Activation of Glycogen Synthase by Insulin in 3T3-L1 Adipocytes Involves c-Cbl-associating Protein (CAP)-dependent and CAP-independent Signaling Pathways*

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In adipose and muscle, insulin stimulates glucose uptake and glycogen synthase activity. Phosphatidylinositol 3-kinase (PI3K) activation is necessary but not sufficient for these metabolic actions of insulin. The insulin-stimulated translocation of phospho-c-Cbl to lipid rafts, via its association with CAP, comprises a second pathway regulating GLUT4 translocation. In 3T3-L1 adipocytes, overexpression of a dominant negative CAP mutant (CAPΔSH3) completely blocked the insulin-stimulated glucose transport and glycogen synthesis but only partially inhibited glycogen synthase activation. In contrast, CAPΔSH3 expression did not affect glycogen synthase activation by insulin in the absence of extracellular glucose. Moreover, CAPΔSH3 has no effect on the PI3K-dependent activation of protein phosphatase-1 or phosphorylation of glycogen synthase kinase-3. These results indicate blockade of the c-Cbl/CAP pathway directly inhibits insulin-stimulated glucose uptake, which results in secondary inhibition of glycogen synthase activation and glycogen synthesis.

Insulin stimulates the storage of glucose as glycogen in muscle and fat through a coordinate increase in glucose uptake and glycogen synthesis (1). Glycogen synthase (GS), the rate-limiting enzyme for glycogen synthesis, is regulated by covalent and allosteric modification (2). Insulin promotes the net dephosphorylation of GS through the activation of glycogen-targeted protein-phosphatase-1 (PP1) and inhibition of glycogen synthase kinase-3 (GSK-3). This action of insulin is facilitated by the stimulation of glucose uptake, since the resulting increase in glucose-6-phosphate leads to the allosteric activation of GS and increased susceptibility to PP1-mediated dephosphorylation (3, 4).

The stimulation of glucose disposal by insulin requires the activation of phosphatidylinositol 3-kinase (PI3K), which leads to the increased activity of PI3K-dependent kinases such as Akt and protein kinase Cζ (5, 6). The PI3K inhibitor wortmannin blocks many of the metabolic actions of insulin, including the effects of the hormone on glucose transport and GS activities (7–9). Moreover, both the inactivation of GSK-3 and the activation of PP1 are blocked by wortmannin (10, 11). However, the activation of PI3K alone is not sufficient for insulin action, since other hormones and adhesion molecules can stimulate PI3K activity without reproducing the metabolic effects of insulin (12, 13). Furthermore, cell-permeable derivatives of PI3K do not fully mimic the effects of insulin on glucose transport (14). Thus, although the PI3K pathway is necessary, there is at least one additional pathway required for glucose transport regulation by insulin.

The PI3K-independent pathway might involve the tyrosine phosphorylation of the proto-oncogene c-Cbl (15). The phosphorylation of c-Cbl by insulin requires the c-Cbl-associated protein (CAP), an adapter molecule that recruits c-Cbl to the insulin receptor (16). CAP expression correlates with c-Cbl phosphorylation and insulin sensitivity. Moreover, expression of the CAP gene is increased by insulin-sensitizing activators of the nuclear receptor PPARγ (17). These data suggest a potential link between CAP/Cbl and the metabolic actions of insulin.

Upon phosphorylation, Cbl translocates to a Triton-insoluble lipid raft microdomain (18), where CAP binds to the caveolar protein flotillin (19). In 3T3-L1 adipocytes, expression of a CAP mutant in which the SH3 domains have been deleted (CAPΔSH3) disrupted the formation of a CAP-flotillin-Cbl complex in lipid rafts, blocked translocation of the insulin-stimulated glucose transporter GLUT4, and inhibited glucose transport, glycogen, and lipid synthesis (19). However, it remained unclear whether CAPΔSH3 directly blocked the signaling pathways involving GS dephosphorylation that are independent of glucose transport. We demonstrate here that the CAP/Cbl pathway regulates GS activation exclusively through a glucose-dependent mechanism.

