observed between PI 3-kinase activity and glucose transport activity.

Analysis of Expressions of GLUT4 Glucose Transporter

I next evaluated the effect of expressing the dominant negative form of p85α or Ras on cellular expression levels of GLUT4 glucose transporter. As shown in Fig. 4A, no apparent differences in cellular expression levels of GLUT4 were observed among control, N17Ras-expressing, and Δp85α-expressing 3T3-L1 adipocytes.

Determination of Subcellular Distribution of Glucose Transporters

To address the effects of the expression of either Δp85α or N17Ras on the subcellular distribution of GLUT4 protein, immunoblot analyses of the plasma membrane (PM) fraction and the intracellular low-density

Fig. 3

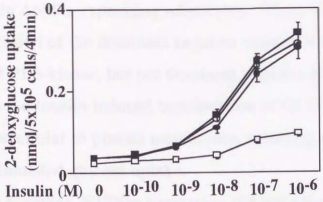

Fig. 3. Effect of Δp85α or N17Ras overexpression on 2-deoxy-D-glucose uptake in 3T3-L1 adipocytes. The uptake of 2-deoxy-D-glucose after incubation with indicated concentrations of insulin for 15 min was assayed in parental (closed squares), lacZ-expressing (open circles), Δp85α-overexpressing (open squares), and N17Ras-expressing (closed circles) 3T3-L1 adipocytes for 4 min. Values represent the means ± S. D.of three independent experiments, each performed in triplicate.

microsome (LDM) fraction were performed (Fig. 4B and 4C). As expected, insulin caused a 6-fold increase in the amount of GLUT4 in the PM fraction and a corresponding decrease in the LDM fraction of control adipocytes. Similar results were observed in N17Ras-expressing cells, whereas insulin-induced translocation of GLUT4 protein was markedly inhibited in Δp85α-expressing adipocytes. These findings demonstrate that expression of the dominant negative mutant of the p85α regulatory subunit of PI 3-kinase, but not dominant negative Ras, exerts inhibitory effects on the insulin-induced translocation of GLUT4 glucose transporter from intracellular to plasma membranes, resulting in inhibition of insulin-stimulated glucose uptake.

Taken together, N17Ras expression did not affect glucose transport activity, whereas Δp85α expression inhibited insulin-stimulated glucose transport with impairment of GLUT4 translocation, although inhibition of glucose transport activity is less remarkable than that of PI 3-kinase activity in Δp85α-expressing cells.

Fig. 4.

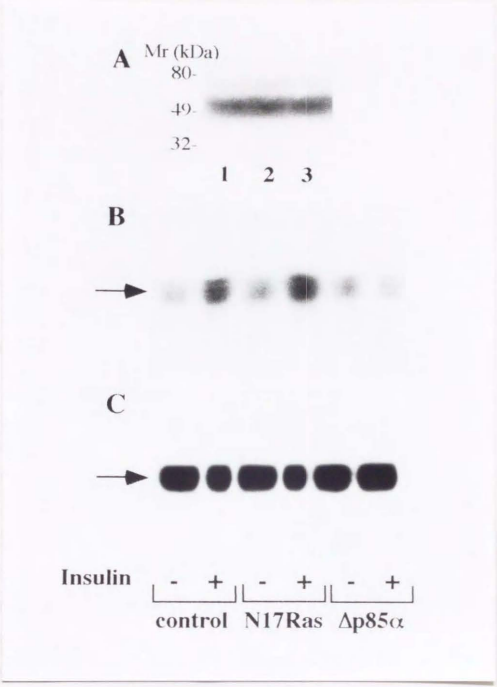

Fig. 4. Effects of Δp85a or N17Ras overexpression on cellular expression levels and subcellular distribution of GLUT4 glucose transporter protein in 3T3-L1 adipocytes. A, total cellular proteins prepared from control (lane 1), N17Ras-expressing (lane 2), and Δp85a-overexpressing (lane 3) 3T3-L1 adipocytes were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-GLUT4 antiserum. B and C, the PM (B) and LDM (C) fractions prepared from control (lanes 1 and 2), N17Ras-expressing (lanes 3 and 4), and Δp85a-overexpressing (lanes 5 and 6) 3T3-L1 adipocytes after incubation with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) 10^{-6} M insulin were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-GLUT4 antiserum.

