Osmotic Shock Stimulates GLUT4 Translocation in 3T3L1 Adipocytes by a Novel Tyrosine Kinase Pathway*

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Similar to insulin, osmotic shock of 3T3L1 adipocytes stimulated an increase in glucose transport activity and translocation of GLUT4 protein from intracellularly localized vesicles to the plasma membrane. The docking/fusion of GLUT4 vesicles with the plasma membrane appeared to utilize a similar mechanism, since expression of a dominant interfering mutant of syntaxin-4 prevented both insulin- and osmotic shock-induced GLUT4 translocation. However, although the insulin stimulation of GLUT4 translocation and glucose transport activity was completely inhibited by wortmannin, activation by osmotic shock was wortmannin-insensitive. Furthermore, insulin stimulated the phosphorylation and activation of the Akt kinase, whereas osmotic shock was completely without effect. Surprisingly, treatment of cells with the tyrosine kinase inhibitor, genistein, or microinjection of phosphotyrosine antibody prevented both insulin- and osmotic shock-stimulated translocation of GLUT4. In addition, osmotic shock induced the tyrosine phosphorylation of several discrete proteins, including Cbl, p130cas, and the recently identified soluble tyrosine kinase, calcium-dependent tyrosine kinase (CADTK). In contrast, insulin had no effect on CADTK but stimulated the tyrosine phosphorylation of Cbl and the tyrosine dephosphorylation of pp125FAK and p130cas. These data demonstrate that the osmotic shock stimulation of GLUT4 translocation in adipocytes occurs through a novel tyrosine kinase pathway that is independent of both the phosphatidylinositol 3-kinase and the Akt kinase.

The facilitative glucose transporters are a family of related integral membrane proteins that are responsible for the regulation of whole body and cellular glucose homeostasis. Unlike other members of this family, the insulin-responsive glucose transporter isoform (GLUT4) is predominantly expressed in adipose tissue and in skeletal and cardiac muscle (1, 2). In these tissues, insulin increases glucose uptake by regulating the intracellular trafficking of the GLUT4 protein. In the basal state, GLUT4 cycles continuously between the plasma membrane and one or more intracellular compartments, with the vast majority of the transporter residing within the cell interior (3, 4). Activation of the insulin receptor triggers a large increase in the rate of GLUT4 vesicle exocytosis in addition to a smaller decrease in the rate of internalization by endocytosis. This insulin-dependent shift in the cellular dynamics of GLUT4 vesicle trafficking results in a net increase of GLUT4 protein level on the cell surface, thereby increasing the rate of glucose uptake (for recent reviews, see Refs. 5–8).

Activation of the insulin receptor by ligand binding initiates a cascade of signaling events, which include activation of the intrinsic receptor tyrosine kinase, autophosphorylation of the receptor, and phosphorylation of cellular substrates such as insulin receptor substrate (IRS)-1/2 and Shc (for recent reviews, see Refs. 9 and 10). Phosphorylation of these substrates provides docking sites for several intracellular signaling complexes. For example, tyrosine-phosphorylated IRS-1 provides a docking site for p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase), resulting in activation of its enzymatic activity (11–14). Activation of PI 3-kinase has recently been shown to be necessary for insulin-mediated translocation of GLUT4 protein to the plasma membrane (15–26). Furthermore, growth factor activation of the serine/threonine kinase Akt, also known as protein kinase B or RAC-PK, is a downstream target of the activated PI 3-kinase (27–30). In this regard, stable expression of a constitutively active membrane bound form of Akt in 3T3L1 adipocytes resulted in increased glucose transport and persistent localization of GLUT4 to the plasma membrane (31, 32).

In addition to growth factors, other extracellular stimuli can activate Akt kinase activity. For example, osmotic shock of fibroblasts has recently been reported to stimulate Akt kinase activity in a manner analogous to growth factor activation (33). This finding is of particular interest, since early studies in the 1960s and 1970s demonstrated that hyperosmolality had potent insulin-like properties on glucose metabolism, including activation of glucose transport in adipocytes and skeletal muscle (34–38). Based upon these data, we hypothesized that a comparison between the effect of insulin and osmotic shock may provide an important approach to identify common and unique signaling pathways leading to increased glucose transport activity. In the present study we demonstrate that, similar to insulin, osmotic shock of 3T3L1 adipocytes stimulates glu-
cose transport activity through increased GLUT4 translocation to the plasma membrane. Insulin- and osmotic shock-stimulated GLUT4 translocation also share a common docking/fusion mechanism requiring syntaxin-4 as a functional t-SNARE. However, in contrast to insulin, osmotic shock stimulates GLUT4 translocation by activation of a tyrosine kinase signaling pathway independent of the PI 3-kinase and the Akt kinase. These data demonstrate the presence of a novel tyrosine kinase signaling pathway that converges with the insulin-stimulated pathway for GLUT4 translocation downstream from both the PI 3-kinase and Akt kinase in adipocytes.

