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Requirement for phosphoinositide 3-kinase in insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes

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SUMMARY : Insulin stimulates glucose transport in muscle and fat cells by inducing the redistribution of a specific glucose transporter, GLUT4, from intracellular vesicles to the cell surface. Phosphoinositide (PI) 3-kinase has been implicated as a key intermediate in insulin-stimulated glucose transport by studies that have examined the effects of wortmannin and LY294002, which are thought to be specific inhibitors of this enzyme. However, the specificity of these compounds for PI 3-kinase has recently been questioned. Epidermal growth factor, which activates mitogen-activated protein kinase in mouse 3T3-L1 adipocytes, has now been shown to have no effect on PI 3-kinase activity or GLUT4 translocation in these cells. Furthermore, microinjection of a dominant negative mutant of the 85-kDa subunit of PI 3-kinase, which lacks a binding site for the catalytic 110-kDa subunit, inhibited GLUT4 translocation induced by insulin in 3T3-L1 adipocytes; microinjection of the wild-type protein had no effect. These observations indicate that PI 3-kinase is necessary for insulin-induced GLUT4 translocation and glucose transport in adipocytes.

The ability of insulin to increase glucose transport into muscle and fat cells is a critical determinant of glucose homeostasis (1). This effect is mediated by the insulin-induced translocation of a specific glucose transporter, GLUT4, from intracellular vesicles to the cell surface (2, 3). Despite marked progress in our understanding of the cell biology of this process, relatively little is known concerning the signaling pathway (or pathways) that links the insulin receptor to GLUT4 translocation.

The binding of insulin to its cell surface receptor activates an intrinsic tyrosine kinase that phosphorylates the receptor itself as well as downstream substrates, such as insulin receptor substrate-1 (IRS-1) and Shc (4). The tyrosine phosphorylation sites on these latter molecules act as docking sites for the Src homology 2 (SH2) domains of other signaling

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proteins (5). However, many of these signaling molecules are activated by multiple growth factors, whereas GLUT4 translocation appears to be a specific effect of insulin. For example, both insulin and epidermal growth factor (EGF) activate mitogen-activated protein (MAP) kinase in the 3T3-L1 adipocyte cell line, but only insulin stimulates GLUT4 translocation (6).

Recently, phosphoinositide (PI) 3-kinase has been implicated as one of the key signal transducers in insulin-stimulated glucose uptake, on the basis of experiments with the PI 3-kinase inhibitors wortmannin and LY294002 (7-10) and with a dominant negative mutant regulatory subunit of this enzyme (11). However, the specificity of PI 3-kinase inhibitors has been questioned (12). The best-characterized mammalian PI 3-kinase exists as a heterodimer comprised of a regulatory subunit (p85), capable of binding phosphorylated IRS-1, and a catalytic subunit (p110) (13). Recently, a dominant negative p85 subunit (Δp85) has been constructed that, although able to bind IRS-1, cannot interact with p110 (11). We have previously generated a stable Chinese hamster ovary (CHO) cell line that overexpresses both human insulin receptors and Δp85. Insulin-stimulated GLUT1 translocation was markedly decreased in these cells (11). However, it is not clear whether the mechanism of insulin-stimulated translocation of GLUT1 in CHO cells is the same as that of insulin-induced GLUT4 translocation in adipocytes.

We have now investigated the role of PI 3-kinase in GLUT4 translocation in 3T3-L1 adipocytes by comparing the abilities of insulin and EGF to activate PI 3-kinase with their abilities to induce GLUT4 translocation, and by examining the effect of microinjected Δp85 on GLUT4 translocation.

MATERIALS AND METHODS

Cell culture: 3T3-L1 mouse fibroblasts were maintained in Dulbecco's minimum essential medium (DMEM) containing 4.5 mg/ml glucose supplemented with 10% newborn calf serum, 2 mM L-glutamine, penicillin (50 units/ml), and streptomycin sulfate (50 mg/ml). The cells were grown to confluence in 35-mm tissue culture dishes containing glass coverslips (24 by 24 mm) and then induced to differentiate into adipocytes as described previously (14). Cells were used 10 to 15 days after the initiation of differentiation.
PI 3 -kinase assay: Cells were incubated in Krebs-Ringer phosphate solution [12.5 mM Hepes (pH 7.4), 120 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM sodium phosphate] containing 2% bovine serum albumin for 3 h at 37°C and then with growth factor (1 μM) for 15 min at 37°C. After washing once rapidly with phosphate-buffered saline (PBS), the cells were frozen in liquid N_2 and stored at -20°C. Cells were lysed in buffer A [137 mM NaCl, 20 mM Tris-HCl (pH 7.6), 1 mM $CaCl_2$, 10% glycerol, 1% Nonidet P-40, 1 mM dithiothreitol, 250 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate] and subjected to immunoprecipitation with antibodies to p85 (F12) (15). Immunobeads (15 μl per dish)(Bio-Rad) were incubated with F12 hybridoma supernatant (200 μl) for 2 h at 4°C, washed with PBS, and incubated with cell extracts for 2 h at 4°C. Beads were washed and used for measurement of PI 3-kinase activity as described previously (15). The protein concentration of extracts was determined with the BCA protein assay (Pierce), and relative activities were quantitated by excising radioactive spots from thin-layer chromatography plates and subjecting them to liquid scintillation counting.
Expression and purification of recombinant proteins: Recombinant wild-type p85 and mutant Δp85 were expressed in Escherichia coli as glutathione S-transferase fusion proteins, purified with glutathione-agarose (Pharmacia), and cleaved from the carrier with thrombin as described previously (16). The proteins were dialyzed against a solution

containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.6), and 5 mM $MgCl_2$, and stored at -80°C. Protein concentrations were determined with bovine serum albumin as standard, by densitometric scanning of protein bands stained with Coomassie brilliant blue on an SDS-polyacrylamide gel, also as described previously (17). Recombinant proteins were used at concentrations of 0.5 to 1 mg/ml.

Microinjection of 3T3-L1 adipocytes: Microinjection was performed as previously described (16), with a slight modification. The micropipet (GD-1, Narishige), prepared with a pipet puller (PU-7, Narishige), was controlled with a Narishige micromanipulator (MW-2) attached to an Olympus microscope (IMT-2). Cells were deprived of serum for 4 h at 37°C in DMEM containing 20 mM Hepes (pH 7.4) prior to microinjection of recombinant proteins into the cytoplasm. Cells were allowed to recover at 37°C for 30 min before incubation in the absence or presence of 0.1 μM insulin for 10 min and GLUT4 translocation was measured by the plasma membrane lawn assay.

Plasma membrane lawn assay and immunofluorescence labeling of GLUT4: GLUT4 translocation to the plasma membrane was measured by the plasma membrane (PM) lawn assay as previously described (18). Samples were examined with a fluorescence microscope (BX-2, Olympus), and photographed with Fuji Super Presto 1600 film. Samples were also examined with a model Axiovert 35 Zeiss fluorescence microscope equipped with a Bio-Rad MRC-500 laser scanning confocal imaging system. Fluorescence intensities of PM lawns were determined with image analysis software (CoMOS) (Bio-Rad) as previously described (18).

RESULTS

PI 3 -kinase activity in growth factor-treated cells:

Extracts of growth factor-treated 3T3-L1 adipocytes were subjected to immunoprecipitation with antibodies to p85, and the washed immunoprecipitates were assayed for PI 3-kinase activity with phosphatidylinositol as substrate. Insulin stimulated PI 3-kinase activity approximately three fold, whereas EGF had no significant effect (Figure 1). To establish that the lack of an effect of EGF was not attributable to the absence of functional EGF receptors, we reconfirmed previously published data (18) showing that EGF and insulin stimulated MAP kinase activity to similar extents (data not shown).

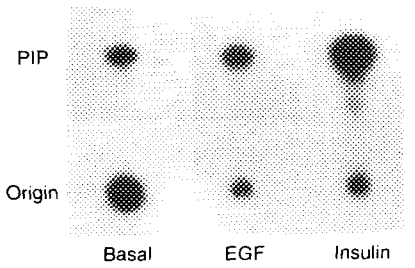

Figure 1. Effects of insulin and EGF on PI 3-kinase activity in 3T3-L1 adipocytes. Cells were incubated in the absence (basal) or presence of 1 μM insulin or EGF for 15 min, lysed, and subjected to immunoprecipitation with antibodies to p85. The washed immunoprecipitates were assayed for PI 3-kinase activity with phosphatidylinositol as substrate, and the labeled PI 3-phosphate product (PIP) was resolved by thin-layer chromatography and visualized by autoradiography. This experiment was repeated on three separate occasions with identical results.

Effects of microinjection of wild-type and mutant p85 on insulin-induced GLUT4 translocation:

To assess the role of PI 3-kinase in insulin-stimulated GLUT4 translocation, we microinjected wild-type p85 and mutant Δp85 proteins into 3T3-L1 adipocytes grown on coverslips. A fluorescent dye (lucifer yellow, 0.1 mg/ml) was microinjected together with the recombinant proteins to facilitate identification of injected cells. Cells were then incubated in the presence or absence of insulin, and plasma membrane lawns were prepared, labeled with antibodies to GLUT4, and examined with a fluorescence microscope (Figure 2A). Fluorescence intensities were also quantitated by image analysis (Figure 2B). Insulin alone increased GLUT4 translocation ~10-fold. Microinjection of cells with wild-type p85 had no significant effect on the degree of insulin-stimulated GLUT4 translocation. However, microinjection of Δp85 significantly inhibited GLUT 4 translocation by ~80%. Microinjection of fluorescent dye alone had no effect on GLUT4 translocation (data not shown).