2. Overexpression of p110α^{PI3K} in 3T3-L1 Adipocytes

Next, to investigate whether the activation of PI 3-kinase is sufficient for insulin-stimulated glucose transport, the epitope-tagged p110α subunit of PI 3-kinase was overexpressed in 3T3-L1 adipocytes.

Expression of p110α^{PI3K} in 3T3-L1 Adipocytes

As shown in Fig. 5, overexpression of p110α^{PI3K} was also achieved utilizing an adenovirus-mediated gene transduction system in 3T3-L1 adipocytes, as demonstrated by immunoblotting with the antibody against the tagged epitope.

Effect of p110α^{PI3K} Expression on PI 3-kinase activity in 3T3-L1 Adipocytes

Control (lacZ-expressing) and p110α^{PI3K}-overexpressing 3T3-L1 (p110α-L1) adipocytes were incubated with or without insulin (10^{-10}-10^{-6} M) 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 (max. approximately 35-fold) (Fig. 6A) and that with the regulatory subunits of PI 3-kinase (max. approximately 2-fold) (Fig. 6B) in a dose-dependent manner. Similar results were obtained in parental 3T3-L1 adipocytes. Overexpression of p110α^{PI3K} induced only a very modest elevation of PI 3-kinase activity in precipitates with the anti-phosphotyrosine antibody in the basal condition (Fig. 6A). Thus, a very small portion of exogenously expressed p110α^{PI3K} bound to the tyrosine-phosphorylated protein in the basal state. Insulin addition to p110α-L1 adipocytes markedly and dose-dependently increased the PI 3-kinase activity in the precipitates with the anti-phosphotyrosine antibody, and activity was significantly

Fig. 5

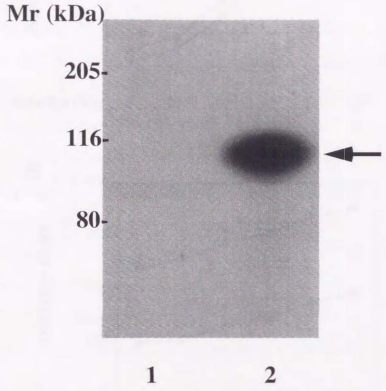

Fig. 5. Overexpression of p110α^{PI3K} in 3T3-L1 adipocytes determined by immunoblot analysis. Total cellular proteins were prepared from control (lane 1) and p110α^{PI3K}-overexpressing 3T3-L1 (lane 2) adipocytes, subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-tagged epitope antiserum.

Fig. 6

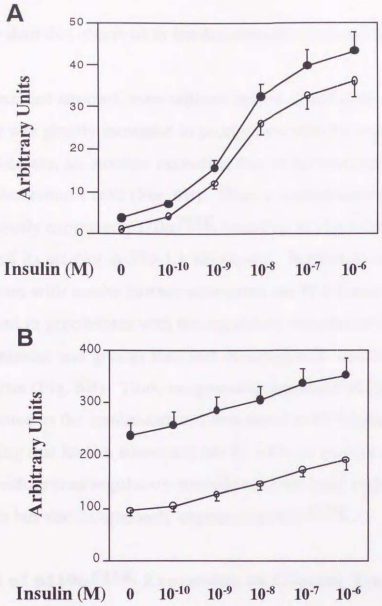

Fig. 6. PI 3-kinase activity associated with tyrosine-phosphorylated proteins (A) and regulatory subunits of PI 3-Kinase (B) in p110α^{PI3K} overexpressed 3T3-L1 adipocytes. Lysates were prepared from control (open circles) and p110α^{PI3K}-overexpressing 3T3-L1 adipocytes (closed circles) after incubation with the indicated concentrations of insulin, and were immunoprecipitated with anti-phosphotyrosine (A) or anti-regulatory subunits of PI 3-kinase (B). PI 3-kinase activity was then assayed in the immunoprecipitates. The dried thin layer chromatography plates were visualized for the radioactivities. Results were quantitated using an image analyzer. The values are means ± S.D. for triplicate measurements in a representative experiment. Three other separate experiments yielded similar results.

greater than that observed in insulin-stimulated control adipocytes (Fig. 6A).