EXPERIMENTAL PROCEDURES

Cell Culture—3T3L1 preadipocytes were maintained in Dulbecco’s modified Eagle’s medium containing 25 mM glucose (DMEM) plus 10% fetal bovine serum and 200 nM insulin. After an additional 4 days, the cells were then placed into DMEM containing 10% fetal bovine serum and 200 nM insulin. After an additional 4 days, the cells were placed into DMEM containing 10% fetal bovine serum without any additives and were used between 8 and 14 days postinduction of the differentiation protocol.

Preparation of Plasma Membrane Sheet—Plasma membrane sheets were prepared by the method of Robinson et al. (40). Differentiated 3T3L1 adipocytes were washed with phosphate-buffered saline (PBS; 5 mM Na2HPO4, pH 7.4, 150 mM NaCl) containing 0.5 mg/ml poly-D-lysine followed by three washes with hypotonic buffer (25 mM KCl, 10 mM HEPES, pH 7.5, 1.7 mM MgCl2, 1 mM EGTA). The cells were then covered with sonication buffer (3 mM HEPES, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride) and sonicated with a Fisher probe membrane disruptor. Following sonication, the plasma membrane sheets were washed three times with sonication buffer and were used either for immunoblotting or immunofluorescence as described below.

 Immunofluorescence—The plasma membrane sheets were fixed in paraformaldehyde (2% paraformaldehyde in 3 x hypotonic buffer) for 20 min, followed by a 15-min incubation in a quenching solution (100 mM glycine in PBS). The membranes were then washed three times with PBS and blocked in 5% serum at 4°C overnight. After blocking, the plasma membrane sheets were incubated with a rabbit polyclonal GLUT4 antibody (1:100) at 37°C for 1 h. The plasma membrane sheets were then washed three times with PBS, followed by incubation with a lissamine rhodamine-conjugated donkey anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories) solution for 30 min at 37°C. The plasma membrane sheets were again washed three times with PBS, once with water, and the samples were visualized by confocal microscopy.

Glucose Transport Assay—3T3L1 adipocytes were placed in DMEM containing 25 mM glucose plus 0.5% bovine serum albumin for 2 h at 37°C. Cells were then washed with KRPH buffer (5 mM Na2HPO4, 20 mM HEPES, pH 7.4, 1 mM MgSO4, 1 mM CaCl2, 136 mM NaCl, 4.7 mM KCl, and 1% bovine serum albumin) and either untreated or stimulated as described in the figure legend. Glucose transport was determined at 37°C by incubation with 50 μCi 2-deoxy[3H] glucose in the absence or presence of 10 μM cytochalasin B. The reaction was stopped after 10 min by washing the cells three times with ice-cold PBS. The cells were then solubilized in 1% Triton X-100 at 37°C for 30 min, and aliquots were subjected to scintillation counting.

Vaccinia Virus Infection—Recombinant Vaccinia virus encoding β-galactosidase or the cytoplasmic domain of syntaxin-4 (amino acids 1–274) fused with the Myc epitope tag was prepared as described previously (41). Quantitative infection of differentiated 3T3L1 adipocytes was performed by infecting 35-mm plates of cultured 3T3L1 adipocytes at a multiplicity of infection of 10 plaque-forming units/cell for 4 h in DMEM containing 25 mM glucose and 0.5% bovine serum. Infection/expression in 100% of the cell population was confirmed by an in situ assay for β-galactosidase activity (42). Forty h postinfection, the adipocytes were either left untreated or treated as described in each figure legend. The cells were then used for the preparation of plasma membrane sheets and subsequently analyzed by GLUT4 immunoblotting as described below.

Immunoblotting—Whole cell detergent extracts of 3T3L1 adipocytes were prepared as described previously (43). Briefly, following experimental treatment, the cells were solubilized in 50 mM HEPES, pH 7.4, 1% Triton X-100, 100 mM sodium fluoride, 2.5 mM EDTA, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg of pepstatin, 0.5 τm phenylalanine inhibitors of aprotinin, and 10 μM leupeptin. The extracts were then microcentrifuged at 13,000 × g for 10 min, and 50 μg of the infranatants were resolved by SDS-polyacrylamide gel electrophoresis. Immunoblotting was performed by electrophoretic transfer to nitrocellulose membranes using a polyclonal GLUT4 antibody (East Araces Biologicals), a polyclonal pp125FAK antibody (Upstate Biotechnology Inc.), or the monoclonal phosphorysosfotyrosine antibody PY20-HRP (Transduction Laboratories).

