Effect of the Activation of Phosphatidylinositol 3-Kinase by a Thiophosphotyrosine Peptide on Glucose Transport in 3T3-L1 Adipocytes*  

(Received for publication, July 10, 1995)  

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Insulin causes the activation of phosphatidylinositol 3-kinase (PI 3-kinase) through complexation of tyrosine-phosphorylated YMXX motifs on insulin receptor substrate 1 with the Src homology 2 domains of PI 3-kinase. Previous studies with inhibitors have indicated that activation of PI 3-kinase is necessary for the stimulation of glucose transport in adipocytes. Here, we investigate whether this activation is sufficient for this effect. Short peptides containing two tyrosine-phosphorylated or thiophosphorylated YMXX motifs potently activated PI 3-kinase in the cytosol from 3T3-L1 adipocytes. Introduction of the phosphatase-resistant thiophosphorylated peptide into 3T3-L1 adipocytes through permeabilization with Staphylococcus aureus α-toxin stimulated PI 3-kinase as strongly as insulin. However, under the same conditions the peptide increased glucose transport into the permeabilized cells only 20% as well as insulin. Determination of the distribution of the glucose transporter isotype GLUT4 by confocal immunofluorescence showed that GLUT4 translocation to the plasma membrane can account for the effect of the peptide. These results suggest that one or more other insulin-triggered signaling pathways, besides the PI 3-kinase one, participate in the stimulation of glucose transport.

A major metabolic effect of insulin is the stimulation of glucose transport into muscle and adipose cells. The immediate basis of this effect is the insulin-induced increase in the amount of the glucose transporter isotype GLUT4 in the plasma membrane of these cells (1). This increase results from an enhanced rate of trafficking of GLUT4 to the plasma membrane and possibly also some slowing of the rate of endocytosis of GLUT4 from the plasma membrane (2). The trafficking of GLUT4 to the cell surface may proceed by the docking and fusion of specialized secretory vesicles enriched in GLUT4 (1–3).

An important unsolved issue is the identity of the signaling pathway(s) from the insulin receptor that triggers this translocation of GLUT4 to the plasma membrane. The insulin receptor is a tyrosine kinase. Binding of insulin to the extracellular domain activates the kinase function in the intracellular domain, and the receptor both autophosphorylates and phosphorlates substrate proteins. Among the latter is insulin receptor substrate 1 (IRS-1). IRS-1 is phosphorylated on multiple tyrosine residues, and through these, binds and so regulates at least three Src homology 2 (SH2) domain-containing proteins (4, 5). Phosphatidylinositol 3-kinase (PI 3-kinase) is one of the proteins that associates with IRS-1. It consists of an 85-kDa regulatory subunit with two SH2 domains and a 110-kDa catalytic subunit. The binding of two tyrosine-phosphorylated YMXX motifs present in IRS-1 to the two SH2 domains markedly stimulates PI 3-kinase activity (6, 7). This signal transduction pathway from the insulin receptor to PI 3-kinase thus accounts for the rapid elevation of PI 3,4-bisphosphate and 3,4,5-trisphosphate seen in vivo in response to insulin (8, 9). The downstream components of the pathway have not yet been elucidated. The finding that PI 3,4,5-trisphosphate stimulates some isotypes of protein kinase C in vitro suggests a role for these kinases (10).

Several lines of evidence have indicated that the activation of PI 3-kinase by insulin is required for GLUT4 translocation to the plasma membrane. First, wortmannin, a potent inhibitor of the enzyme, blocks insulin stimulation of glucose transport in rat and 3T3-L1 adipocytes and in muscle tissue (11–13). Second, the compound LY294002, another inhibitor that is structurally unrelated to wortmannin, has also been found to prevent the stimulation of transport in 3T3-L1 adipocytes (9). In each study, except for the one with muscle, it was directly shown that GLUT4 translocation was blocked. Lastly, the microinjection of a dominant negative mutant of the 85-kDa subunit of PI 3-kinase into 3T3-L1 adipocytes prevented GLUT4 translocation in response to insulin (14). The mutant form of this subunit is one that is unable to bind to the catalytic subunit of PI 3-kinase but can still bind to IRS-1, since it retains both SH2 domains.