EXPERIMENTAL PROCEDURES

Materials—All cell culture reagents were purchased from Life Technologies, Inc. with the exception of serum, which was obtained from Summit Biotechnology (Ft. Collins, CO). Insulin, 2-deoxy-glucose, and differentiation agents were supplied by Sigma. UDP-[3H]glucose (308 mCi/mmol) was from by ICN, and d-[14C]glucose (3.4 mCi/mmol) and 2-deoxy-d-[14C]glucose (323 mCi/mmol) were obtained from PerkinElmer Life Sciences. Anti-phospho-Akt (serine 473) and anti-phospho-GSK3 (serine 21/serine 9) antibodies were purchased from New England Biolabs, and horseradish peroxidase goat anti-mouse and goat anti-rabbit IgGs were from Bio-Rad. CAP and CAPΔSH3 expression vectors were constructed as previously described (16, 19). ECL reagent was purchased from Amersham Pharmacia Biotech, and GFA filters were supplied by Whatman.

Cell Culture and Experimental Treatment—3T3-L1 fibroblasts were maintained and differentiated into adipocytes as previously reported (20). Electroporation of adipocytes was performed as described (21). Prior to insulin stimulation, cells were washed two times with low serum medium (Dulbecco's modified Eagle's medium containing 5 mM glucose, 0.5% fetal bovine serum, 25 mM Hepes (pH 7.4), 100 units/ml penicillin, 100 units/ml streptomycin, and 0.29 mg/ml glutamine) and incubated in the same medium for 3 h.
Enzymatic and Metabolic Assays—Glucose transport measurements were done as described previously (22) with minor modifications. Briefly, following serum starvation, adipocytes were washed three times with PBS and placed in 0.5 ml/well Krebs-Ringer buffer with 30 mM Hepes (pH 7.4) and 0.5% bovine serum albumin in the absence and presence of 100 nM insulin. After 30 min at 37 °C, 20 μM 2-deoxy-D-[14C]glucose (20 cpm/pmol) was added to all wells. After 5 min at room temperature, the assay was terminated by the adding 50 μl of 200 mM 2-deoxyglucose and washing the cells 3 times with PBS on ice. Adipocytes were collected in 0.5 ml of distilled water. 10 μl of the cell suspension was retained to determine protein concentration and the remainder was subject to scintillation counting.

Glycogen synthase assays were performed as described (22). In some experiments, the cells were washed and incubated in glucose-free, low serum media immediately prior to insulin treatment. Measurement of PP1 activity was performed as described previously (23), with 1–2 μg of cell extract assayed for 2 min at 30 °C. Glycogen synthesis rate was measured in 6-well dishes as reported (22). Briefly, cells were treated in the absence and presence of 100 nM insulin for 15 min, and then 1 μCi of [14C]glucose (20 cpm/pmol) was added to all wells. After 5 min, cells were washed on ice with PBS, and glycogen was precipitated with 30% potassium hydroxide and quantitated by scintillation counting.

Other Procedures—Immunoblotting (22) and immunoprecipitations (15) were performed as described previously. Protein measurements were determined by the method of Bradford.

RESULTS

CAPΔSH3 Expression Blocks Glucose Uptake and Glycogen Synthesis in 3T3-L1 Adipocytes—CAPΔSH3 overexpression in 3T3-L1 adipocytes significantly inhibits the translocation of the insulin-stimulated glucose transporter GLUT4 to the plasma membrane, without affecting PI3K-dependent signals (19). To explore the implications of this result in more detail, we examined the effects of CAPΔSH3 expression on glucose uptake and glycogen synthesis over a range of insulin concentrations. In control LacZ-transfected 3T3-L1 adipocytes, insulin produced a 15-fold increase in glucose transport (Fig. 1A) and a 10-fold increase in glycogen synthesis (Fig. 1B). Overexpression of CAPΔSH3 decreased maximal glucose uptake by 40–50% (Fig. 1A). The construct blocked the responsiveness to insulin without effecting the EC50 for the hormone. Not surprisingly, CAPΔSH3 expression also blocked insulin-stimulated glycogen synthesis (Fig. 1B), which is dependent on increased glucose uptake under these conditions. Extrapolating for an average 50% transfection efficiency (data not shown), these results demonstrate that expression of CAPΔSH3 in 3T3-L1 adipocytes produces a nearly complete inhibition of insulin-stimulated glucose uptake and glycogen synthesis.