RESULTS

Sorbitol Treatment Stimulates Glucose Transport and GLUT4 Translocation in 3T3L1 Adipocytes—As expected, insulin-stimulated pathway for 15 min typically resulted in an approximate 18-fold increase in 2-deoxyglucose uptake (Fig. 1A, open and solid bars). Consistent with previous studies (34–38), hyperosmolarity induced by sorbitol treatment also resulted in a significant increase in 2-deoxyglucose uptake (Fig. 1A, open and...
examined the effect of two different D- and L-monosaccharides on GLUT4 translocation (Fig. 2). Similar to D-sorbitol, incubation of 3T3L1 adipocytes with either D-xylose or D-glucose for 30 min resulted in GLUT4 translocation to the plasma membrane compared with control cells (Fig. 2, A, C, and E). In addition, treatment with the nonmetabolizable L-sugars (L-xylose and L-glucose) also resulted in an equivalent extent of GLUT4 translocation (Fig. 2, D and F). Consistent with Fig. 1, insulin stimulation resulted in a greater extent of GLUT4 translocation compared with the effect of these other sugars (Fig. 2B). Together, these data demonstrate that acute osmotic shock and not changes in carbohydrate metabolism in 3T3L1 adipocytes stimulates GLUT4 translocation to the plasma membrane.

**Syntaxin-4 Functions as the t-SNARE for Both Osmotic Shock- and Insulin-stimulated GLUT4 Translocation**—We and others have recently reported that insulin-stimulated GLUT4 translocation utilizes syntaxin-4 as a functional plasma membrane t-SNARE for GLUT4, but not GLUT1, vesicle docking (39, 50–53). To assess whether osmotic shock stimulation of GLUT4 translocation was also dependent upon docking to syntaxin-4, we expressed the syntaxin-4 cytosolic domain using recombinant Vaccinia virus (Fig. 3). As controls for virus infection, GLUT4 immunoblotting of plasma membrane sheets prepared from unstimulated 3T3L1 adipocytes infected with the empty virus resulted in a low level of GLUT4 protein associated with the plasma membrane (Fig. 3, lane 1). In contrast, cells infected with the empty virus and stimulated by osmotic shock demonstrated a marked increase in the amount of plasma membrane-associated GLUT4 protein (Fig. 3, lane 2). Consistent with our previous immunoblotting and immunofluorescence results (Figs. 1 and 2), insulin stimulation resulted in a greater increase in plasma membrane-associated GLUT4 protein compared with the osmotic shock-treated cells (Fig. 3, lanes 2 and 3). These data demonstrate that Vaccinia virus infection per se does not interfere with either osmotic shock or insulin-stimulated GLUT4 translocation.

Previous studies have observed that the cytoplasmic domain of syntaxin-4 functions as a dominant interfering mutant for granule fusion with the plasma membrane in PC12 cells, and we have observed a similar property for syntaxin-4 in adipocytes (39, 54). This presumably results from the competition of the soluble syntaxin fragment with the plasma membrane-bound syntaxin for the vesicle-associated membrane proteins (VAMPs). To compare the role of syntaxin-4 in osmotic shock and insulin-stimulated GLUT4 translocation, the 3T3L1 adi-
Recently, the serine/threonine kinase Akt has been identified as an intermediate kinase functioning downstream of the PI 3-kinase and upstream of p70S6 kinase and GSK3 (29, 55). In addition, stable overexpression of a constitutively active membrane-targeted mutant of Akt results in the apparent decrease in Akt SDS-polyacrylamide gel electrophoretic mobility (Fig. 5). As previously reported (56), insulin stimulation resulted in a marked decrease in electrophoretic mobility compared with unstimulated cells (Fig. 5A, lanes 1–3). In contrast, osmotic shock stimulation had no significant effect on the electrophoretic mobility of Akt compared with control cells (Fig. 5A, lanes 4–6).

The Akt kinase has also been reported to be activated by phosphatidylinositol phosphates, particularly phosphatidylinositol-3,4-bisphosphate, in the absence of any direct serine/threonine phosphorylation (57–59). In addition, activation of Akt protein kinase activity, although not as strongly, has been observed in the absence of any apparent changes in SDS-polyacrylamide gel electrophoretic mobility (45, 57). Therefore, to assess the potential activation of Akt in the absence of detectable change in SDS-polyacrylamide gel electrophoretic mobility, we directly determined Akt protein kinase activity (Fig. 5B). Immunoprecipitation of Akt from unstimulated cells demonstrated a basal level of Akt kinase activity as detected by the phosphorylation of histone H2B (Fig. 5B, lane 1). Insulin treatment for 5 and 30 min resulted in the stimulation of the Akt protein kinase activity, which correlated with the decrease in Akt SDS-polyacrylamide gel electrophoretic mobility (Fig. 5B, lanes 2 and 3). Consistent with the inability of osmotic shock to alter Akt mobility, osmotic shock treatment for either 5 or 30 min had no significant effect on Akt protein kinase activity (Fig. 5B, lanes 4 and 5).

