Guanosine 5′-O-(3-thiotriphosphate) (GTPγS) Stimulation of GLUT4 Translocation is Tyrosine Kinase-dependent*

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Guanosine 5′-O-(3-thiotriphosphate) (GTPγS) treatment of permeabilized adipocytes results in GLUT4 translocation similar to that elicited by insulin treatment. However, although the selective phosphatidylinositol 3-kinase inhibitor, wortmannin, completely prevented insulin-stimulated GLUT4 translocation, it was without effect on GTPγS-stimulated GLUT4 translocation. In addition, insulin was an effective stimulant, whereas GTPγS was a very weak activator of the downstream Akt serine/threonine kinase. Consistent with an Akt-independent mechanism, guanosine 5′-O-2-(thio) diphosphate inhibited insulin-stimulated GLUT4 translocation without any effect on the Akt kinase. Surprisingly, two functionally distinct tyrosine kinase inhibitors, genistein and herbimycin A, as well as microinjection of a monoclonal phosphotyrosine specific antibody, inhibited both GTPγS- and insulin-stimulated GLUT4 translocation. Phosphotyrosine immunoblotting and specific immunoprecipitation demonstrated that GTPγS did not elicit tyrosine phosphorylation of insulin receptor or insulin receptor substrate-1. In contrast to insulin, proteins in the 120–130-kDa and 55–75-kDa range were tyrosine-phosphorylated following GTPγS stimulation. Several of these proteins were identified and include protein-tyrosine kinase 2 (also known as CAKβ, RAFTK, and CADTK), pp125 focal adhesion tyrosine kinase, pp130 Crk-associated substrate, paxillin, and Cbl. These data demonstrate that the GTPγS-stimulated GLUT4 translocation utilizes a novel tyrosine kinase pathway that is independent of both the phosphatidylinositol 3-kinase and the Akt kinase.

It has been well established that the insulin stimulation of glucose uptake primarily results from the translocation of the GLUT4 glucose transporter isoform from intracellular storage sites to the cell surface membrane in muscle and adipose tissues. Although the molecular mechanism and signaling cascade(s) regulating the intracellular trafficking of GLUT4-containing vesicles have not been completely elucidated, several important effector molecules have recently been identified. Insulin binding to the insulin receptor results in tyrosine autophosphorylation of the β-subunit and activation of its intrinsic tyrosine kinase (1). Subsequently, the insulin receptor tyrosine kinase phosphorylates several intracellular proteins on tyrosine residues, most notably insulin receptor substrate 1 (IRS1) (1). The phosphorylation of these substrates creates recognition sites for additional effector proteins containing Src homology 2 (SH2) domains, thereby generating multisubunit signaling complexes (1, 2). In particular, the tyrosine phosphorylation of IRS1 induces the association and activation of phosphatidylinositol (PI) 3-kinase (3). The targeting and/or activation of PI 3-kinase is well documented to be necessary for GLUT4 translocation (4, 5). Recently, it has been shown that insulin stimulates the Akt kinase, also known as protein kinase B or RAC-PK (6). Insulin-stimulated Akt kinase activity is dependent on PI 3-kinase activation, and expression of a membrane-targeted and constitutively active Akt kinase results in persistent GLUT4 translocation (7, 8). Consistent with a role for GLUT4 trafficking, expression of a dominant interfering Akt mutant inhibited insulin-stimulated GLUT4 translocation (9).

