To identify potential proteins interacting with the insulin-responsive glucose transporter (GLUT4), we generated fusion proteins of glutathione S-transferase (GST) and the final 30 amino acids from GLUT4 (GST-G4) or GLUT1 (GST-G1). Incubation of these carboxyl-terminal fusion proteins with adipocyte cell extracts revealed a specific interaction of GLUT4 with fructose 1,6-bisphosphate aldolase. In the presence of aldolase, GST-G4 but not GST-G1 was able to co-pellet with filamentous (F)-actin. This interaction was prevented by incubation with the aldolase substrates, fructose 1,6-bisphosphate or glyceraldehyde 3-phosphate. Immunofluorescence confocal microscopy demonstrated a significant co-localization of aldolase and GLUT4 in intact 3T3L1 adipocytes, which decreased following insulin stimulation. Introduction into permeabilized 3T3L1 adipocytes of fructose 1,6-bisphosphate or the metabolic inhibitor 2-deoxyglucose, two agents that disrupt the interaction between aldolase and actin, inhibited insulin-stimulated GLUT4 exocytosis without affecting GLUT4 endocytosis. Furthermore, microinjection of an aldolase-specific antibody also inhibited insulin-stimulated GLUT4 translocation. These data suggest that aldolase functions as a scaffolding protein for GLUT4 and that glucose metabolism may provide a negative feedback signal for the regulation of glucose transport by insulin.

The insulin-responsive glucose transporter GLUT4 is expressed primarily in adipose tissue, skeletal, and cardiac muscle (1–4). Under basal conditions, GLUT4 slowly recycles between poorly defined intracellular compartments and the plasma membrane with the vast majority sequestered in these intracellular storage sites. Insulin stimulates a large increase in the rate of GLUT4 exocytosis concomitant with a smaller decrease in the rate of GLUT4 endocytosis (5–7). The overall insulin-induced changes in GLUT4 trafficking kinetics result in a 10–20-fold increase in the number of cell surface GLUT4 proteins that accounts for the majority of insulin-stimulated increases in glucose transport activity (8, 9).

Recently, several laboratories have begun to examine the subcellular distribution of GLUT4 to identify the mechanism responsible for the intracellular sequestration of the GLUT4 protein. Steady-state and kinetic analysis of various expressed GLUT4 chimeric proteins have indicated that both the amino- and carboxyl-terminal domains are important in GLUT4 internalization from the plasma membrane (10–15). In particular, the carboxyl-terminal dileucine motif (SLL) was found to substantially alter GLUT4 trafficking kinetics and steady-state localization (16, 17). Although a specific GLUT4 sequence responsible for intracellular localization has yet to be identified, the presence of a carboxyl-terminal retention signal is consistent with the observation that expression of the GLUT4 carboxyl-terminal domain results in the translocation of the endogenous GLUT4 protein to the plasma membrane (18). In addition, the presence of a GLUT4 carboxyl-terminal binding protein can also account for the apparent increase in GLUT4 carboxyl-terminal antibody immunoreactivity following insulin stimulation (19, 20).

To address these issues, we have used GST fusion proteins to identify and characterize GLUT4 carboxyl-terminal specific binding proteins. In this manuscript, we demonstrate that the carboxyl-terminal domain of GLUT4 can specifically associate with the bifunctional glycolytic enzyme fructose-1,6-bisphosphate aldolase. This in vitro interaction appears to be physiologically important as glycolytic intermediates that disrupt aldolase-actin interactions also inhibit insulin-stimulated GLUT4 translocation.

**EXPERIMENTAL PROCEDURES**

cDNA Constructs—DNA fragments encoding the carboxyl-terminal sequences of GLUT1 (G1), IASFGREQGASQSAKTPEELPHPLGADSOQ, GLUT4 (G4), SATFRRTPSLEEQEYKPESTLEYLGPDEND; and GLUT4* (G4*), SATFRRTPSASEEQEYKPESTLEYLGPDEND were provided by Dr. Morris Birnbaum and subcloned into pGEX-KG vector. The resultant glutathione S-transferase (GST) fusion proteins were purified by glutathione affinity chromatography as described by the manufacturer (Amersham Pharmacia Biotech).

