Insulin-mediated Targeting of Phosphatidylinositol 3-Kinase to GLUT4-containing Vesicles*

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Phosphatidylinositol (PI) 3-kinase is hypothesized to be a signaling element in the acute redistribution of intracellular GLUT4 glucose transporters to the plasma membrane in response to insulin. However, some receptors activate PI 3-kinase without causing GLUT4 translocation, suggesting specific cellular localization may be critical to this PI 3-kinase function. Consistent with this idea, complexes containing PI 3-kinase bound to insulin receptor substrate 1 (IRS-1) in 3T3-L1 adipocytes are associated with intracellular membranes (Heller-Harrison, R., Morin, M. and Czech, M. (1995) J. Biol. Chem. 270, 24442–24450). We report here that in response to insulin, activated complexes of IRS-1-PI 3-kinase can be immunoprecipitated with anti-IRS-1 antibody from detergent extracts of immobilized GLUT4-containing vesicles prepared from 3T3-L1 adipocytes. The targeting of PI 3-kinase to rat adipocyte GLUT4-containing vesicles using vesicles prepared by sucrose velocity gradient ultracentrifugation was also demonstrated. Insulin treatment caused a 2.3-fold increase in immunoreactive p85 protein in these GLUT4-containing vesicles while anti-p85 immunoprecipitates of PI 3-kinase activity in GLUT4-containing vesicle extracts increased to a similar extent. HPLC analysis of the GLUT4 vesicle-associated PI 3-kinase activity showed insulin-mediated increases in PI 3-P, PI 3,4-P2, and PI 3,4,5-P3 when PI, PI 4-P, and PI 4,5-P2 were used as substrates. Our data demonstrate that insulin directs the association of PI 3-kinase with GLUT4-containing vesicles in 3T3-L1 and rat adipocytes, consistent with the hypothesis that PI 3-kinase is involved in the insulin-regulated movement of GLUT4 to the plasma membrane.

One of the major physiological effects of insulin is its ability to stimulate glucose uptake in adipose tissue and muscle (1, 2). The acute stimulation of glucose transport rates in response to insulin is associated with the rapid translocation of GLUT4 transporters to the cell surface from an intracellular location, most likely within the tubulovesicular endosomal membrane system (for reviews see Refs. 3 and 4). Rapid appearance of GLUT4 on the cell surface in response to insulin has been well documented utilizing a variety of techniques including biochemical cell fractionation (1, 2), photoaffinity labeling (5–7), tryptic cleavage (8), immunoelectron microscopy (9, 10), and antibody binding assays of expressed epitope-tagged transporters (11, 12). However, it is still not known how early events initiated by insulin binding to its cell surface receptor lead to increased glucose uptake.

One early step in the insulin signaling pathway is the activation of PI 3-kinase secondary to the phosphorylation of IRS-1 on tyrosine residues (for reviews see Refs. 13–16). Phosphorylation of tyrosine residues specifically found within multiple YXXM motifs on IRS-1 have been demonstrated to bind Src homology 2 (SH2) domains present on the p85 regulatory subunit of PI 3-kinase following insulin stimulation (17–19). The binding of p85 to IRS-1 activates the p110 catalytic subunit of PI 3-kinase which catalyzes the phosphorylation of phosphoinositides at the D3 position of the inositol ring (17, 20). A key role for 3'-phosphoinositides in membrane trafficking is suggested by studies on yeast PI3-kinase, denoted VPS34, which is required for the proper delivery of soluble hydrolases to the yeast vacuole (21). Similarly, mutant platelet-derived growth factor and colony stimulating factor receptors lacking binding sites for PI 3-kinase are defective in their sorting to a degradative pathway (22, 23). That PI 3-kinase may also participate in insulin-dependent membrane trafficking of GLUT4 is suggested by the demonstration that inhibitors of PI 3-kinase activity abolish insulin-mediated translocation of GLUT4 to the plasma membrane in both 3T3-L1 and rat adipocytes (24, 25). However, activation of PI 3-kinase by insulin cannot fully account for the effects of the enzyme on glucose transport since other receptor signaling systems activate PI 3-kinase activity to the same extent without causing translocation of GLUT4 (26). A critical aspect of this function of PI 3-kinase may therefore be its directed localization to the specialized intracellular membranes that sequester GLUT4.