In marked contrast, even without insulin stimulation, PI 3-kinase activity was greatly increased in precipitates with the regulatory subunits of PI 3-kinase, an increase exceeding that of the maximally insulin-stimulated control cells (Fig. 6B). Thus, a considerable portion of exogenously expressed p110α^{PI3K} bound to its regulatory subunits and exhibited its activity in 3T3-L1 adipocytes. Incubation of p110α-L1 adipocytes with insulin further stimulated the PI 3-kinase activity measured in precipitates with the regulatory subunits of PI 3-kinase, and the increment was greater than that observed with insulin in control adipocytes (Fig. 6B). Thus, exogenously expressed p110α^{PI3K} contributed to the insulin-induced increment in PI 3-kinase activity, indicating that insulin stimulated the PI 3-kinase activity in the complex of the endogenous regulatory subunits with not only endogenous catalytic subunits but also exogenously expressed p110α^{PI3K}.

Effect of p110α^{PI3K} Expression on Glucose Transport in 3T3-L1 Adipocytes

2-Deoxy-[^{3}H]glucose uptake in response to a 15-min incubation with various concentrations (10^{-10}-10^{-6} M) of insulin was measured in control and p110α-L1 adipocytes. Insulin dose-dependently stimulated 2-deoxy-glucose uptake in control 3T3-L1 adipocytes (Fig. 7) and the maximally stimulated values were similar (approximately 12-fold) in control 3T3-L1 adipocytes. Overexpression of p110α^{PI3K} induced a marked increase (approximately 14-fold) in the basal glucose transport rate. Insulin further stimulated the glucose uptake in p110α-L1 in a dose dependent manner (max. approximately 1.4-fold) (Fig. 7).

Fig. 7

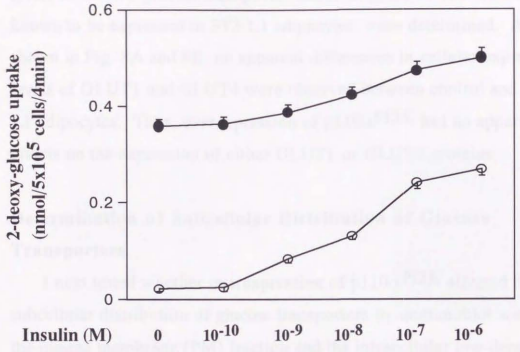

Fig. 7. Effect of p110α^{PI3K} overexpression on 2-deoxy-D-glucose uptake in 3T3-L1 adipocytes. The uptake of 2-deoxy-D-glucose after incubation with the indicated concentrations of insulin was assayed in control (lacZ-expressing) (open circles) and p110α^{PI3K}-overexpressing (closed circles) 3T3-L1 adipocytes for 4 min. Values represent the means ± S. D. in three independent experiments, each performed in triplicate.

Analysis of Expressions of Glucose Transporter Isoforms

To begin addressing the mechanism whereby overexpression of $p110\alpha^{PI3K}$ 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. 8A and 8B, no apparent differences in cellular expression levels of GLUT1 and GLUT4 were observed between control and p110α-L1 adipocytes. Thus, overexpression of $p110\alpha^{PI3K}$ had no apparent effects on the expression of either GLUT1 or GLUT4 proteins.

Determination of Subcellular Distribution of Glucose Transporters

I next tested whether overexpression of $p110\alpha^{PI3K}$ 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. 9A and 9B). As expected, 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 protein were observed in the LDM fraction of these cells. Strikingly, $p110\alpha^{PI3K}$ overexpression induced translocation of GLUT1 and GLUT4 glucose transporter proteins 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 proteins.

I further confirmed the translocation of GLUT4 protein to the plasma membrane using the membrane sheet assay method. Fig. 8 demonstrates cell surface GLUT4 expression in control and p110α-L1 adipocytes treated in the absence or presence of insulin (10^{-7} M) for 15 min. In

Fig. 8

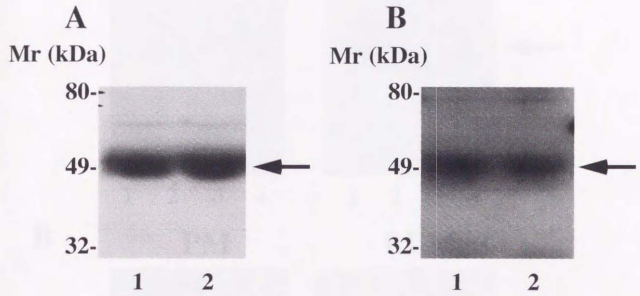

Fig. 8. Effect of p110α^{PI3K} overexpression on cellular expression levels of glucose transporter isoforms in 3T3-L1 adipocytes. Total cellular proteins prepared from control (lane 1) and p110α^{PI3K} -overexpressing (lane 2) 3T3-L1 adipocytes were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-GLUT1 (A) or anti-GLUT4 (B) antiserum.