**GST Fusion Protein Pull-down Experiments—**3T3L1 fibroblasts were differentiated into adipocytes as described (21). In labeling experiments, 3T3L1 adipocytes were incubated with 10 μCi/ml Tran35S label (ICN) for 24 h before isolation of cell extracts in 50 mM Hepes, pH 7.4, 1% Triton X-100, 10% glycerol. Insoluble material was separated by microcentrifugation and extracts precleared by incubation with glutathione-Sepharose beads for 1 h at 4°C followed by a second 1 h incubation with the GST fusion protein. The beads were then washed three times with 50 mM Hepes, pH 7.4, 0.1% Triton-100, 10% glycerol, bound proteins eluted using Laemmli sample buffer and samples subjected to SDS-PAGE, and autoradiography or aldolase immunoblotting. For large scale preparation of the GST-GLUT4 binding proteins, rat epididymal fat pads were isolated from 50 rats. Cell extracts were incubated with the GST fusion proteins, eluants transferred onto polyvinylidene difluoride membrane and N-terminal sequences determined using an automated protein sequencer at the University of Michigan
Aldolase Mediates the Association of F-actin and GLUT4

To identify proteins that interact with the GLUT4 carboxyl-terminal domain, we expressed the GLUT1 and GLUT4 carboxyl-terminal 30 amino acids as GST fusions, GST-G1 and GST-G4, respectively. An additional fusion construct of the GLUT4 carboxyl terminus was generated containing a substitution of two residues shown to be important in GLUT4 endocytosis, leucines 488 and 489, with alanine and serine (GST-G4*), respectively (15–17). Incubation of GST and GST-G4 with extracts from [35S]methionine-labeled 3T3L1 adipocytes resulted in the binding of multiple proteins. However, there were two faint bands at 40 and 43 kDa that appeared to specifically precipitate with GST-G4 and GST-G4* but not with GST or GST-G1 (Fig. 1A). Pretreatment of 3T3L1 adipocytes with insulin before isolation of the cell extracts had no significant effect on the in vitro association of the 40 and 43 kDa protein with GST-G4 and GST-G4* (Fig. 1A).

Cell extracts were also prepared from primary isolated rat adipocytes and incubated with the GST fusion proteins (Fig. 1B). Similar to 3T3L1 adipocytes, Coomassie Blue staining of these precipitates demonstrated that the 40- and 43-kDa proteins were specifically precipitated with GST-G4 and GST-G4* but not by GST or GST-G1. Although other more intense bands were detected on the Coomassie Blue stained gel, these were also seen in the absence of lysate and were therefore nonspecific. We could not obtain a defined amino acid sequence following purification of the 43-kDa protein; however, the 40-kDa protein was partially sequenced and identified as fructose 1,6-bisphosphate aldolase. To confirm that the 40-kDa protein that bound GST-G4 and GST-G4* was aldolase, immunoblotting with an aldolase-specific antibody was performed (Fig. 1C). Incubation of cell extracts with GST-G4 and GST-G4* resulted in the co-precipitation of a 40-kDa protein that demonstrated specific immunoreactivity with an aldolase antibody. In contrast, no immunoreactive proteins were detected in the GST or GST-G1 precipitates. Furthermore, as observed in the [35S]methionine-labeled 3T3L1 adipocytes, cell extracts from insulin-stimulated cells also precipitated aldolase immunoreactivity in the presence of GST-G4 and GST-G4* but not GST or GST-G1. These data demonstrate that the 40-kDa protein that specifi-
Aldolase Mediates the Association of F-actin and GLUT4

Aldolase is an unusual protein in that it not only possesses enzymatic activity but also plays a structural role in the assembly of the actin cytoskeleton (26). The interaction of aldolase with actin can be modulated by aldolase substrates and products (27, 28). We therefore sought to determine the allosteric effect of these and related molecules on the binding of aldolase to GST-G4 (Fig. 2). Dose-response relationships demonstrated that 1 mM FBP, a concentration similar to the $K_m$ of the enzyme for this substrate, completely inhibited the binding of aldolase to GST-G4 in vitro (Fig. 2A). The aldolase reaction product, G3P, also inhibited GST-G4 binding to aldolase, although at a significantly higher concentration, 10 mM (Fig. 2A). In contrast, the aldolase product dihydroxyacetone phosphate, as well as two other structurally related sugars, fructose 1-phosphate and fructose 6-phosphate, had no effect on the interaction between GST-G4 and aldolase (Fig. 2, C, D, and E).