The above considerations have led us to hypothesize that insulin receptor activation causes the rapid delivery of IRS-1-PI 3-kinase complexes to intracellular GLUT4-enriched membranes where 3'-phosphoinositides may accumulate and function in the membrane-associated events which accompany the translocation of GLUT4 to the cell surface. The high concentration of tyrosine-phosphorylated IRS-1 present in intracellular low density microsomes (27, 28) and our recent finding that membrane association of IRS-1 can be regulated by insulin in 3T3-L1 adipocytes (29) reinforce this hypothesis. Two separate studies have reported that GLUT4-containing vesicles prepared from rat adipocytes do not contain PI 3-kinase, IRS-1, or insulin receptors (27, 30). However, the insulin stimulations in these studies were for 10 min or longer. In the present studies, we tested the possibility that rapid association of activated

1 The abbreviations used are: PI, phosphatidylinositol; IRS-1, insulin receptor substrate 1; SH, Src homology; BCA, bicinchoninic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.
IRS-1-PI 3-kinase complexes with the GLUT4-containing vesicles might occur. Our data demonstrate that insulin-activated PI 3-kinase indeed specifically associates with GLUT4-containing vesicles of both 3T3-L1 adipocytes and rat adipocytes. These results are consistent with the hypothesis that PI 3-kinase and its reaction products play a functional role in the movement of intracellular GLUT4 to the cell surface in response to insulin.

EXPERIMENTAL PROCEDURES

Materials—Rabbit anti-p85 PI 3-kinase for both immunoblotting and immunoprecipitation was purchased from UBI and was used according to manufacturer’s specifications. Horseradish peroxidase-conjugated anti-rabbit antibody was purchased from Boehringer Mannheim. Non-immune rabbit IgG and Protein A-Sepharose were from Sigma. [γ-32P]ATP, [32P]-Protein A, [3H]P PI 4-P, and [3H]PI 4,5-P2, and Renaissance enhanced chemiluminescence reagent were purchased from DuPont NEN. Bicinchoninic acid (BCA) protein determination kit was from Pierce. The rabbit COOH-terminal peptide anti-GLUT4 antisera and the rabbit anti-IRS-1 antisera were described previously (29, 31). Both were utilized as an IgG fraction prepared by Protein A-Sepharose chromatography.

Preparation of Cell Fractions—Differentiated 3T3-L1 adipocytes were grown in 15-cm2 dishes and were serum-starved for 12–18 h in Dulbecco’s modified Eagle’s medium supplemented with 0.5% bovine serum albumin. Cells were then stimulated with the indicated concentrations of insulin for the indicated times. The insulin containing medium was aspirated, and cells monolayers were washed once with warm Bubonic 100 mM Tris-HCl, pH 7.5, 1 mM EDTA, 255 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 100 μM Na3VO4, 1 mM Na2VO4, 1 mM NaPiP, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. Cells were prepared using previously described methods (31). The final plasma membrane pellet was resuspended in ice cold Buffer A to a concentration of approximately 1–2 mg/ml. The final low density microsome pellet was resuspended in Buffer B (50 mM Hepes, 1 mM EDTA, 150 mM NaCl, 0.5 mM EGTA, 10 mM MgCl2, PI, PI4-P, PI, PI4,5-P2, 100 μM Na2VO4, 1 mM NaPiP, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) to a concentration of approximately 1–2 mg/ml. Protein was quantitated using the BCA protein determination kit with bovine serum albumin as standard.