Although these studies indicate that activation of PI 3-kinase is required for insulin stimulation of glucose transport via GLUT4 translocation, they do not address the question of whether it is sufficient. The present study examines this issue. Previously, we have shown that simple peptides containing two tyrosine-phosphorylated YMXX motifs bind to and activate PI 3-kinase as well as IRS-1 itself does (7). Here, these peptides have been used to activate PI 3-kinase selectively in permeabilized 3T3-L1 adipocytes, and the effect on glucose transport has been determined.

1 The abbreviations used are: IRS-1, insulin receptor substrate 1; GTPγS, guanosine 5′-O-(3-thiotriphosphate); MAP, mitogen-activated protein; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; Z, norleucine; SH2, Src homology 2; Tyr(s), thiophosphotyrosine.
EXPERIMENTAL PROCEDURES

Materials—2-Deoxy-o-[2,6-3H]Glucose and [U-14C]Glucose were from Amer sham. [γ-32P]ATP was from ICN. Phosphatidylinositol was from Avanti Polar Lipids. GTP-S was from Sigma. Staphylococcus aureus α-toxin (catalog no. 616385) was from Calbiochem.

Peptide Synthesis—The non-phosphorylated and Tyr(P) peptides were synthesized as described previously (7). The Tyr(S) peptide was synthesized on an Applied Biosystems model 433 peptide synthesizer using Fmoc (N-(9-fluorenyl)methoxycarbonyl) procedures. Upon completion of peptide synthesis, the amino-terminal 9-fluorenylmethoxy-carbonyl group was removed, and the resin was acetylated (7). The peptide was then thio phosphorylated by the method of Kitas et al. (15) and was then deacetylated and deprotected according to the method of King et al. (16).

Peptide Binding—The relative affinities of peptides for binding to both SH2 domains of PI 3-kinase were determined by a Biacore assay as described in detail previously (7). In this assay, the Tyr(P) peptide YZPZSPK, biotinylated on the α-amino group of lysine, was bound to streptavidin covalently linked to the dextran surface of the flow cell of the Biacore instrument (Pharmacia Biotech Inc.). A glutathione S-transferase fusion protein containing both SH2 domains of PI 3-kinase (residues 312–722 of the 85-kDa subunit), either alone or after mixture with various concentrations of the peptide being analyzed, was injected into the flow cell. Values dependent upon the refractive index, which varies with the amount of glutathione S-transferase-SH2 domain bound to the immobilized peptide, were measured in arbitrary reflectance units.

Cell Culture—3T3-L1 cells were grown in Dulbecco’s modified Eagle’s medium. Collagen-coated dishes were used because permeabilization of the plasma membranes. The cells were rinsed quickly with IC buffer, fixed in 3.5% paraformaldehyde for 5 min, and then permeabilized in 5 ml of TBSB (20 m Tris, 150 m NaCl, 1% bovine serum albumin, 0.025% NaN3, pH 7.4) containing 0.2% saponin for 5 min. The cells were then incubated with 5 mCi of [3H]glucose, followed by separation of the PI 3-kinase protein and that as the result of insulin treatment, the enzyme in the cytosol is activated due to complexation with the Tyr(P) form of IRS-1 (6). This method was applied to both intact cells and to cells that had been permeabilized with α-toxin and exposed to insulin, GTP-S, peptide, or IC buffer alone for 15 min. To ensure reduction of the peptide in the medium below a concentration that activates PI 3-kinase, the cells in each well were washed three times with 2 ml of IC buffer and once with 20 m Tris-Cl, 140 m NaCl, pH 7.6, over a 0.5-min period at 20 °C before being scrapped and homogenized in 0.3 ml of the homogenization buffer.