Previous studies have demonstrated that aldolase is associated with the actin cytoskeleton and can cross-link actin fibers (27, 29). Because the actin cytoskeleton has also been implicated in the regulation of GLUT4 trafficking (30, 31), we next examined the ability of aldolase to function as a molecular scaffold linking the carboxyl-terminal domain of GLUT4 with filamentous actin.

To determine whether aldolase and GLUT4 were co-localized in vivo, double immunofluorescence labeling was performed. 3T3L1 adipocytes were treated with or without insulin, fixed, and incubated with aldolase and GLUT4-specific antibodies followed by labeling with complimentary fluorescent-conjugated secondary antibodies (Fig. 4). Under basal conditions, the membrane bound GLUT4 protein was found primarily in a peri-nuclear distribution and in small vesicles scattered throughout the cytoplasm. Following insulin stimulation, GLUT4 moved to the cell surface generating a “rim”-like fluorescence (Fig. 4, panel A). Conversely, the soluble protein aldolase stained in a diffusely cytosolic pattern although it was also concentrated in the perinuclear region of the cell (Fig. 4, panels B and E). When their respective labels are superimposed, GLUT4 and aldolase exhibited a high degree of co-localization (Fig. 4, panels C and F), suggesting an in vivo association between aldolase and the GLUT4-containing compartments.

To further investigate the in vivo significance of these findings, we took advantage of the glycolytic inhibitor 2DG, which induces the dissociation of perinuclear aldolase from the actin cytoskeleton (32). In adipocytes, the insulin-induced translocation of GLUT4 to the plasma membrane can be detected by the fluorescent detection of a GLUT4 antibody on the cytoplasmic face of isolated plasma membrane sheets. Pretreatment of 3T3L1 adipocytes with 5 mM 2DG slightly reduced the amount of basal plasma membrane GLUT4 immunofluorescence (Fig. 5A, panels 1 and 3). In contrast, pretreatment with 2DG markedly attenuated the insulin-stimulated translocation of GLUT4 to the cell surface (Fig. 5A, panels 2 and 4). The 2DG inhibition of insulin-stimulated GLUT4 translocation occurred in a dose-dependent manner with an EC$_{50}$ of approximately 100 mM (data not shown). This inhibition of GLUT4 translocation was also observed in GLUT4 immunoblots of isolated plasma membranes without any effect on the plasma membrane association of the clathrin heavy chain (Fig. 5B).

The blockade of insulin-stimulated GLUT4 translocation by 2DG could be due to either an inhibition of exocytosis or an increase in endocytosis. To determine whether 2DG affected the rate of GLUT4 endocytosis, we stimulated cells with insulin, removed the insulin by washing with an acidic buffer, and then assayed plasma membrane GLUT4 at various times after insulin removal in the presence of 2DG. Disappearance of GLUT4 from the cell surface after insulin removal was not inhibited by 2DG treatment but rather seemed to be slightly enhanced (data not shown). This small increase in the rate of GLUT4 endocytosis in the presence of 2DG likely reflected the inhibition of further GLUT4 exocytosis following insulin removal. This effect was similar to that reported for wortmannin.
which also inhibits GLUT4 exocytosis but appears to enhance endocytosis due to the inhibition of residual exocytosis (33). These data demonstrate that the rate of GLUT4 endocytosis was not significantly affected by 2DG pretreatment and is consistent with an inhibition of GLUT4 exocytosis.

Although cells were incubated with glutamine and pyruvate as alternative energy sources during 2DG treatment, cellular ATP levels were found to decrease by 40% (data not shown). Because endocytosis and exocytosis may have different sensitivities to ATP depletion, the inhibition of insulin-stimulated GLUT4 exocytosis could have been due to this decrease in ATP.