Inhibition of GS Activation by CAPΔSH3 Is a Result of Reduced Glucose Uptake—We next examined the effects of CAPΔSH3 expression on the regulation of GS activity by insulin. Electroporation of 3T3-L1 adipocytes with CAPΔSH3 resulted in a 50% decrease in the activation of GS by insulin, compared with both LacZ- and CAP-expressing cells (Fig. 2A). In these experiments the electroporation efficiency was ~50–60% (data not shown), indicating that CAPΔSH3 overexpression reduced GS activation by half. Previous studies in 3T3-L1 adipocytes indicated that removal of extracellular glucose also decreased the insulin-stimulated GS activity by 40–50% (20), suggesting that the effect of CAPΔSH3 on GS activation may be secondary to inhibition of glucose transport. To evaluate this possibility directly, the electroporated adipocytes were incubated in glucose-free medium during the insulin treatment, and GS activity was assayed in vitro. Removal of extracellular glucose decreased insulin-stimulated GS activity by 40% in LacZ- and CAP-expressing cells (Fig. 2B; compare with Fig. 2A). However, CAPΔSH3 expression had no further inhibitory effect on GS activation by insulin. Thus, the glucose-independent pathway that mediates the dephosphorylation and subsequent activation of GS by insulin appears not to involve the CAP/Cbl pathway.
Wells were pretreated for 30 min with 200 nM wortmannin (indicated constructs and treated as in Fig. 2, except the indicated B. B. B. I.B. Samples were then analyzed by anti-phosphotyrosine and c-Cbl immuno blotting (I.P.). I.P. were performed.

After a 15 min treatment with 100 nM insulin (Ins), glycogen synthase activity was determined in vitro. Results are representative of three independent experiments (A) or the average of three independent experiments performed in duplicate (B). *p < 0.01, n = 3 by Student’s t test.

The PI3K inhibitor wortmannin completely blocked the regulation of GS, PP1, and GSK-3 by insulin (10, 11). In contrast, pretreatment of 3T3-L1 adipocytes with 200 nM wortmannin did not significantly change the insulin-stimulated tyrosine phosphorylation of c-Cbl (Fig. 3A). However, wortmannin pretreatment of transfected cells incubated in glucose-free media completely blocked the residual activation of GS by insulin (Fig. 3B). Thus, a PI3K-dependent pathway is likely to trigger the glucose-independent activation of GS.

CAPΔSH3 Has No Effect on the Regulation of PP1 and GSK-3 by Insulin—Insulin promotes the activation of GS by decreasing the phosphorylation state of the enzyme. Both the activation of glycogen-targeted PP1 and the inactivation of GSK-3 have been proposed to mediate this effect of the hormone (5). We next examined the effect of CAPΔSH3 expression on the regulation of these enzymes by insulin. Electroporation of LacZ, CAP, or CAPΔSH3 into 3T3-L1 adipocytes had no effect on the insulin-stimulated phosphorylation of GSK3 (Fig. 4A), which results in enzymatic inactivation (24). Insulin treatment of LacZ-electroporated cells also caused a 50% increase in PP1 activity (Fig. 4B). Overexpression of either CAP or CAPΔSH3 had no effect on PP1 activity by insulin in 3T3-L1 adipocytes (Fig. 4B). These results cumulatively indicate that the CAP/Cbl pathway is not necessary for the insulin-mediated regulation of enzymes that covalently modify GS.