In addition to insulin, various other stimuli display insulinomimetic properties and can induce the translocation of GLUT4-containing vesicles to the plasma membrane. For example, introduction of guanosine 5′-O-(3-thiotriphosphate) (GTPγS), a nonhydrolyzable GTP analogue, into adipocytes rapidly stimulates GLUT4 translocation to a similar extent as insulin (10, 11). In addition, GTPγS can stimulate GLUT4 translocation in the absence of ATP, suggesting that ATP is required at an early step(s) in the insulin-signaling pathway and that a GTP-binding protein(s) functions at a more distal step(s) (11). The stimulatory effect of GTPγS can also be mimicked by treatment with AlF4−, which is characteristic of the involvement of a heterotrimeric GTP binding protein (12, 13). Consistent with this interpretation, adenrenergic stimulation can induce GLUT4 translocation and glucose uptake in both cardiac myocytes (14, 15) and brown adipocytes (16, 17). Furthermore, in transfected Chinese hamster ovary cells and 3T3L1 adipocytes, activation of receptors coupled to Gi1, also stimulate GLUT4 translocation (18). In this regard, skeletal muscle appears to have two distinct pathways mediating GLUT4 translocation. Similar to adipocytes, insulin-stimulated glucose transport in muscle is PI 3-kinase dependent (19–21). In contrast, muscle contraction/exercise or hypoxia stimulates glucose transport through a PI 3-kinase-independent pathway, which may utilize a distinct and separate pool of GLUT4 intracellular vesicles (22). It has been suggested that this alternative pathway leading to GLUT4 translocation may be mediated by an increase in cytoplasmatic calcium levels (23–25), consistent with the known functional role of G proteins in stimulating increases in intracellular calcium (26–30).

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The abbreviations used are: GLUT4, the insulin-responsive glucose transporter isoform; PI 3-kinase, phosphatidylinositol 3-kinase; IRS1, insulin receptor substrate-1; SH2, Src homology 2; PYK2, protein-tyrosine kinase 2 (also known as CAKβ, RAFTK, and CADTK); pp125 FAK, pp125 focal adhesion tyrosine kinase; pp130 Crk-associated substrate; DMEM, Dulbecco’s modified Eagle’s medium; SL-O, streptolysin-O; PBS, phosphate-buffered saline; GTPγS, guanosine 5′-O-(3-thiotriphosphate); GDPγS, guanosine 5′-O-2-(thio)phosphate; IC, intracellular; MOPS, 4-morpholinepropanesulfonic acid; MBP, maltose-binding protein; GST, glutathione S-transferase.
In the present study, we have examined the mechanism by which GTP\(\gamma\)S stimulates GLUT4 translocation by comparing its signaling properties with those of insulin. Our data demonstrate that GTP\(\gamma\)S-stimulated GLUT4 translocation is independent of the PI 3-kinase, the Akt kinase, and changes in intracellular calcium ion concentration. However, GTP\(\gamma\)S-stimulated GLUT4 translocation occurred through the activation of a novel tyrosine kinase pathway, which does not involve the insulin receptor or IRS1 but may require the tyrosine phosphorylation of protein-tyrosine kinase 2 (PYK2, also known as CAKβ, RAFTK, and CADTK), pp125 focal adhesion tyrosine kinase (pp125\(^{FAK}\)), pp130 Crk-associated substrate (pp130\(^{Cas}\)), paxillin, and Cbl. 

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation of 3T3L1 Adipocytes—Marine 3T3L1 preadipocytes were obtained from the American Type Tissue Culture repository and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose and 10% calf serum at 37 °C. Confluent cultures were induced to differentiate into adipocytes by incubation of the cells with DMEM containing 25 mM glucose, 10% fetal bovine serum, 1 mg/ml bovine serum albumin, 1 mM dithiothreitol and enolase. Upon incorporation of propidium iodide. Following SL-O permeabilization, the cells were washed two times with ICR buffer (ICR buffer containing 23 mM KCl, 10 mM Hepes, pH 7.5, 2 mM MgCl\(_2\), 5 mM EGTA, 5 mM NaCl, 0.2% Triton X-100, 0.1% Nonidet P-40, 0.2 mM phenethylsulfonyl fluoride, and 0.2 mM sodium vanadate), and boiled in Laemml sample buffer. The precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting (as described below).