Fig. 9

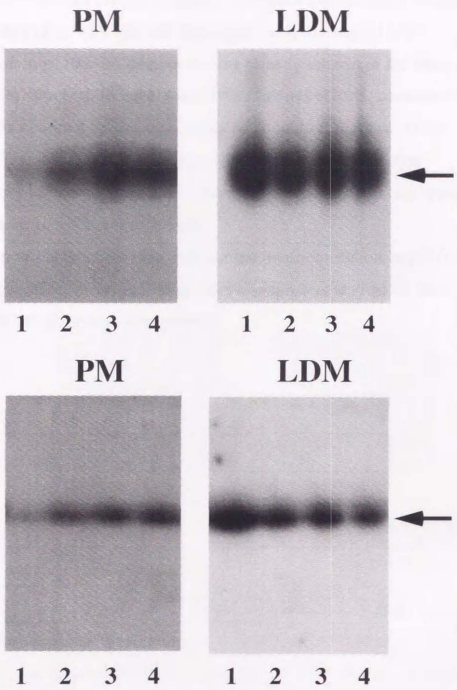

Fig. 9. Effect of p110 $p110\alpha^{PI3K}$ overexpression on subcellular distribution of glucose transporter isoforms in 3T3-L1 adipocytes. The PM and LDM fractions prepared from lacZ-expressing (control) (lanes 1 and 2) and $p110\alpha^{PI3K}$-overexpressing (p110α-L1) (lanes 3 and 4) 3T3-L1 adipocytes after incubation with (lanes 2 and 4) or without (lanes 1 and 3) 10^{-6}M insulin were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-GLUT1 (A) or anti-GLUT4 (B) antiserum.

control cells, very little GLUT4 staining was observed on the plasma membrane, whereas insulin treatment increased surface GLUT4 staining substantially (Fig. 10). 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 $p110\alpha^{PI3K}$ exerts stimulatory effects on the translocation of glucose transporters from intracellular to plasma membranes.

Taken together, these data indicate that overexpression of $p110\alpha^{PI3K}$ mimics the effect of insulin on glucose transport as well as on the translocation of glucose transporters.

Fig. 10.

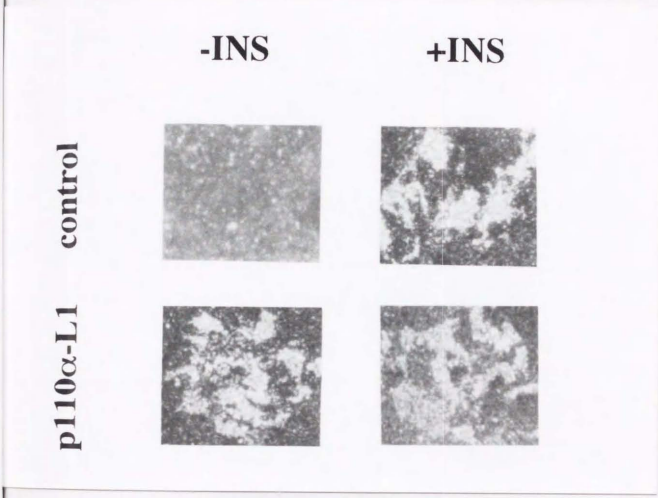

Fig. 10. Immunofluorescence of GLUT4 in plasma membrane sheets after stimulation with insulin. LacZ-expressing (control) and p110α^{PI3K}-overexpressing (p110α-L1) 3T3-L1 adipocytes were treated in the absence (-INS) or presence of 10^{-6}M of insulin for 15 min (+INS). Plasma membrane sheets were prepared by sonication and subjected to immunofluorescence using anti-GLUT4 antiserum.