DISCUSSION

Insulin stimulates glycogen synthesis in muscle and fat by a coordinate increase in GLUT4 vesicle translocation and covalent modification of glycogen metabolizing enzymes (1). Insulin promotes the dephosphorylation of glycogen synthase and phosphorylase, resulting in enzymatic activation and inactivation, respectively. Both phosphatase activation and kinase inactivation contribute to the dephosphorylation of both proteins in response to insulin. Additionally, increased glucose-6-phosphate levels allosterically activate GS, overriding inhibition caused by phosphorylation (2). Finally, glucose metabolites induce the translocation of cytosolic GS to glycogen-containing fractions in primary hepatocytes (25), skeletal muscle (26), and 3T3-L1 adipocytes (20). Insulin therefore activates GS through covalent modification, allosteric activation, and enzymatic translocation. However, the complex interplay between insulin signaling cascades, elevated levels of glucose, and its metabolites and glycogen stores in the regulation of GS activity and glycogen synthesis rates remains unclear.

The activation of PI3K and downstream serine/threonine kinases plays an important role in the hormonal regulation of glucose uptake and storage. Pharmacological inhibition of PI3K blocked the stimulation of glucose transport (7) and the enzymes involved in glycogen metabolism, such as GS, PP1, and GSK-3 (10, 11). Further, overexpression of PI3K or downstream enzymatic effectors in cells partially increased glucose uptake (27, 28). However, PI3K activation is not sufficient to mediate changes in glucose metabolism produced by insulin (12–14), implicating a second signaling pathway.

The PI3K-independent arm of insulin action may result from the phosphorylation of c-Cbl via its association with CAP. Insulin stimulated c-Cbl phosphorylation and translocation of the Cbl-CAP complex to lipid rafts, where CAP directly bound flotillin (15, 18, 19). The insulin-dependent localization of phospho-Cbl to subdomains of the plasma membrane resulted in the generation of signaling pathways involved in GLUT4 translocation. In 3T3-L1 adipocytes, overexpression of the dominant negative CAP mutant, CAPΔSH3, specifically blocked the insulin-mediated translocation of phospho-Cbl into lipid rafts and completely inhibited insulin-stimulated GLUT4 translocation (19). Interestingly, the insulin-mediated phosphorylation and translocation of Cbl were not blocked by PI3K inhibitors (Fig. 3A, data not shown), indicating that the Cbl-CAP-flotillin...
axis may comprise the PI3K-independent pathway regulating glucose transport.

Overexpression of the dominant negative mutant CAPΔSH3 completely blocked insulin-stimulated glucose transport and glycogen synthesis in 3T3-L1 adipocytes, correcting for transfection efficiency (Fig. 1, A and B). Further, CAPΔSH3 overexpression also reduced insulin-mediated GS activation by 50% (Fig. 2A). Interestingly, this effect was solely the result of reduced glucose uptake, because CAPΔSH3 did not block GS activation in the absence of extracellular glucose (Figs. 2B and 3B). Moreover, the inhibition of GSK-3 and activation of PP1 by insulin were unaffected by CAPΔSH3 expression (Fig. 4, A and B), whereas the glucose-independent activation of GS by insulin was completely suppressed by the PI3K inhibitor wortmannin (Fig. 3B).

In primary adipocytes, insulin and glucose synergistically promote the dephosphorylation and activation of GS (29). Removal of extracellular glucose decreases insulin-stimulated GS activation by 40% in 3T3-L1 adipocytes (Fig. 2B) (20). Similarly, blockade of glucose transport by CAPΔSH3 overexpression caused a 50% reduction in GS activation by insulin. However, the regulation of GSK-3 and PP1 by insulin was unaffected by CAPΔSH3, indicating that these enzymes may constitute the other 50% of GS activation. In agreement, the inhibition of GSK-3 and activation of PP1 by insulin were unaffected by CAPΔSH3 expression (Fig. 4, A and B), whereas the glucose-independent activation of GS by insulin was completely suppressed by the PI3K inhibitor wortmannin (Fig. 3B).

Whether insulin also utilizes a PI3K-independent pathway to regulate PP1 and GSK-3 activities is presently under investigation.

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