Although the physiological kinase(s) responsible for Akt phosphorylation and activation have not been identified, Akt can be phosphorylated in vitro by mitogen-activated protein kinase-activated protein kinase 2 (27). On this basis, it has been speculated that signaling pathways leading to mitogen-activated protein kinase-activated protein kinase 2 activation may also result in Akt activation (60). Since mitogen-activated protein kinase-activated protein kinase 2 is an immediate downstream target for activated p38 MAP kinase, we determined the ability of insulin and osmotic shock to stimulate the p38 MAP kinase (Fig. 5C). This was assessed by immunoblotting with a phospho-specific p38 MAP Kinase antibody that only detects the activated form of this enzyme. As is readily apparent, there was a low level of activated p38 MAP kinase in unstimulated cells (Fig. 5C, lane 1). Insulin stimulation for either 5 or 30 min resulted in a relatively poor activation of the p38 MAP kinase (Fig. 5C, lanes 2 and 3), whereas osmotic shock was an effective activator of the p38 MAP kinase (Fig. 5C, lanes 4 and 5). In contrast, insulin was a strong activator of Akt phosphorylation, whereas osmotic shock was not (Fig. 5A). Thus, taken together these data demonstrate that the osmotic shock stimulation of GLUT4 translocation can occur in a path-

**Fig. 3.** Syntaxin-4 functions as the plasma membrane t-SNARE for both osmotic shock and insulin-stimulated GLUT4 vesicle translocation. Differentiated 3T3L1 adipocytes were infected with the empty vaccinia virus (lanes 1–3) or vaccinia virus encoding for the cytoplasmic domain of syntaxin-4 (Syn4ΔTM) (lanes 4–6). Following infection for 4 h, the cells were either unstimulated (C, lanes 1 and 4) or stimulated with 600 nM d-sorbitol (S, lanes 2 and 5) or 100 nM insulin (I, lanes 3 and 6) as described in the Fig. 1 legend. Plasma membrane sheets were prepared, and the resultant membranes were solubilized and subjected to GLUT4 immunoblotting as described under “Experimental Procedures.”

**Fig. 4.** Osmotic shock stimulation of GLUT4 translocation is wortmannin-independent. Differentiated 3T3L1 adipocytes were incubated in the absence (−Wort; panels A, C, and E) or presence of 100 nM wortmannin (+Wort; panels B, D, and F) for 15 min at 37 °C. The cells were either left untreated (panels A and B) or stimulated with 100 nM insulin (panels C and D) or 600 nM d-sorbitol (panels E and F) as described under “Experimental Procedures.” Plasma membrane sheets were then prepared and subjected to GLUT4 immunofluorescence microscopy.
cells were either left untreated (C, lanes 1 and 6) or stimulated with 100 nM insulin (I, lanes 2 and 3) or with 600 mM D-sorbitol (S, lanes 4 and 5) for the times indicated at 37 °C. Whole cell detergent extracts were prepared and immunoblotted for Akt as described under “Experimental Procedures.” B, differentiated 3T3L1 adipocytes were either left untreated (C, lane 1) or stimulated with 100 nM insulin (I, lanes 2 and 3) or with 600 mM D-sorbitol (S, lanes 4 and 5) for the times indicated at 37 °C. Whole cell detergent extracts were prepared, immunoprecipitated with an Akt antibody, and subjected to in vitro phosphorylation of histone 2B (H2B) as described under “Experimental Procedures.” C, differentiated 3T3L1 adipocytes were either left untreated (C, lane 1) or stimulated with 100 nM insulin (I, lanes 2 and 3) or with 600 mM D-sorbitol (S, lanes 4 and 5) for the times indicated at 37 °C. Whole cell detergent extracts were prepared and immunoblotted with a phosphospecific p38 antibody as described under “Experimental Procedures.”

**FIG. 5.** Insulin but not osmotic shock stimulates Akt kinase activity. A, differentiated 3T3L1 adipocytes were either left untreated (C, lanes 1 and 6) or stimulated with 100 nM insulin (I, lanes 2 and 3) or with 600 mM D-sorbitol (S, lanes 4 and 5) for the times indicated at 37 °C. Whole cell detergent extracts were prepared and immunoblotted for Akt as described under “Experimental Procedures.” B, differentiated 3T3L1 adipocytes were either left untreated (C, lane 1) or stimulated with 100 nM insulin (I, lanes 2 and 3) or with 600 mM D-sorbitol (S, lanes 4 and 5) for the times indicated at 37 °C. Whole cell detergent extracts were prepared, immunoprecipitated with an Akt antibody, and subjected to in vitro phosphorylation of histone 2B (H2B) as described under “Experimental Procedures.” C, differentiated 3T3L1 adipocytes were either left untreated (C, lane 1) or stimulated with 100 nM insulin (I, lanes 2 and 3) or with 600 mM D-sorbitol (S, lanes 4 and 5) for the times indicated at 37 °C. Whole cell detergent extracts were prepared and immunoblotted with a phosphospecific p38 antibody as described under “Experimental Procedures.”

**FIG. 6.** Insulin and osmotic shock-stimulated GLUT4 translocation is independent of calcium. Differentiated 3T3L1 adipocytes were incubated in medium containing 2 mM CaCl2 (Vehicle; panels A, C, and E) or in calcium-free medium containing 50 mM BAPTA-AM and 5 mM EGTA (BAPTA/EGTA; panels B, D, and F) for 15 min at 37 °C. The cells were either left untreated (panels A and B) or stimulated with 100 nM insulin (panels C and D) or with 600 mM D-sorbitol (panels E and F) as described under “Experimental Procedures.” Plasma membrane sheets were then prepared and subjected to GLUT4 immunofluorescence microscopy.