DISCUSSION

Expression of the dominant negative mutant of the p85α regulatory subunit of PI 3-kinase, but not dominant negative Ras, exerted inhibitory effects on the translocation of glucose transporters from intracellular to plasma membranes, resulting in inhibition of insulin-stimulated glucose uptake. These findings indicates that the tyrosine-phosphorylated IRSs-heterodimeric PI 3-kinase complex plays a pivotal role in the signaling pathway from insulin receptor through the translocation of glucose transporters. Cross-talk between Ras-mediated and PI 3-kinase-mediated signaling pathways was recently reported. Several growth factors were shown to stimulate Ras-mediated activation of PI 3-kinase in PC12 and COS cells, presumably due to direct binding between Ras-GTP and a catalytic subunit of PI 3-kinase (18). On the contrary, blocking PI 3-kinase recruitment to the plasma membrane in response to PDGF treatment prevented Ras activation in some cell types (20, 21). In addition, expression of a constitutively active mutant of the catalytic subunit of PI 3-kinase induced Ras-dependent cellular responses in NIH 3T3 cells and *Xenopus laevis* oocytes (19). Thus, it is possible that PI 3-kinase lies both upstream and downstream of Ras in the signaling pathway. However, the results obtained in the present study indicate that the Ras-mediated pathway, including the Ras-MAP kinase cascade and Ras-PI 3-kinase cross-talk, is not involved in either the translocation or intrinsic activity of glucose transporters in adipocytes. However, several investigators have reported that the stable (38) or transient (39) expression or microinjection (40) of the activated mutants of Ras induces the translocation of GLUT4 glucose transporters to the plasma membranes. In the present study, expression of the dominant negative Ras did not alter either the insulin-stimulated glucose transport activity

or GLUT4 translocation, suggesting that overexpression of the activated Ras induced GLUT4 translocation by a different mechanism which is not involved in physiological activation of glucose transport by insulin.

Several studies have recently been reported on blocking of the signaling pathway from insulin receptor to PI 3-kinase using the dominant negative mutant of the p85α regulatory subunit of PI 3-kinase. The stable overexpression of Δp85α protein in a Chinese hamster ovary cell line inhibited the insulin-stimulated glucose uptake which occurs with translocation of GLUT1 glucose transporters (41). In addition, microinjection of glutathione S-transferase-p85α subunit fusion protein (16) or Δp85α (17) inhibited the GLUT4 translocation induced by insulin in 3T3-L1 adipocytes. The transient overexpression of Δp85α inhibited insulin-stimulated translocation of co-expressed GLUT4 in 3T3-L1 adipocytes (39). However, microinjection study or the transient co-transfection study does not reveal the effects of induction of the dominant negative protein on either PI 3-kinase activity or glucose transport activity. In the present study, an adenovirus-mediated gene transduction system enables us to investigate the effects of Δp85α overexpression on glucose transport activity as well as phosphotyrosine-associated PI 3-kinase activity in 3T3-L1 adipocytes. Overexpression of Δp85α markedly inhibited insulin-stimulated PI 3-kinase activity, whereas the inhibition of insulin-stimulated glucose transport activity was less remarkable than that of PI 3-kinase activity and GLUT4 translocation. A discrepancy between PI 3-kinase activity and glucose transport activity was also observed between control cells stimulated with 10^{-9} M insulin and Δp85α-expressing cells stimulated maximally with insulin. Furthermore, in p110α^{PI3K} overexpressing 3T3-L1 cells, insulin produced no apparent increase in the amount of glucose transporter protein in the PM fraction, despite further stimulating glucose transport

activity in these cells. These observations suggest the possible presence of signaling pathway(s) to glucose transport activity, in addition to those occurring via PI 3-kinase activation, involved in increasing in the intrinsic activity of glucose transporters. In addition, it was reported that 100 nM of wortmannin almost completely inhibited insulin-stimulated glucose transport activity in rat adipocytes (15). I also observed that the same concentration of wortmannin exerted a further inhibition on the insulin-stimulated glucose transport activity in Δp85α-expressing 3T3-L1 adipocytes (data not shown). Thus, the possible pathway(s) may be also wortmannin-sensitive.

In addition, overexpression of $p110\alpha^{PI3K}$ induced greater glucose transport activity than that observed in maximally insulin-stimulated control cells. A slightly larger amount (1.3-fold) of GLUT1 glucose transporter protein was observed in the PM fraction of p110α-L1 cells as compared with that in insulin-stimulated control cells. However, it may be difficult that the small difference in the amount of GLUT1 protein in the PM fraction can entirely explain the difference in glucose transport activity between basal p110α-L1 cells and insulin-stimulated control cells. These findings suggest that a further increase in PI 3-kinase activity also stimulates the intrinsic activity of glucose transporters. Mechanisms whereby insulin stimulates the intrinsic activity of glucose transporters in addition to their translocation is a key point which remains to be clarified.