Preparation of Total Cell Extracts and Immunoprecipitation—Total cell extracts were prepared from 60- or 150-mm plates of 3T3L1 adipocytes following the appropriate treatment as described in each figure legend. Cells from each plate were washed two times with ice-cold PBS and scraped in (50 ml of lysis buffer containing 50 mM HEPES, pH 7.8, 1% Triton X-100, 100 mM NaF, 10 mM Na\(_2\)PO\(_4\), 2.5 mM EDTA containing 1.0 mM phenethylsulfonyl fluoride, 2 mM Na\(_3\)VO\(_4\), 1 mg/ml aprotinin, 10 mM leupeptin, and 1 mM pepstatin A by rotation for 15 min at 4 °C. Insoluble material was separated from the soluble extract by microcentrifugation for 15 min at 4 °C. Protein concentration was determined, and samples were either subjected directly to SDS-polyacrylamide gel electrophoresis (as described below) or immunoprecipitated for IRS1 or PYK2. Briefly, 3–5 μg of cellular protein were immunoprecipitated with 5 μg of IRS1 polyclonal antibody (aIRS1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or PYK2 polyclonal antibody (31) for 2 h at 4 °C. Immune complexes were recovered by the addition of protein A-Sepharose (Amersham Pharmacia Biotech) and subjected to SDS-polyacrylamide gel electrophoresis and Western blotting (as described below).

Electrophoresis and Immunoblotting—Plasma membrane sheets were prepared as described previously (32). Soluble GST fusion proteins were covalently linked to agarose beads with Amino-Link (Pierce). 100 μg of fusion proteins bound to the beads were incubated for 2 h at 4 °C with 4 μg of cell extracts isolated from control, insulin-stimulated, or GTP\(\gamma\)S-stimulated cells. The beads were subsequently pelleted, washed with buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Triton X-100, 0.1% Nonidet P-40, 0.2 mM phenethylsulfonyl fluoride, and 0.2 mM sodium vanadate), and boiled in Laemml sample buffer. The precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting (as described below).

Plasma Membrane Sheet Assay—Preparation of plasma membrane sheets from the adipocytes was performed essentially by the method of Robinson et al. (11) with minor modifications. Briefly, 3T3L1 adipocytes were washed three times with intracellular (IC) buffer (140 mM potassium glutamate, 20 mM Hepes, pH 7.15, 7.5 mM MgCl\(_2\), 5 mM EGTA, 5 mM NaCl, 2 mM CaCl\(_2\)) and incubated in IC buffer containing 0.8 IU/ml of SL-O (Murex Diagnostics Inc., Atlanta, GA) for 5 min at 37 °C. Under these conditions, more than 95% of the cell population morphologically differentiated into adipocytes. All studies were performed on adipocytes between 8 and 12 days after initiation of differentiation (day 0). Prior to all experimental treatments, the differentiated adipocytes were serum-starved in DMEM containing 25 mM glucose and 0.1% bovine serum albumin for 2 h at 37 °C. Streptolysin-O Permeabilization of 3T3L1 Adipocytes—3T3L1 adipocytes were permeabilized with streptolysin-O (SL-O) as described by Robinson et al. (11) with minor modifications. Briefly, 3T3L1 adipocytes were washed three times with intracellular (IC) buffer (140 mM potassium glutamate, 20 mM Hepes, pH 7.15, 7.5 mM MgCl\(_2\), 5 mM EGTA, 5 mM NaCl, 2 mM CaCl\(_2\)) and incubated in IC buffer containing 0.8 IU/ml of SL-O (Murex Diagnostics Inc., Atlanta, GA) for 5 min at 37 °C. Under these conditions, more than 95% of the cells were permeabilized based upon incorporation of propidium iodide. Following SL-O permeabilization, the cells were washed two times with IC buffer (IC buffer containing 0.1% bovine serum albumin), and the cells were enriched with either 10 mM MgATP or an ATP-regenerating system: 40 IU/ml creatine phosphokinase, 5 mM creatine phosphate, and 1 mM ATP). Unless otherwise indicated, all experimental treatments were performed by incubating the cells in IC buffer containing various additions for 15 min at 37 °C.

Single Cell Microinjection—3T3L1 adipocytes used for microinjection were grown on 60- or 100-mm tissue culture dishes. The cells were incubated in Krebs-Ringer bicarbonate Hepes buffer (pH 7.4), containing 2 mM pyruvate, 0.5% bovine serum albumin, and 2.5 mM glucose for 45 min prior to microinjection. Adipocytes were microinjected with antibodies over a 45-min period using an Eppendorf model 5171 micromanipulator and given injections of approximately 0.1 nl directly into the cell cytoplasm with an Eppendorf model 5246 transjector. Following microinjection, the cells were allowed to recover for 90 min at 37 °C, prior to permeabilization and treatment.