**FIG. 7.** Insulin and osmotic shock-stimulated GLUT4 translocation is prevented by the tyrosine kinase inhibitor genistein. Differentiated 3T3L1 adipocytes were incubated in the absence (− Gen; panels A, C, and E) or presence of 300 μM genistein (+ Gen; panels B, D, and F) for 15 min at 37 °C. The cells were either left untreated (panels A and B) or stimulated with 100 nM insulin (panels C and D) or with 600 mM D-sorbitol (panels E and F) for 30 min as described under “Experimental Procedures.” Plasma membrane sheets were then prepared and subjected to GLUT4 immunofluorescence microscopy.

Osmotic Shock Stimulates GLUT4 Translocation by a Calcium-independent Pathway—Several studies in skeletal muscle have observed that exercise/contraction-stimulated GLUT4 translocation occurs by a wortmannin-insensitive pathway (61–63). In addition, various receptor agonists that are coupled to Gq have been reported to induce GLUT4 translocation in both transfected fibroblasts and adipocytes (64). Since all these stimuli result in elevated intracellular calcium, we therefore compared the effect of calcium chelation on both insulin- and osmotic shock-stimulated GLUT4 translocation (Fig. 6). Incubation of 3T3L1 adipocytes in the presence of EGTA and the cell-permeant calcium chelator BAPTA-AM, conditions that block calcium-dependent signaling (65), had no effect on the basal steady-state level of plasma membrane-associated GLUT4 protein (Fig. 6, A and B). The insulin-stimulated appearance of GLUT4 on the plasma membrane was unaffected by the presence of EGTA and BAPTA-AM, consistent with a calcium-independent mechanism of translocation (Fig. 6, C and D). Similarly, the osmotic shock-stimulated translocation of GLUT4 was also not affected by EGTA and BAPTA-AM calcium chelation (Fig. 6, E and F). In addition, insulin and osmotic shock stimulation did not have any effect on intracellular calcium ion concentrations as determined by Fura 2 fluorescence (data not shown).

Osmotic Shock Stimulates GLUT4 Translocation by a Tyrosine Kinase-dependent Pathway—Having ruled out both the Akt and PI 3-kinases, as well as changes in intracellular calcium, we next assessed whether osmotic shock stimulated GLUT4 translocation in a phosphotyrosine kinase-dependent pathway (Fig. 7). This was initially examined using the relatively specific tyrosine kinase inhibitor genistein. Unstimulated cells, either in the absence or presence of genistein, had a low level of GLUT4 immunofluorescence from isolated plasma membrane sheets (Fig. 7, A and B). As expected, insulin stimulation increased plasma membrane sheet GLUT4 immunofluorescence, which was inhibited by pretreatment with genistein (Fig. 7, C and D). Surprisingly, however, the osmotic shock-stimulated increase in GLUT4 plasma membrane immunofluorescence was also inhibited by genistein pretreatment (Fig. 7, E and F).

Although genistein is a relatively specific inhibitor of tyrosine kinase activity, it is possible that the prevention of GLUT4 translocation was due to some other nonspecific effect of this inhibitor. To address this issue, we next utilized single cell microinjection of a monoclonal phosphotyrosine antibody to examine the effect of insulin and osmotic shock on GLUT4 translocation (Fig. 8). To identify the specific 3T3L1 adipocytes that were microinjected following preparation of the plasma membrane sheets, the cells were co-injected with the carboxyl-
terminal domain of Ras fused to the maltose-binding protein (MBP-Ras) (39). The identification of plasma membrane sheets isolated from microinjected cells was detected by MBP-Ras immunofluorescence (Fig. 8, A, C, and E). Co-injection with a nonspecific mouse monoclonal antibody had no significant effect on insulin-stimulated GLUT4 translocation compared with the surrounding cells that were not microinjected (Fig. 8, A and B). As expected, the monoclonal PY20 phosphotyrosine antibody inhibited the insulin-stimulated translocation of GLUT4 in the cells that were microinjected but not in the neighboring noninjected cells (Fig. 8, C and D). Similarly, microinjection of the monoclonal PY20 antibody also prevented the osmotic shock-stimulated translocation of GLUT4 (Fig. 8, E and F). The fact that two independent methods to inhibit tyrosine kinase activity (genistein treatment and PY20 microinjection) prevent both insulin- and osmotic shock-stimulated GLUT4 translocation provides compelling evidence that osmotic shock utilizes a tyrosine kinase pathway to mediate GLUT4 translocation.