To ensure adequate cellular ATP levels while directly assessing the uncoupling of aldolase from GLUT4 in vivo, 3T3L1 adipocytes were permeabilized with SLO in the presence of 10 mM MgATP (Fig. 5, C and D). Under these experimental conditions, cellular metabolism did not deplete local concentrations of ATP (data not shown). In the SLO-permeabilized adipocytes, insulin induced an increase in the translocation of GLUT4 to the plasma membrane (Fig. 5C, panels 1 and 2). As in the intact cells, preincubation with 2DG prevented GLUT4 translocation (data not shown). In addition, although preincubation with FBP slightly enhanced the basal amount of GLUT4 associated...
with the plasma membrane, FBP pretreatment markedly inhibited the insulin-stimulated translocation of GLUT4 (Fig. 5C, panels 3 and 4). It should be noted that the small increase in plasma membrane-associated GLUT4 following SLO permeabilization has also been previously observed by others (25). In agreement with the immunofluorescence analysis, immunoblotting of isolated plasma membranes demonstrated that FBP preincubation partially increased the basal plasma membrane-associated GLUT4 and inhibited the insulin-stimulated increase without affecting the plasma membrane association of the clathrin heavy chain (Fig. 5D).

To examine the metabolite specificity for the inhibition of insulin-stimulated GLUT4 translocation, we compared the effects of several aldolase substrates, products and related sugars on GLUT4 translocation (data not shown). Consistent with their effect on aldolase-actin binding, pretreatment of SLO-permeabilized adipocytes with dihydroxyacetone phosphate, fructose 1-phosphate, fructose 6-phosphate, or glucose had no significant effect on insulin-stimulated GLUT4 translocation compared with control cells. To further confirm a specific requirement for aldolase in insulin-stimulated GLUT4 translocation, we next microinjected cells with control or aldolase antibodies and assessed single cell GLUT4 translocation (Fig. 6). Specific identification of the microinjected cells was accomplished by co-injection with a carboxyl-terminal domain of Ras fused to the MBP as a plasma membrane marker. In unstimulated cells, microinjection of either pre-immune IgG or the aldolase IgG had no effect on GLUT4 translocation, which remained at low basal levels (Fig. 6A, panels 1-4). Microinjection of pre-immune IgG did not inhibit the insulin-stimulated translocation of GLUT4 (Fig. 6B, panels 1 and 2). In contrast, the aldolase-specific IgG reduced the extent of GLUT4 translocation only in the cells that were microinjected but not in the surrounding nonmicroinjected cells (Fig. 6B, panels 3 and 4). Quantitation of these data demonstrated that insulin stimulated GLUT4 translocation in 49% of cells microinjected with pre-immune IgG and 29% of cells microinjected with aldolase-specific IgG (Fig. 6C). Together, these data provide compelling evidence for a specific functional role of fructose 1,6-bisphosphate aldolase in the insulin stimulation of GLUT4 translocation in adipocytes.

Previous studies have demonstrated that aldolase exhibits functional duality. In addition to its enzymatic activity, this protein plays a structural role in the binding and polymerization of actin (28, 29, 34). Depolymerization of the actin cytoskeleton with cytochalasin D or latrunculin B has been shown to attenuate insulin-stimulated GLUT4 translocation, suggesting that an intact actin cytoskeleton is required for insulin-stimulated GLUT4 translocation (30, 31). Consistent with this model, our data demonstrate that aldolase, a functional tetramer, may also serve as a scaffolding protein linking GLUT4 and hence GLUT4 vesicles, to the actin cytoskeleton. In 2D experiments, aldolase not only specifically interacted with the carboxyl-terminal domain of GLUT4, but linked GLUT4 to
polymerized actin. This interaction was disrupted by specific substrates of aldolase that inhibit GLUT4-aldolase or aldolase-actin interactions. These specific substrates not only prevented aldolase-actin interaction but also prevented GLUT4-aldolase binding in vitro and in a manner consistent with their ability to interfere with insulin-stimulated GLUT4 translocation in vivo. Furthermore, aldolase and GLUT4 exhibited an overlapping subcellular distribution, and microinjection of an aldolase-specific antibody reduced the insulin-stimulated translocation of GLUT4. Together, these data strongly suggest that aldolase plays a critical role in the dynamic association of GLUT4 vesicles with the actin cytoskeleton. The ability of aldolase substrates to disrupt the actin-aldolase-GLUT4 complex, as well as GLUT4 vesicle trafficking, suggests that the metabolism of glucose may provide a negative feedback signal to prevent further exocytosis of GLUT4 vesicles and potentially contribute to the mechanism of glucotoxicity.

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