Plasma Membrane Sheet Assay—Preparation of plasma membrane sheets from the adipocytes was performed essentially by the method of Robinson et al. (11). Briefly, cells cultured on 35- or 60-mm dishes, following the appropriate treatment as described in each figure legend, were rinsed once in ice-cold phosphate-buffered saline (PBS) and incubated with 0.5 mg/ml of poly-l-lysine in PBS for 30 s. The cells were then swollen in a hypotonic buffer (23 mM KCl, 10 mM Hepes, pH 7.5, 2 mM MgCl\(_2\), 1 mM EGTA) by three successive rinses. The swollen cells were sonicated for 3 s at power setting 4.5 with a model 550 Fisher sonic dismembrator fitted with a 5-mm microtip set 1 cm above the surface of the cell monolayer in 10 ml of sonication buffer (70 mM KCl, 30 mM Hepes, pH 7.5, 5 mM MgCl\(_2\), 3 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenethylsulfonyl fluoride). The bound plasma membrane sheets were washed two times with sonication buffer and used for either indirect immunofluorescence or immunoblot analysis as described below.
RESULTS

GTPγS, but Not Insulin, Stimulation of GLUT4 Translocation in 3T3L1 Adipocytes Is Wortmannin-insensitive—As previously observed (10, 11), treatment of SL-O permeabilized 3T3L1 adipocytes with insulin resulted in the translocation of GLUT4 to the plasma membrane as detected by GLUT4 immunoblotting of isolated plasma membrane sheets (Fig. 1A, lanes 1 and 2). Similarly, the addition of GTPγS to the permeabilized cells also induced the translocation of GLUT4 (Fig. 1A, lanes 3 and 4). Since it is well established that activation and/or appropriate intracellular targeting of the PI 3-kinase is necessary for insulin-stimulated GLUT4 translocation, we next examined the role of PI 3-kinase in GTPγS-stimulated GLUT4 translocation. In the control, unstimulated cells, there was a low level of GLUT4 immunofluorescence detected from the isolated plasma membrane sheets (Fig. 1B, panel 1). Pretreatment with the selective PI 3-kinase inhibitor wortmannin (100 nM) slightly reduced the amount of GLUT4 present in the isolated plasma membrane sheets from unstimulated cells (Fig. 1B, panel 4). As expected, insulin stimulated a large increase in the translocation of GLUT4 to the plasma membrane, which was completely inhibited by pretreatment with wortmannin (Fig. 1B, panels 2 and 5). In contrast, although GTPγS stimulated a similar extent of GLUT4 translocation compared with insulin, pretreatment with wortmannin was without effect (Fig. 1B, panels 3 and 6). In addition, pretreatment of the 3T3L1 adipocytes with higher concentrations of wortmannin (1 μM) also inhibited insulin-stimulated GLUT4 translocation but did not reduce the translocation induced by GTPγS (data not shown).

GTPγS Is an Ineffective Activator of the Akt Protein Kinase—Recently, it has been reported that insulin stimulation increases Akt protein kinase activity in a PI 3-kinase-dependent manner, and stable overexpression of a constitutively active membrane-bound form of Akt kinase resulted in the persistent translocation of GLUT4 (7, 8). We therefore examined the ability of GTPγS to stimulate Akt kinase as a potential common point of convergence between the insulin and GTPγS signaling pathways leading to GLUT4 translocation. Activation of Akt protein kinase activity was assessed by immunoprecipitation and analysis of in vitro protein kinase activity (Fig. 2). Isolation of Akt from unstimulated cells demonstrated a basal level of Akt protein kinase activity that was reduced by pretreatment with wortmannin (Fig. 2, lanes 1 and 2). Insulin stimulation resulted in a marked activation of Akt protein kinase activity, which was also attenuated by wortmannin (Fig. 2, lanes 5 and 6). Treatment with GTPγS resulted in an intermediate stimulation of Akt protein kinase activity (Fig. 2, lane 3). Nevertheless, pretreatment with wortmannin completely inhibited the GTPγS-stimulated activation of Akt protein kinase activity (Fig. 2, lane 4). Since Akt kinase activation is accompanied by dual phosphorylation on serine and threonine residues (34), we also examined the effect of insulin and GTPγS on the SDS-polyacrylamide gel electrophoretic mobility of Akt. Consistent
with Akt kinase activity, insulin stimulation resulted in a marked reduction of Akt electrophoretic mobility, whereas GTPγS stimulation had little effect (data not shown). Furthermore, pretreatment of the cells with wortmannin completely prevented the reduction in Akt electrophoretic mobility. Thus, together these data demonstrate that GTPγS stimulation of GLUT4 translocation occurs in a pathway that is independent of both PI 3-kinase and Akt kinase activation.