Osmotic Shock Stimulates Tyrosine Phosphorylation of Several Distinct Proteins—Based upon the inhibition of both insulin- and osmotic shock-stimulated GLUT4 translocation by genistein and the PY20 phosphotyrosine antibody, we next compared the tyrosine phosphorylation of proteins induced by insulin and osmotic shock treatment (Fig. 9). Insulin treatment resulted in the tyrosine phosphorylation of several proteins detected in phosphotyrosine immunoblots of whole cell detergent extracts (Fig. 9A, lanes 1 and 2). The two most predominant bands at approximately 185 and 95 kDa represent IRS-1 and the insulin receptor β subunit, respectively. In unstimulated cells, there were also predominant tyrosine-phosphorylated bands in the 120–130-kDa region that did not appear to change significantly following insulin stimulation. However, this region of the gel contains multiple protein bands, and we cannot exclude the possibility that a subset of tyrosine-phosphorylated proteins within this molecular weight range are affected by insulin. Nevertheless, pretreatment with genistein not only inhibited the insulin-stimulated tyrosine phosphorylation of IRS-1 and the insulin receptor β subunit but also markedly reduced the extent of basal tyrosine phosphorylation of the 120–130-kDa proteins (Fig. 9A, lanes 3 and 4). In comparison with insulin stimulation, osmotic shock stimulated a dramatic increase in the tyrosine phosphorylation of proteins in the 120-kDa region as well as several proteins ranging from 55–75 kDa (Fig. 9A, lanes 5 and 6). However, osmotic shock did not induce the tyrosine phosphorylation of either the insulin receptor or IRS-1 as determined by a phosphotyrosine immunoblotting of whole cell extracts (Fig. 9A, lanes 5 and 6) and by specific immunoprecipitation of the insulin receptor or IRS-1 (data not shown). As expected, genistein pretreatment of the 3T3L1 adipocytes inhibited both the basal and osmotic shock-stimulated increase in protein tyrosine phosphorylation (Fig. 9A, lanes 7 and 8).

To determine whether any of these tyrosine-phosphorylated proteins were specifically localized to the plasma membrane, we performed phosphotyrosine immunoblotting on isolated plasma membrane sheets (Fig. 9B). Insulin stimulation resulted in the tyrosine phosphorylation of the insulin receptor β subunit without any detectable tyrosine phosphorylation of plasma membrane-associated IRS-1 protein (Fig. 9B, lanes 1 and 2). However, there was a basal tyrosine-phosphorylated protein at approximately 125 kDa that was dephosphorylated following insulin treatment. We and others have previously reported that in fibroblast cell lines the focal adhesion kinase (pp125FAK) is tyrosine-dephosphorylated in response to insulin and osmotic shock (66–69). Consistent with these data, the insulin-stimulated tyrosine dephosphorylation of the 125-kDa plasma membrane-associated protein co-migrated with pp125FAK (Fig. 9B, lanes 4–6). Although insulin stimulation resulted in the tyrosine phosphorylation of the insulin receptor β subunit and tyrosine dephosphorylation of pp125FAK, under the same experimental conditions osmotic shock had no significant effect on the tyrosine phosphorylation of any detectable plasma membrane-associated proteins (Fig. 9B, lane 3). As a control, the identical plasma membrane sheets were subjected to GLUT4 immunoblotting, which demonstrated the typical insulin- and osmotic shock-induced translocation of GLUT4 protein (Fig. 9B, lanes 7–9).

Osmotic Shock Stimulates the Tyrosine Phosphorylation of p130cas, Cbl, and CADTK—In the 120–130-kDa range, there are several known tyrosine-phosphorylated proteins, in particular p130cas, Cbl, and CADTK. CADTK is a newly identified member of the pp125FAK family of tyrosine kinases also known as Pyk2, CAKβ, or RAFTK (44, 70–72). These proteins undergo tyrosine phosphorylation and/or kinase activation in response...
to various growth factors, Src transformation, cell adhesion, increased intracellular calcium, and/or protein kinase C activation. CADTK is expressed in a number of epithelial, neural, and hematopoietic cell types but has not been detected in NIH3T3 fibroblasts (44). However, CADTK was apparently expressed in both 3T3L1 fibroblasts and 3T3L1 adipocytes (Fig. 10, lanes 1 and 2). In contrast, p130-cas and pp125FAK were both down-regulated during adipocyte differentiation (Fig. 10, lanes 3–6), whereas Cbl was up-regulated (Fig. 10, lanes 7 and 8).

To examine the possibility that these proteins were potential targets for the osmotic shock-stimulated tyrosine phosphorylation in 3T3L1 adipocytes, we immunoprecipitated these proteins followed by phosphotyrosine immunoblotting (Fig. 11). Similar to pp125FAK, p130-cas was tyrosine-phosphorylated in the basal state (Fig. 11A, lane 1) and, following insulin stimulation, underwent tyrosine dephosphorylation (Fig. 11A, lanes 2 and 3). The insulin-stimulated tyrosine dephosphorylation of p130-cas is consistent with previous studies, indicating that p130-cas is a substrate for pp125FAK and that pp125FAK tyrosine kinase activity is dependent upon pp125FAK tyrosine phosphorylation (73–76). Although osmotic shock had no apparent effect on pp125FAK tyrosine phosphorylation (Fig. 9), there was a marked increase in the tyrosine phosphorylation of p130-cas (Fig. 11A, lanes 4 and 5).