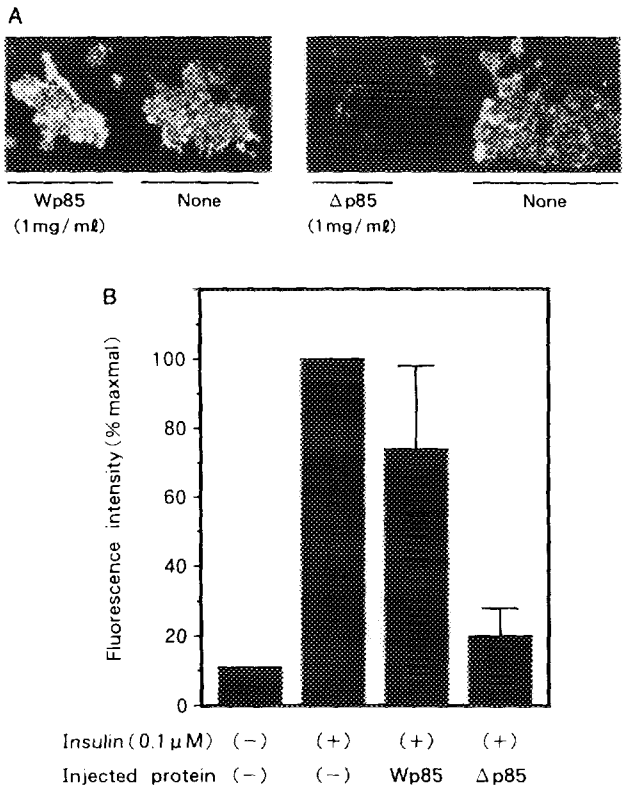

Figure 2. Effects of microinjection of wild-type and mutant p85 on insulin-induced GLUT4 translocation in 3T3-L1 adipocytes.
(A) Cells were microinjected with wild-type p85 (Wp85), mutant Δp85, or neither (None), as indicated, and subsequently stimulated with 0.1 μM insulin for 10 min. Plasma membrane fragments were prepared for immunofluorescence microscopy with antibodies to GLUT4 and tetramethyl rhodamine isothiocyanate-labeled secondary antibodies.
(B) Microinjected cells were incubated in the absence or presence of insulin as indicated and the fluorescence intensity of plasma membrane lawns of injected cells was quantified with image analysis software. Data are means ± SD of three independent experiments.

DISCUSSION

Wortmannin and LY294002 inhibit insulin-stimulated glucose transport and GLUT4 translocation in CHO cells overexpressing GLUT4 (7) and adipocytes (8,9,10) with similar dose-response relations to those apparent for inhibition of PI 3-kinase. However, the specificity of wortmannin and LY294002 for PI 3-kinase has been challenged (12). Overexpression of the dominant negative Δp85 mutant protein inhibited insulin-stimulated glucose transport and GLUT1 translocation in CHO cells that also overexpress insulin receptors (11). However, the mechanism of insulin action in these latter cells may differ from that in other cell types. We have now provided evidence in support of a central role for PI 3-kinase in insulin-stimulated GLUT4 translocation in adipocytes. Firstly, we showed that insulin, but not EGF, increased PI 3-kinase activity in adipocytes, correlating with the ability of only insulin to regulate glucose transport. PI 3-kinase is thus the first insulin-specific signaling intermediate that has been described in 3T3-L1 adipocytes. Both EGF and insulin activate p42 and p44 MAP kinases (18) as well as the two major ribosomal S6 kinases (19), RSK2 and p70 S6 kinase, to the same extent in adipocytes. Furthermore, insulin and EGF induce similar increases in the GTP bound form of p21 Ras in 3T3-L1 adipocytes (20). Given that multiple PI 3-kinase isoforms have been detected, however, we cannot exclude the possibility that the antibody used in our experiments may be specific for an isoform that is responsive to insulin as opposed to an isoform that is sensitive to EGF. Our second observation that supports a role for PI 3-kinase in insulin regulation of glucose transport is that microinjection of Δp85 into 3T3-L1 adipocytes resulted in a marked inhibition of insulin-stimulated GLUT4 translocation to the plasma membrane. This result thus suggests that wortmannin and LY294002 exert their effects on insulin-stimulated glucose transport via inhibition of PI 3-kinase and not some other, as yet unidentified, target.

Collectively, these data indicate that PI 3-kinase may be an important component of the insulin signaling cascade that regulates glucose transport in adipocytes. However, we cannot exclude the possibility that this enzyme has a more general function in protein sorting, receptor-mediated endocytosis, or assembly of a signaling complex involving IRS-1. Indeed, other studies with Δp85 support a more generalized role for PI 3-kinase in insulin action. Thus, microinjection of Δp85 inhibited insulin-stimulated membrane ruffling in human epidermal carcinoma KB cells (16) as well as insulin-induced membrane ruffling and pinocytosis in CHO cells (21). Furthermore, many growth factors activate PI 3-kinase in a variety of cell types. Nevertheless, the growth factor specificity observed in the present study suggests that these signaling molecules may mediate specific events in specific cellular contexts.

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