In this study, overexpression of $p110\alpha^{PI3K}$ 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. Treatment of 3T3-L1 adipocytes with PDGF caused no significant stimulation of

glucose transport activity, despite PDGF increasing PI 3-kinase activity to a level approaching that elicited by insulin (23, 24). I obtained similar results in PDGF-treated 3T3-L1 adipocytes (data not shown). In addition, introduction of a thiophosphotyrosine 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 (42). 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 thiophosphotyrosine peptide, including the possibility that insulin activates PI 3-kinase in different subcellular locations than do the peptide and PDGF. A previous study (43) showed that activated PI 3-kinase has the same intracellular location as tyrosine-phosphorylated IRS1 in insulin-stimulated adipocytes, whereas the thiophosphotyrosine 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 (43). 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 (44). 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 fractionated on a sucrose-gradient (data not shown). Subcellular redistribution of PI 3-kinase activity may be the key determinant in signal generation.

The increase in PI-3 kinase activity induced by overexpressing $p110\alpha^{PI3K}$ is due to an increased amount of the protein, whereas insulin stimulates enzyme activity. In this respect, the increased activity resulting from overexpression is quantitative, rather than qualitative, and thus, might be nonphysiological. Overexpression of ras (38) or raf-1 (45) also increased the glucose transport activity. However, levels of total GLUT4 and GLUT1 expression were changed in stable cell lines of 3T3-L1 adipocytes overexpressing ras and raf-1, respectively. In contrast, in the present study, transient overexpression of $p110\alpha^{PI3K}$ in 3T3-L1 adipocytes exerted essentially no effect on the total expression level while having a marked effect on the subcellular distribution of glucose transporters, which is qualitatively similar to the physiological effect of insulin.

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 (46) and the small G-protein rac (47). However, rac does not couple PI 3-kinase to insulin-stimulated glucose transport in 3T3-L1 adipocytes (48). Other as yet unknown pathway(s) may be involved in insulin-stimulated glucose transport. p110-L1 adipocytes may serve as a model for studying the steps downstream from PI 3-kinase.

CONCLUSION

1. The Ras-mediated pathway, including the Ras-MAP kinase cascade and Ras-PI 3-kinase cross-talk, is not involved in either the translocation or intrinsic activity of glucose transporters in adipocytes.
2. The tyrosine-phosphorylated IRSs-heterodimeric PI 3-kinase complex plays a pivotal role in the signaling pathway from insulin receptor through the translocation of glucose transporters.
3. The possibility was suggested that a different signaling pathway(s) is involved in increasing in the intrinsic activity of glucose transporters in addition to those occurring via PI 3-kinase activation.
4. Overexpression of $p110\alpha^{PI3K}$ 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, i. e. mimics the effect of insulin. Subcellular redistribution of PI 3-kinase activity may be the key determinant in signal generation.

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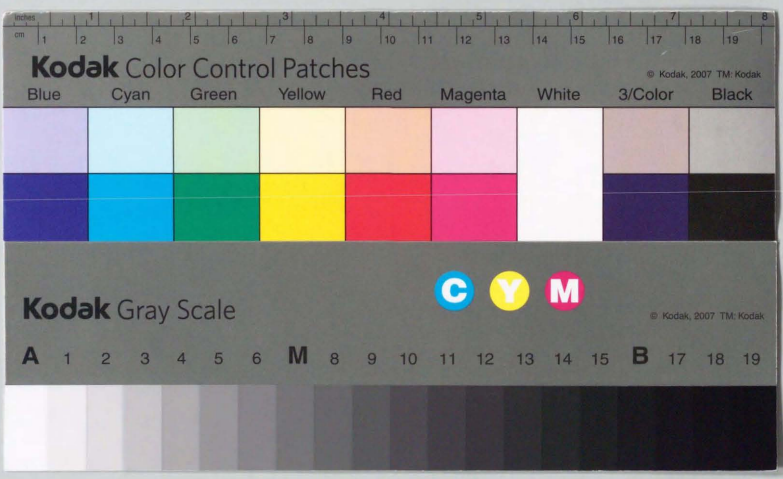
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