**GDPβS Inhibits Insulin Stimulation of GLUT4 Translocation**—The data presented above suggest that the GTPγS stimulation of GLUT4 translocation occurs in a pathway(s) downstream to and/or in parallel with the PI 3-kinase and Akt kinase. To further characterize the relationship between insulin and GTPγS stimulation, we next incubated the SL-O-permeabilized cells with 100 μM GDPβS prior to insulin treatment (Fig. 3). In the absence of insulin, GDPβS had no significant effect on GLUT4 translocation as detected by GLUT4 immunofluorescence of isolated plasma membrane sheets (Fig. 3A, panels 1 and 5). Insulin stimulation resulted in a dose-dependent increase in the amount of plasma membrane-associated GLUT4 protein (Fig. 3A, panels 1–4). Pretreatment with GDPβS resulted in the inhibition of GLUT4 translocation at low (1 and 10 nM) insulin concentrations but not at high (100 nM) insulin concentrations (Fig. 3A, panels 5–8). Insulin stimulation also resulted in a dose-dependent decrease in Akt SDS-polyacrylamide gel electrophoretic mobility (Fig. 3B, lanes 1, 2, 5, and 8). However, pretreatment with GDPβS had no effect on the insulin stimulation of the Akt gel shift and, hence, phosphorylation and presumably protein kinase activation (Fig. 3B, lanes 3, 6, and 9). These data further support a model in which GTPγS stimulation of GLUT4 translocation is mediated by a mechanism downstream of the Akt kinase.

**GTPγS Stimulation of GLUT4 Translocation Is Tyrosine Kinase-dependent**—Recently, several studies have suggested that G protein-coupled receptors can modulate tyrosine kinase signaling pathways. Having ruled out any potential role for PI 3-kinase and Akt protein kinase, we next assessed whether GTPγS-stimulated GLUT4 translocation was mediated by a tyrosine kinase-dependent mechanism. This possibility was initially examined using the relatively specific tyrosine kinase inhibitors genistein and herbimycin A (Fig. 4). In unstimulated cells, there was little GLUT4 detectable in the isolated plasma membrane sheets, and pretreatment with either genistein or herbimycin A had no effect (Fig. 4, panels 1, 4, and 7). As expected, both genistein and herbimycin A were effective inhibitors of insulin-stimulated GLUT4 translocation (Fig. 4, panels 2, 5, and 8), as both of these agents inhibit the insulin receptor tyrosine kinase and IRS1 tyrosine phosphorylation (data not shown). However, to our surprise, pretreatment with these tyrosine kinase inhibitors also prevented the GTPγS-stimulated translocation of GLUT4 (Fig. 4, panels 3, 6, and 9).

Although genistein and herbimycin A are relatively specific tyrosine kinase inhibitors, it is possible that the prevention of GLUT4 translocation was due to some other nonspecific effect. To further confirm the requirement for tyrosine kinase activity, we next utilized single cell microinjection of a monoclonal phosphotyrosine antibody (Fig. 5). To identify the plasma membrane sheets derived from the small fraction of microinjected cells, the carboxyl-terminal domain of Ras fused to the maltose-binding protein (MBP-Ras) was co-injected (35). Plasma membrane sheets isolated from cells that were microinjected with MBP-Ras and a nonspecific mouse IgG were detected by MBP-Ras immunofluorescence (Fig. 5, panel 1). Insulin stimulation increased GLUT4 immunofluorescence in the plasma membrane sheets derived from both mouse IgG microinjected and noninjected cells (Fig. 5, panel 4). As expected, microinjection of the monoclonal PY20 phosphotyrosine antibody inhibited the insulin-stimulated translocation of GLUT4 compared with the GLUT4 translocation in the neighboring noninjected cells (Fig. 5, panel 2). These observations strongly support the idea that tyrosine kinase activity is required for the GTPγS-stimulated translocation of GLUT4.