Immunoadsorption of Glucose Transporter Vesicles—Vesicles carrying GLUT4 were specifically adsorbed from the low density microsomes using an IgG fraction prepared from rabbit polyclonal antiserum generated against a COOH-terminal peptide of GLUT4 (31). 100–200 μg of low density microsomes were preadsorbed with 20 μl of packed Protein A-Sepharose in Buffer B for 12 h. Samples were centrifuged for 3 s at 16,000 × g and supernatants were transferred to fresh tubes. Anti-GLUT4 IgG was incubated with 20 μl of packed Protein A-Sepharose for 12 h, the beads were washed with Buffer B, and the antibody-bound beads were then added to the preadsorbed low density microsomes and allowed to incubate for 24 h. Samples were centrifuged for 3 s at 16,000 × g, the supernatants were transferred to fresh tubes, and the immunoadsorbed vesicles were washed several times with Buffer B. Immunoadsorbed vesicles were then analyzed by SDS-PAGE (polyacrylamide gel electrophoresis) and immunoblotting.

In experiments designed to demonstrate the specificity of the immunoadsorption of GLUT4-containing vesicles (Fig. 2), 1 μl of low density microsome COOH-terminal peptide was included in Buffer B during the incubation of anti-GLUT4 IgG with Protein A-Sepharose. The beads were washed with Buffer B, and then antibody-bound beads in Buffer B containing 1 mM MgCl2 were added to the preadsorbed low density microsomes and allowed to incubate for 24 h. The supernatants were collected and subjected to immunoblot analysis. The beads were washed in Buffer B, and immunoadsorbed vesicles were solubilized in Buffer B containing 1% Nonidet P-40. The detergent-solubilized vesicles were then immunoprecipitated with anti-IRS-1 and subjected to a PI 3-kinase assay as described (32) using exogenous PI as substrate followed by thin layer chromatography (33).

Preparation of GLUT4-enriched Vesicles by Sucrose Velocity Gradient Centrifugation—GLUT4-enriched vesicles were prepared from rat adipocyte low density microsomes utilizing the sedimentation velocity gradient centrifugation method exactly as described (34). The resultant vesicles were either subjected to immunoblot analysis with anti-p85 PI 3-kinase or were solubilized in Buffer B containing 1% Nonidet P-40, immunoprecipitated with anti-p85 PI 3-kinase, and subjected to a PI 3-kinase assay as described above.

3T3-L1 adipocytes were stimulated with insulin for times ranging from 1–15 min. Immunoblot analysis of low density microsome fractions and plasma membrane fractions prepared from these cells using anti-GLUT4 (Fig. 1A) demonstrates that GLUT4 decreases in the low density microsome fraction and increases in the plasma membrane fraction in a time-dependent manner, as previously published (36). A slight increase in GLUT4 (~30%) can be consistently detected in the plasma membrane fraction as early as 1 min, and further increases are observed at later time points, as also reported for rat adipocytes (28).

GLUT4-containing vesicles were prepared from basal and insulin-stimulated 3T3-L1 adipocytes by immunoadsorption from the low density microsome fraction using anti-GLUT4 IgG. Results presented in Fig. 1B demonstrate that this method of preparing GLUT4-containing vesicles is highly specific in that no detectable GLUT4 protein is observed in the immunoprecipitates when nonimmune IgG is utilized to immunoadsorb vesicles instead of anti-GLUT4 IgG. GLUT4 protein is recovered in the supernatants from the nonimmune IgG immunoadsorption reactions while there is little detectable
GLUT4 protein in the anti-GLUT4 supernatants (data not shown). Approximately 85% of the GLUT4-containing vesicles are immunoadsorbed with our anti-GLUT4 IgG following the initial clearing of low density microsomes with Protein A-Sepharose alone. Also shown in Fig. 1B is the decrease in GLUT4-containing vesicles in the low density microsome fractions with prolonged exposure to insulin.