GLUT4 Immunofluorescence—3T3-L1 adipocytes on coverslips were serum starved, permeabilized, and incubated under the conditions described for the assay of hexose transport. Following these treatments, the cells were rinsed quickly with IC buffer, fixed in 3.5% paraformaldehyde for 5 min, and then permeabilized in 5 ml of TBSB (20 m Tris, 150 m NaCl, 1% bovine serum albumin, 0.025% NaN3, pH 7.4) containing 0.2% saponin for 5 min. The cells were then incubated with 5 mglm affinity-purified rabbit antibodies against the carboxyl-terminal peptide of GLUT4 (18) in TBSB for 30 min, washed twice in 5 ml of TBSB for 5 min, and treated with goat antibodies against rabbit immunoglobulin at 6 mglm conjugated to fluorescein (Vector Laboratories) in TBSB for 30 min. The coverslips were washed twice with 5 ml of TBSB for 5 min, mounted in fluorescein anti-fade reagent (Testog, Inc.), and sealed at the edges with nail varnish. The slides were then viewed on a Bio-Rad M/C 1000 confocal microscope, and the immunofluorescent images were captured and printed.

RESULTS

Peptides Used—In our previous study, we found that peptides containing two tyrosine-phosphorylated YXXM motifs potently activate PI 3-kinase by binding simultaneously to the two SH2 domains on the 85-kDa regulatory subunit of this enzyme (7). For ease of synthesis and stability, norleucine (designated Z), an isomorph of methionine, was used instead of methionine. In this study, we have employed one of these peptides, YZPZSGYSYPSZ, in which both tyrosines were phosphorylated, thiophosphorylated, or nonphosphorylated (Table I). The thiophosphorylated peptide was examined because phosphothiroyrine tyrosine peptides have been found to be resistant to hydrolysis by several tyrosine phosphatases (19, 20). The nonphosphorylated peptide served as a control.

Peptide Binding to PI 3-Kinase SH2 Domains—To determine the effect of substituting the thiophosphoryl group for the phosphoryl group on binding to the PI 3-kinase SH2 domains, the Tyr(S) and Tyr(P) peptides were compared in a Biacore assay in which the effectiveness of the peptide to compete with immobilized Tyr(P) YZPZSPK peptide for binding a glutathione S-transferase fusion protein containing both SH2 domains was measured. The Tyr(S) peptide was almost as effective as the Tyr(P) peptide in blocking binding of the SH2 domains to the immobilized peptide (Fig. 1). Thus, substitution of the thi-
phosphoryl groups had no major effect on the affinity for the SH2 domains. In contrast, as expected, the nonphosphorylated peptide did not bind to the SH2 domains.

Stimulation of PI 3-Kinase by Peptides—To determine whether the strong binding of the Tyr(S) peptide caused a stimulation of PI 3-kinase activity, we measured its effect on the activity of PI 3-kinase in the cytosol of 3T3-L1 adipocytes. The Tyr(S) peptide stimulated activity as potently as the Tyr(P) peptide (Fig. 2). Maximal stimulation was 4.5-fold, with half-maximal effect at 5 nM. This degree of stimulation was approximately the same as that found upon insulin treatment of the cells, which is due to the complexation of Tyr(P) form IRS-1 with PI 3-kinase (6). On the other hand, GTPγS, a compound that stimulates glucose transport in permeabilized cells (see below), did not enhance PI 3-kinase activity.

Effect of Peptides on Glucose Transport—The effect of the Tyr(P) and Tyr(S) peptides on glucose transport was examined in α-toxin permeabilized 3T3-L1 adipocytes. α-toxin is a 34-kDa protein that inserts into the plasma membrane and then oligomerizes to form a 3-nm-diameter aqueous pore that allows passage of molecules of about 5 kDa and less (21). It has been previously used to introduce membrane-impermeant molecules into rat and 3T3-L1 adipocytes (22, 23). An appropriate concentration of α-toxin for permeabilization was determined by allowing intact cells to take up 2-deoxy-[^3H]glucose, which is trapped by phosphorylation as 2-deoxyglucose 6-phosphate (24), and then incubating cells with various concentrations of α-toxin in the ICR buffer (see “Experimental Procedures”) and measuring the appearance of radiolabel in the medium. We chose a concentration of α-toxin that caused a release of 50% of the 2-deoxyglucose 6-phosphate in 5 min.