**GTPγS Stimulation of GLUT4 Translocation but not Akt Phosphorylation**—A differentiated 3T3L1 adipocytes were serum-starved for 2 h, permeabilized in the absence (control; panels 1–4) or presence of 100 μM GDPβS (panels 5–8) with 0.8 IU/ml SL-O for 5 min at 37 °C. The cells were then incubated with 0 (panels 1 and 5), 1 (panels 2 and 6), 10 (panels 3 and 7), or 100 nM (panels 4 and 8) insulin for 15 min. Plasma membrane sheets were prepared and subjected to immunofluorescence microscopy as described under “Experimental Procedures.” B, differentiated 3T3L1 adipocytes were serum-starved for 2 h and then permeabilized in the absence (−; lanes 1, 2, 4, 5, 7, 8, and 10) or presence (+; lanes 3, 6, and 9) of 100 μM GDPβS with 0.8 IU/ml SL-O for 5 min at 37 °C. The cells were then incubated for an additional 15 min with 0 (lanes 1, 4, 7, and 10), 1 (lanes 2 and 3), 10 (lanes 5 and 6), or 100 nM (lanes 8 and 9) insulin. The cells were detergent-solubilized and subjected to immunoblotting with an Akt antibody as described under “Experimental Procedures.” These are representative observations from two independent experiments.

**Fig. 3.** GDPβS inhibits insulin-stimulated GLUT4 translocation but not Akt phosphorylation. A, differentiated 3T3L1 adipocytes were serum-starved for 2 h, permeabilized in the absence (control; panels 1–4) or presence of 100 μM GDPβS (panels 5–8) with 0.8 IU/ml SL-O for 5 min at 37 °C. The cells were then incubated with 0 (panels 1 and 5), 1 (panels 2 and 6), 10 (panels 3 and 7), or 100 nM (panels 4 and 8) insulin for 15 min. Plasma membrane sheets were prepared and subjected to immunofluorescence microscopy as described under “Experimental Procedures.” B, differentiated 3T3L1 adipocytes were serum-starved for 2 h and then permeabilized in the absence (−; lanes 1, 2, 4, 5, 7, 8, and 10) or presence (+; lanes 3, 6, and 9) of 100 μM GDPβS with 0.8 IU/ml SL-O for 5 min at 37 °C. The cells were then incubated for an additional 15 min with 0 (lanes 1, 4, 7, and 10), 1 (lanes 2 and 3), 10 (lanes 5 and 6), or 100 nM (lanes 8 and 9) insulin. The cells were detergent-solubilized and subjected to immunoblotting with an Akt antibody as described under “Experimental Procedures.” These are representative observations from two independent experiments.
GLUT4 translocation. Differentiated 3T3L1 adipocytes were serum-starved for 2 h, pretreated with 0.25% Me2SO (herbimycin A, prevent both insulin- and GTP S stimulation had no effect on either insulin receptor tyrosine kinase or herbimycin A treatment and PY20 microinjection) pretreatment was used to inhibit tyrosine kinase activity. Tyrosine phosphorylation following GTP S stimulation had no effect on either insulin receptor (Fig. 6, lanes 5 and 6). Similarly, microinjection of the monoclonal PY20 antibody also prevented the GTP S-stimulated translocation of GLUT4 (Fig. 5, panels 2 and 6). The fact that two independent methods to inhibit tyrosine kinase activity (genistein or herbimycin A treatment and PY20 microinjection) prevented both insulin- and GTP S-stimulated GLUT4 translocation provides compelling evidence that GTP S utilizes a tyrosine kinase pathway to mediate GLUT4 translocation. 