In order to determine whether activated complexes of IRS-1-Pi 3-kinase are associated with GLUT4-containing vesicles, anti-GLUT4 immunoadsorbed vesicles were solubilized in detergent-containing buffer, immunoprecipitated with anti-IRS-1 IgG, and the immunoprecipitates were subjected to a PI 3-kinase assay as described under “Experimental Procedures.” B, spots corresponding to PI 3-P on the thin layer plate were quantitated using a Betascope. The data presented are the average values from three independent experiments. C, the supernatants from the immunoadsorption of low density microsomes with anti-GLUT4 IgG bound to Protein A-Sepharose in the presence (+) or absence (-) of 1 mM GLUT4 COOH-terminal peptide were collected, and equal amounts of protein (25 μg) were resolved by SDS-PAGE on a 10% gel and electrophoretically transferred to nitrocellulose for 2 h at 200 mA. The filter was blocked and subsequently incubated with anti-GLUT4 followed by 125I-Protein A. Also shown are the low density microsome fractions (25 μg/lane) for this experiment.

GLUT4-containing vesicles isolated from rat adipocytes by sucrose velocity gradient ultracentrifugation. Low density microsome fraction (1.5 mg of protein) from basal and insulin-treated (3.5 min) rat adipocytes were centrifuged in a 28-ml 10–35% sucrose gradient as described (34). Insulin shows immunoblot analysis of fractions using anti-GLUT4 followed by horseradish peroxidase-anti-rabbit. Graph shows the overall distribution of GLUT4 in basal (closed triangles) and insulin-treated adipocytes (open triangles) and total protein for basal adipocytes (closed circles).

In all three experiments, the insulin effect on PI 3-kinase activity was 2.2-fold or greater relative to basal activity. In order to confirm the effectiveness of this peptide in blocking immunoadsorption of GLUT4-containing vesicles, equal amounts of the supernatants from the immunoadsorption of low density microsome fractions with anti-GLUT4 IgG in the absence or presence of COOH-terminal peptide were immunoblotted for GLUT4. Fig. 2C shows that GLUT4 peptide significantly inhibits immunoadsorption of the GLUT4-containing vesicles by the anti-GLUT4 IgG. The peptide-mediated decreases in PI 3-kinase activity (Fig. 2B) and immunoadsorption of GLUT4-containing vesicles as measured by densitometric scanning of the GLUT4 protein bands (Fig. 2C) were 63% and 68%, respectively. These results demonstrate that the association of IRS-1-PI 3-kinase complexes with GLUT4-containing vesicles is highly specific.

We next tested whether an insulin-mediated stimulation of PI 3-kinase activity also occurs in GLUT4-containing vesicles from rat adipocytes. GLUT4-containing vesicles from freshly isolated rat adipocytes were prepared by fractionating low density microsomes in a sucrose velocity gradient, a procedure recently published for the purification of GLUT4-containing vesicles (34, 37). As observed by Kandror et al. (34) and as depicted in Fig. 3, GLUT4-containing vesicles from rat adipocytes show a distinct sedimentation distribution and sediment faster than most structures present in the low density microsome fraction. Insulin treatment of rat adipocytes (3.5 min) caused a demonstrable reduction in the amount of GLUT4-containing vesicles in the low density microsome fraction, presumably due to their translocation and fusion with the plasma membrane, as well as a reproducible shift (seen inset of Fig. 3) in the sedimentation of GLUT4-containing vesicles when compared with vesicles from control cells.