Insulin treatment of 3T3L1 adipocytes had no effect on the tyrosine phosphorylation of CADTK (Fig. 11B, lanes 1–3). In contrast, osmotic shock stimulation resulted in a rapid and persistent tyrosine phosphorylation of CADTK (Fig. 11B, lanes 4 and 5). Based upon the similarity between pp125FAK and CADTK, and the fact that angiotensin II-dependent p130-cas tyrosine phosphorylation appears to involve CADTK (77), it is likely that the osmotic shock-stimulated tyrosine phosphorylation of p130-cas resulted from the activation of CADTK.

Recently, Cbl has been reported to be tyrosine-phosphorylated in response to insulin in 3T3L1 adipocytes but not in fibroblasts or undifferentiated 3T3L1 cells (78). In addition, the tyrosine phosphorylation of Cbl was relatively rapid but transient. Consistent with these findings, we have also observed a transient insulin-stimulated tyrosine phosphorylation of Cbl in our 3T3L1 adipocytes (Fig. 11C, lanes 2 and 3). Similarly, osmotic shock also resulted in a transient tyrosine phosphorylation of Cbl (Fig. 11C, lanes 4 and 5). All these changes in the tyrosine phosphorylation of p130-cas, CADTK, and Cbl were not due to differences in protein loading based upon the immunoblotting of these immunoprecipitates (data not shown).

**DISCUSSION**

It has been well established that insulin stimulates the translocation of the GLUT4 glucose transporter isoform from intracellular vesicular storage sites to the plasma membrane in both muscle and adipose cells. This translocation process accounts for the majority of the insulin-stimulated increase in glucose transport activity. Although the mechanism by which insulin orchestrates these intracellular trafficking events has not been determined, several important signaling molecules have been implicated in this process. For example, reduction of IRS-1 level by either ribozyme expression in adipocytes or homologous recombination in mice resulted in an impairment of insulin-stimulated glucose transport activity and GLUT4 translocation (79–81). Recently, several studies have observed that specific PI 3-kinase inhibitors and expression of a dominant interfering PI 3-kinase regulatory subunit prevented both insulin-stimulated GLUT4 translocation and glucose transport activity (15–18, 20, 24). Consistent with these findings, expression of a constitutively active PI 3-kinase induced GLUT4 translocation in the absence of insulin (19, 21–26). More recently, it has been shown that growth factor activation of the Akt kinase is PI 3-kinase-dependent, and stable overexpression of a constitutively active membrane-bound form of Akt induces
persistent activation of glucose transport activity and cell surface localization of the GLUT4 protein (31, 32).

In addition to the identification of the signal transduction pathways directly leading from the insulin receptor to downstream targets, there are several insulinomimetic agents that can stimulate glucose transport in adipocytes and muscle. Introduction of nonhydrolyzable GTP analogues, muscle contraction/exercise, okadaic acid, and anoxia can all stimulate glucose transport activity (40, 82–86). Since none of these stimuli result in the activation of the insulin receptor kinase itself, it is presumed that they converge with the insulin signal pathway at a more distal signaling step. In particular, early studies on glucose transport activity observed that osmotic shock is a potent activator of glucose uptake in adipocytes and skeletal muscle (34–38). Thus, we reasoned that the dissection of osmotic shock versus insulin signaling might provide new insight into glucose transport regulation by identifying common and/or distinct pathways leading to GLUT4 translocation.

Based on this speculation, we first determined whether osmotic shock stimulated glucose transport activity by inducing the translocation of GLUT4. Our data demonstrate that, compared with insulin, the relative extent of GLUT4 translocation (approximately 50% of the insulin effect) directly correlated with the relative increase in glucose transport activity. More interestingly, the ability of osmotic shock to stimulate GLUT4 translocation occurred by a PI 3-kinase-insensitive pathway. The presence of a PI 3-kinase-insensitive pool of translocating GLUT4 vesicles has also been observed in other systems. For example, exercise or contraction of skeletal muscle is also a potent activator of glucose transport activity and GLUT4 translocation, which occurs in a wortmannin-insensitive manner (61, 63). Furthermore, GLUT4 translocation induced by other stimuli such as the phosphatase inhibitor, okadaic acid, or uncouplers of oxidative phosphorylation is also independent of PI 3-kinase activation (85, 87, 88).

Since a previous study observed that osmotic shock of COS-7 cells resulted in the activation of Akt (33), we speculated that the Akt kinase could be a potential point of convergence between insulin and osmotic shock signaling downstream of the PI 3-kinase. However, in contrast to insulin, osmotic shock was unable to induce either Akt phosphorylation or protein kinase activation in differentiated 3T3L1 adipocytes. At present, we do not know the basis for the difference between osmotic shock stimulation of Akt in COS-7 cells versus 3T3L1 adipocytes. Nevertheless, our data clearly demonstrate that the ability of osmotic shock to induce GLUT4 translocation is independent of both PI 3-kinase and Akt activation.