Previous studies have found that glucose transport can be measured in adipocytes permeabilized by electric discharge (25) or with the detergent streptolysin O (12), because the rate of hexose uptake catalyzed by the glucose transporter is much greater than the rate of either its uptake or of the loss of the 2-deoxyglucose 6-phosphate product through the pores. This

![FIG. 1. Binding of peptides to the SH2 domains of PI 3-kinase.](Image)

The relative affinities of the peptides for binding to the glutathione S-transferase fusion protein containing both SH2 domains of PI 3-kinase were measured in the Biacore assay described under “Experimental Procedures.” Results are expressed as percent of the binding of the PI 3-kinase SH2 domain protein to the immobilized peptide in the absence of competing peptide. ○, TyrS; ●, TyrP; □, TyrS; 

![FIG. 2. Stimulation of PI 3-kinase activity by Tyr(P) and Tyr(S) peptides.](Image)

The cytosolic fraction from basal 3T3-L1 adipocytes was incubated with peptide in 100 μl at room temperature for 35 min and then assayed for PI 3-kinase by the addition of a mixture of [γ-32P]ATP and PI (60 μl). ○, TyrS; ●, TyrP; □, TyrS. The concentrations given are the ones in the initial 100-μl incubation mixture. The values are the means ± S.D. for triplicate measurements in a representative experiment of three that gave similar results. The activity in the cytosol prepared at the same time from 3T3-L1 adipocytes treated with 100 nM insulin for 3 min and in cytosol to which 200 μM GTPγS was added are also shown.
confocal immunofluorescence. In cells in the basal state, there was strong staining in the perinuclear region and punctate staining through the cell (Fig. 5A). Previously, in brown fat cells GLUT4 has been found by immunoelectron microscopy to be in tubules and vesicles concentrated in the juxtanuclear trans Golgi region and also scattered throughout the cytoplasm (27), and the staining pattern in the 3T3-L1 adipocytes is thus consistent with the same locations in this cell type. Upon treatment with insulin (Fig. 5B) or GTPyS (Fig. 5C), the 3T3-L1 adipocytes developed a distinct ring of staining at the cell border. This finding thus provides direct evidence for translocation of GLUT4 to the cell surface in the α-toxin permeabilized cells. Interestingly, the Tyr(S) peptide also caused a noticeable increase in staining at the cell border (Fig. 5D), even though, as described above, it caused an increase in glucose transport only 20% as large as did insulin and GTPyS. Several possible interpretations of this result are considered under “Discussion.”

**DISCUSSION**

The main finding of this study is that activation of PI 3-kinase is not sufficient to stimulate glucose transport in 3T3-L1 adipocytes to the same extent as insulin. This finding suggests that one or more additional signaling pathways are involved. While this study was in progress, Wiese et al. (28) reached the same conclusion by a different approach. These authors found that treatment of 3T3-L1 adipocytes with PDGF caused no significant stimulation of glucose transport, even though it led to an increase in PI 3-kinase activity similar to that elicited by insulin. Despite the fact that these two lines of investigation point to the same conclusion, it should be noted that alternative explanations for the results remain possible. For example, the stimulation of glucose transport may require that PI 3-kinase be activated at a specific subcellular location, and insulin may, to some extent, activate PI 3-kinase in different subcellular locations than do the Tyr(S) peptide and PDGF. Also, PDGF activates some signaling pathways not activated by insulin (4, 5, 29), and one of these may have inhibited the stimulation of glucose transport.