Insulin and GTP S Stimulate the Tyrosine Phosphorylation of Discrete Proteins—Based upon the inhibition of insulin- and GTP S-stimulated GLUT4 translocation by genistein, herbimycin A, and the PY20 phosphotyrosine antibody, we next compared the tyrosine phosphorylation of proteins induced by insulin and GTP S treatment (Fig. 6). Insulin stimulation resulted in increased tyrosine phosphorylation of the insulin receptor β-subunit and IRS1 (Fig. 6A, lanes 3 and 4). In contrast, GTP S stimulation had no effect on either insulin receptor β subunit or IRS1 tyrosine phosphorylation (Fig. 6A, lanes 1 and 2). Instead, GTP S appeared to induce the tyrosine phosphorylation of several proteins in the molecular mass range of 120–130 kDa and 55–75 kDa (Fig. 6A, compare lanes 1 and 2 with lanes 3 and 4). To more carefully examine the potential for IRS1 tyrosine phosphorylation, we also specifically immunoprecipitated IRS1 from both insulin- and GTP S-stimulated adipocytes. Insulin stimulated the tyrosine phosphorylation of IRS1 in the IRS1 immunoprecipitates (Fig. 6B, lanes 3 and 4), whereas there was no detectable IRS1 tyrosine phosphorylation following GTP S stimulation (Fig. 6B, lanes 1 and 2). IRS1 immunoblotting of that same membrane demonstrated the presence of equal amounts of IRS1 protein (Fig. 6B, lanes 5–8). The apparent increase in molecular weight following insulin and GTP S stimulation most likely reflects the serine/threonine phosphorylation of IRS1.

GTP S Stimulates the Tyrosine Phosphorylation of PYK2, pp130Cas, Paxillin, and Cbl—One interesting tyrosine kinase that has recently been implicated in the intracellular signaling of several trimeric G protein-coupled receptors and by osmotic shock is PYK2 (31, 36–39). Since osmotic shock, like GTP S-stimulated GLUT4 translocation through a PI 3-kinase- and Akt-independent pathway (31), we next examined the tyrosine phosphorylation of PYK2 by immunoprecipitation. As previously reported, in unstimulated cells there was no detectable PYK2 tyrosine phosphorylation, whereas osmotic shock induced a marked tyrosine phosphorylation of PYK2 (Fig. 7A, lanes 1 and 2). In contrast, there was no detectable tyrosine phosphorylation of PYK2 in response to insulin (Fig. 7A, lane 3). Although permeabilization alone resulted in a slight increase in the phosphotyrosine content of PYK2, GTP S was an effective activator of PYK2 tyrosine phosphorylation (Fig. 7A, lanes 4 and 5). Immunoblotting of the same Western blot with a PYK2 antibody indicated the presence of equal amounts of PYK2 in each lane (Fig. 7B, lanes 1–5).

**DISCUSSION**

Over the past several years, significant progress has been made in our understanding of the insulin signal transduction pathway leading to GLUT4 vesicle trafficking. It is generally accepted that insulin stimulation results in the activation of the insulin receptor tyrosine kinase, leading to the tyrosine phosphorylation of the IRS family of intracellular docking proteins (1, 2). The tyrosine phosphorylation of IRS1 induces the association/targeting and/or activation of PI 3-kinase (3). Multiple studies have demonstrated that PI 3-kinase function is necessary for insulin-stimulated GLUT4 translocation (4, 5). Although the downstream target(s) of PI 3-kinase involved in GLUT4 translocation has not been identified, recent studies indicate that activation of PI 3-kinase results in the activation of the Akt serine/threonine-protein kinase (7, 8).

In addition to the identification of this signaling pathway of insulin, there are several insulinoimmetic agents that can also stimulate glucose transport in adipocytes and muscle. In particular, nonhydrolyzable GTP analogues have been reported to increase glucose transport activity by inducing the translocation of GLUT4 (10, 11). We therefore reasoned that examining the GTP S stimulation of glucose transport might provide new insight by identifying common and/or distinct pathways leading to GLUT4 translocation. In this regard, two previous studies have reported contradictory findings for the role of PI 3-kinase in GTP S-stimulated glucose transport regulation. A study by Clarke et al. (40) suggested that the ability of GTP S to stimulate 2-deoxy-D-glucose uptake by permeabilized cells was partially blocked by wortmannin (1 μM). In contrast, it was later reported by Herbst et al. (41) that incubation of permeabilized 3T3L1 adipocytes with GTP S (200 μM) did not increase PI 3-kinase activity over basal level. Although the basis for this difference is not apparent, our data demonstrate that GTP S-stimulated GLUT4 translocation is completely wortmannin-insensitive at concentrations sufficient to inhibit both the receptor tyrosine kinase-activated PI 3-kinase and the Cβγ responsive PI 3-kinase isoform (42).