Surprisingly, however, pretreatment of the 3T3L1 adipocytes with the selective tyrosine kinase inhibitor genistein prevented both insulin- and osmotic shock-stimulated GLUT4 translocation. Consistent with the requirement of a tyrosine kinase signaling cascade, single cell microinjection of phosphotyrosine antibodies also prevented both insulin- and osmotic shock-stimulated GLUT4 translocation. Although there are caveats associated with the use of both kinase inhibitors and high concentrations of microinjected antibodies, the fact that both agents that utilize distinct mechanisms to block protein kinase activity were effective independent inhibitors of insulin- and osmotic shock-stimulated GLUT4 translocation strongly suggests that osmotic shock functions through a tyrosine kinase signaling pathway. The ability of osmotic shock to directly stimulate tyrosine phosphorylation events was confirmed by phosphotyrosine immunoblotting. Several discrete tyrosine-phosphorylated proteins in the range of 115–130 kDa and 55–70 kDa were clearly induced by osmotic shock treatment without any evidence of either insulin receptor or IRS-1 tyrosine phosphorylation. In addition, no detectable plasma membrane proteins were tyrosine-phosphorylated in response to osmotic shock. Consistent with previous studies, we did observe an apparent insulin-induced dephosphorylation of plasma membrane-associated pp125Fak. Furthermore, insulin also induced the tyrosine dephosphorylation of p130cas, which presumably results from the tyrosine dephosphorylation and inactivation of pp125Fak activity. In any case, since osmotic shock had no effect on the phosphorylation state of pp125Fak but enhanced the tyrosine phosphorylation of p130cas, it is unlikely that pp125Fak or p130cas are involved in a convergent pathway leading to GLUT4 translocation.

pp125Fak and CADTK are two members of a new tyrosine kinase family characterized by a centrally located tyrosine kinase domain, at least two proline-rich domains, and a focal adhesion targeting sequence. However, these enzymes do not have intrinsic SH2 or SH3 domains but rather provide tyrosine autophosphorylation sites and SH3 domain recognition sequences with which other SH2 and SH3 domain containing effector proteins can associate. CADTK was originally identified as a trimeric G protein-coupled receptor-stimulated soluble...
tyrosine kinase that can also be activated by both calcium- and protein kinase C-dependent and independent pathways (44–72). One important difference between pp125FAK and CADTK is that in adherent cells pp125FAK is constitutively tyrosine-phosphorylated, whereas CADTK appears to be natively inactive or tyrosine-phosphorylated (77). Rather, CADTK is rapidly activated (within 15 s) by a variety of agonists and growth factors (44). The basal tyrosine phosphorylation state of pp125 FAK has been correlated with pp125 FAK activation, whereas CADTK appears responsible for the agonist-dependent tyrosine phosphorylation of these two cytoskeletal proteins (44, 89, 90). These results are similar to those presented in this manuscript in which the basal and insulin-stimulated tyrosine phosphorylation state of p130cas appears to be controlled by pp125FAK. However, the osmotic shock-dependent increase in CADTK tyrosine phosphorylation correlated with the increase in p130cas tyrosine phosphorylation. Thus, CADTK retains a potential tyrosine kinase candidate that (i) phosphorylates p130cas and (ii) mediates the tyrosine phosphorylation pathway from osmotic shock to GLUT4 translocation. In addition to pp125FAK and p130cas, CADTK, Cbl is another protein in this molecular weight range that undergoes tyrosine phosphorylation by multiple stimuli. Recently, insulin has been reported to induce the tyrosine phosphorylation of Cbl in differentiated 3T3L1 adipocytes but not in 3T3L1 fibroblasts or other cell types transfected to express the insulin receptor (78). We have also observed that both insulin and osmotic shock induced a transient tyrosine phosphorylation of Cbl. However, osmotic shock stimulated a significantly greater extent of Cbl tyrosine phosphorylation than insulin but was not as effective as insulin in promoting GLUT4 translocation. Thus, although it remains possible that Cbl tyrosine phosphorylation is a point of convergence between insulin- and osmotic shock-induced GLUT4 translocation, these differences make this possibility less likely.

In summary, our data demonstrate that there is a novel insulin receptor-independent tyrosine kinase pathway leading to GLUT4 translocation in adipocytes. This signaling pathway does not require the targeting and/or activation of the PI 3-kinase or the Akt kinase. However, it apparently utilizes, at least in part, the same vesicle docking/fusion mechanism (synacin-4) that is used by insulin. Whether CADTK, which is a gene-specific inhibitory tyrosine kinase that is activated by osmotic shock, and/or Cbl tyrosine phosphorylation are causally related to the stimulation of GLUT4 translocation is an important issue for future investigation.

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