Our observations that the PI 3-kinase-activating peptides did increase glucose transport to a modest extent and did cause redistribution of GLUT4 to the cell surface, as assessed by
immunofluorescence, are consistent with the conclusion, derived from the use of inhibitors (see the Introduction), that activation of PI 3-kinase is involved in GLUT4 translocation and transport stimulation. There are, however, complicating factors that should be considered in the interpretation of these observations. With regard to the stimulation of transport, it is possible that translocation of the transporter isotype GLUT1 contributes to part or all of this effect. 3T3-L1 adipocytes contain considerable intracellular GLUT1, part of which is in vesicles with GLUT4 and part of which is in separate vesicles, and a portion of this isotype also translocates to the cell surface in response to insulin (18, 30). Studies with inhibitors indicate that the activation of PI 3-kinase is also necessary for the smaller increases in cell surface GLUT1 and its associated transport activity caused by insulin (12, 31). Moreover, we have previously shown that microinjection of the Tyr(P) peptide into Xenopus oocytes stimulates glucose transport to the same extent as does insulin-like growth factor I (32). The immediate basis of this effect on transport in oocytes has not been determined but may be GLUT1 translocation to the plasma membrane.

In regard to the redistribution of GLUT4 elicited by the Tyr(S) peptide, the qualitative nature of the immunofluorescence methodology precludes a definitive interpretation. It is possible that modest translocation of GLUT4 to the plasma membrane, consistent with the observed increase in transport (about 20% of the insulin effect), appears as a distinct increase in staining at the cell border. Alternatively, the peptide may cause GLUT4 vesicles to migrate to the plasma membrane as effectively as insulin does but not trigger their fusion with the membrane. This situation would probably not be distinguishable from completed translocation by immunofluorescence. Another possibility is that the peptide causes an increase in GLUT4 in the plasma membrane as large as insulin does but that an additional process is required for stimulation of transport. The determination of GLUT4 subcellular distribution by other methods, such as quantitative immunoelectron microscopy (27), will be required to decide which of these possibilities is in fact occurring.

Although our data suggest that a signaling pathway, in addition to the activation of PI 3-kinase, is necessary for the stimulation of GLUT4 translocation and glucose transport, there is at present evidence against any of the other known signaling pathways from the insulin receptor being required for this effect (reviewed in Ref. 33). Most relevant here are studies in which specific inhibitors of signaling pathways were used, since if two signaling mechanisms are necessary, specific inhibition of only one will block the insulin stimulation of transport, whereas specific activation of only one will not elicit it. Insulin treatment of 3T3-L1 adipocytes also causes the stimulation of the 70-kDa ribosomal S6 kinase, the activation of the SH2 domain-containing tyrosine phosphatase PTP2C, the elevation of the GTP form of Ras, and the activation of the mitogen-activated protein (MAP) kinase cascade. Specific inhibition of the activation of the 70-kDa S6 kinase by rapamycin does not block insulin-stimulated GLUT4 translocation or glucose transport (34). Similarly, inhibition of the activation of PTP2C through the microinjection of antibodies or isolated SH2 domains does not prevent insulin-stimulated GLUT4 translocation (35). In the case of Ras, inhibition of GTP loading by microinjection of a dominant negative mutant or a neutralizing antibody or by prevention of Ras isoprenylation does not impair GLUT4 translocation or the increase in glucose transport (36, 37). Although no complete reports have yet appeared regarding specific inhibitors of the MAP kinase cascade, a recent abstract presents data showing that a specific inhibitor of MAP kinase kinase does not inhibit insulin stimulation of glucose transport (38). Moreover, neither PDGF nor epidermal growth factor, both of which activate the MAP kinase cascade in 3T3-L1 adipocytes, increases glucose transport significantly (28, 39, 40). Taken together, these results thus imply that there may be an as yet undiscovered signaling pathway specific to insulin that is necessary for GLUT4 translocation and transport stimulation.

Acknowledgments—We thank Dr. Gwyn Gould for the initial development of the permeabilization procedure, Drs. A. J. Milici and Stuart Ross for assistance with the immunofluorescence, and Drs. J. R. Falck and Lewis Cantley for the dioctanoyl PI 3,4,5-trisphosphate.

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