One recently established function of PI 3-kinase is to couple receptor tyrosine kinase activation to the Akt protein kinase, also known as RAC-PK or protein kinase B (6, 43, 44). The Akt kinase is directly activated by dual phosphorylation on serine and threonine residues (34). In addition, the pleckstrin homology domain of Akt can bind phosphatidylinositol 3,4-bisphosphate in vitro, which also has been shown to stimulate its protein kinase activity (45–47). In either case, insulin activates the Akt kinase in a PI 3-kinase dependent manner. Furthermore, overexpression of a membrane-targeted constitutively active Akt results in the persistent translocation of GLUT4 (7, 8). Since it has been suggested that there are PI 3-kinase-independent pathways leading to Akt activation (48), it remained formally possible that GTP S activated Akt, hence GLUT4 translocation, by a wortmannin-insensitive mecha-
nism. However, our data demonstrated that GTP\textsubscript{γS} is a very weak activator of Akt phosphorylation and protein kinase activity. In addition, wortmannin completely inhibited this small extent of Akt activation, yet had no effect on GTP\textsubscript{γS}-stimulated GLUT4 translocation. Thus, these data clearly establish the presence of a PI 3-kinase- and Akt-independent pathway leading to GLUT4 translocation in 3T3L1 adipocytes.

In an alternative approach to identify the pathway(s) utilized by GTP\textsubscript{γS}, we observed that GDP\textsubscript{βS} was an effective inhibitor of insulin-stimulated GLUT4 translocation at physiological but not at supraphysiological (saturating) insulin concentrations. The inability of GDP\textsubscript{βS} to prevent insulin-stimulated GLUT4 translocation at supraphysiological insulin concentrations has been previously reported, but the effect of GDP\textsubscript{βS} was not examined at physiological insulin levels (49). There are several plausible reasons accounting for the inefficacy of GDP\textsubscript{βS} at high insulin concentrations, including the possibility that insulin can mediate GLUT4 translocation by more than one signaling pathway, i.e. one pathway that is dependent on a GTP binding protein(s) and another one that is not. The former pathway would be the predominant signaling pathway under normal physiological stimulation, whereas the latter pathway would be activated only by high insulin concentrations. Alternatively, at high insulin concentrations, the downstream effectors are maximally activated and would require substantially greater concentrations of GDP\textsubscript{βS} to be inhibited. In any case, the fact that GDP\textsubscript{βS} inhibited the insulin-stimulated translocation of GLUT4 without any effect on Akt kinase is consistent with the presence of at least one GTP-binding protein functioning downstream of Akt. Together, these data provide compelling evidence that neither PI 3-kinase nor Akt kinase are involved in the GTP\textsubscript{γS} stimulation of GLUT4 translocation and that GTP\textsubscript{γS} functions at a site distal to the Akt kinase.

Although it is generally thought that calcium does not play a significant role in the insulin stimulation of GLUT4 translocation, it has been hypothesized to be necessary for the exercise/contraction-stimulated GLUT4 translocation in skeletal mus-
In summary, we have observed that GTPγS-stimulated GLUT4 translocation occurs by a novel tyrosine kinase pathway in 3T3L1 adipocytes. This alternative signaling pathway does not require tyrosine phosphorylation of the insulin receptor, IRS1, or the targeting and/or activation of either PI 3-kinase or Akt kinase. However, GTPγS stimulation results in the tyrosine phosphorylation of several distinct proteins, including PYK2, pp125FRα, pp130Cas, paxillin, and Chl. Whether or not any of these tyrosine-phosphorylated proteins are necessary and/or sufficient for the GTPγS stimulation of GLUT4 translocation is an important future issue for investigation.

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