As shown in panel A of Fig. 4, insulin treatment (3.5 min) caused a 1.6-fold increase in immunoreactive p85 protein in the low density microsome fraction while a 2.3-fold increase in the level of p85 was observed in the GLUT4-containing vesicles. PI 3-kinase activities measured in anti-p85 immunoprecipitates of the low density microsome fraction and GLUT4-containing vesicles were increased to corresponding levels (Fig. 4B). However, only a fraction of the total PI 3-kinase activity present in the low density microsome fraction was in the GLUT4-containing vesicle preparation since equal amounts of protein were used in
the assays (Fig. 4B). The PI 3-kinase activity associated with both the low density microsome fraction and the GLUT4-containing vesicles was abolished by 100 nM wortmannin (data not shown). Similar results were obtained by preparing GLUT4-containing vesicles from unstimulated or insulin-stimulated (3.5 min) rat adipocytes by sucrose velocity gradient ultracentrifugation. A, protein (25 μg) from each preparation was resolved by SDS-PAGE on a 7.5% gel and was electrophoretically transferred to nitrocellulose for 8 h at 150 mA. The filter was blocked, incubated with anti-p85 PI 3-kinase and then horseradish peroxidase-anti-rabbit followed by detection by chemiluminescence. Bands corresponding to p85 were quantitated using a scanning densitometer. B, GLUT4-containing vesicles (25 μg) were solubilized, immunoprecipitated with anti-p85 PI 3-kinase, and the immunoprecipitates were then subjected to PI 3-kinase assay as described under “Experimental Procedures.” Spots corresponding to PI 3-P on the thin layer plate were quantitated using a Betascope. The data presented for A and B are the average values from three independent experiments ± S.E.

GLUT4-containing vesicles prepared from control and insulin-stimulated rat adipocytes were also assayed directly for PI 3-kinase activity using PI, PI 4-P, and PI 4,5-P2 as substrates (Fig. 5). The HPLC profiles of the deacylated polyphosphoinositides from the assay reaction demonstrate insulin-stimulated increases of approximately 6-fold, 3-fold, and 3.3-fold in PI 3-P, PI 3,4-P2, and PI 3,4,5-P3, respectively (Fig. 5C). The presence of 100 nM wortmannin in the assay completely abolished the insulin-stimulated increases in polyphosphoinositides associated with the GLUT4-containing vesicles (Fig. 5B). Large amounts of PI 4-P, which were not stimulated by insulin treatment, were also generated confirming PI 4-kinase is present in the GLUT4-containing vesicles (30). In addition, when GLUT4-containing vesicles prepared by the sucrose velocity gradient procedure were immunoadsorbed with anti-GLUT4 and then assayed for PI 3-kinase activity, HPLC analysis demonstrated a 2-fold increase in insulin-stimulated PI 3-P (data not shown).

The association of activated PI 3-kinase with intracellular membranes enriched in GLUT4 reported here combined with other data presented in this study and our previous work (29) suggests the following hypothesis: membrane-bound IRS-1 becomes tyrosine-phosphorylated upon insulin stimulation, associates with PI 3-kinase, and is internalized on endosomal vesicles. A population of these IRS-1-PI 3-kinase complexes are delivered to the intracellular storage pool of GLUT4-containing vesicles. The products of PI 3-kinase catalytic action in GLUT4-containing vesicles cause budding, fusion, or movement of these membranes which regulates GLUT4 translocation to the plasma membrane. The data in this study do not exclude the possibility that other mechanisms are involved in mediating increased PI 3-kinase activity in GLUT4-containing membranes in response to insulin nor do they address the mechanism by which PI 3-kinase and its 3-phosphoinositide products trigger GLUT4 translocation, an important question for future investigation. However, a conceptual framework for the hypothesized activation of ADP-ribosylation factor-regulated vesicular fusion events by phosphoinositides is currently being developed (38, 39).

Recent data obtained from other laboratories also support the hypothesis that activated PI 3-kinase is involved in regulating GLUT4 trafficking in adipocytes. For example, IRS-1 antisense constructs introduced into rat adipocytes transfected with epitope-tagged GLUT4 decreased the sensitivity to insulin of GLUT4 translocation to the cell surface (40). When IRS-1 was co-expressed in these cells, this effect was abolished. The specific insulin-mediated localization of PI 3-kinase to the specialized compartment that sequesters GLUT4 might also explain why activation of PI 3-kinase by other receptor signaling systems do not cause GLUT4 translocation (26). Taken together, the data presented in this study and the findings of others are consistent with the hypothesis that PI 3-kinase is an essential component in the insulin signaling pathway leading to glucose uptake and GLUT4 translocation.
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