The Roles of Cbl-b and c-Cbl in Insulin-stimulated Glucose Transport

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Previous studies suggest that the stimulation of glucose transport by insulin involves the tyrosine phosphorylation of c-Cbl and the translocation of the c-Cbl/CAP complex to lipid raft subdomains of the plasma membrane. We now demonstrate that Cbl-b also undergoes tyrosine phosphorylation and membrane translocation in response to insulin in 3T3-L1 adipocytes. Ectopic expression of APS facilitated insulin-stimulated phosphorylation of tyrosines 665 and 709 in Cbl-b. The phosphorylation of APS produced by insulin drove the translocation of both c-Cbl and Cbl-b to the plasma membrane. Like c-Cbl, Cbl-b associates constitutively with CAP and interacts with Crk upon insulin stimulation. Cbl proteins formed homo- and heterodimers in vivo, which required the participation of a conserved leucine zipper domain. A Cbl mutant incapable of dimerization failed to interact with APS and to undergo tyrosine phosphorylation in response to insulin, indicating an essential role of Cbl dimerization in these processes. Thus, both c-Cbl and Cbl-b can initiate a phosphatidylinositol 3-kinase/protein kinase B-independent signaling pathway critical to insulin-stimulated GLUT4 translocation.

Molecular protein adapters have emerged as essential components of signal transduction pathways. The Cbl family of adapters, which comprises c-Cbl, Cbl-b, and Cbl-c/Cbl-3, has been implicated in receptor tyrosine kinase signaling. These related gene products all have a tyrosine kinase-binding (TKB) domain, a RING finger domain, and a proline-rich region (1, 2). In 3T3-L1 adipocytes, the TKB domain, also called the Cbl-N domain, is an integrated phosphopeptide-binding platform composed of a four-helical bundle, a Ca
+ -binding EF hand, and an SH2 domain (3). The Cbl family proteins are tyrosine-phosphorylated in response to a wide variety of stimuli, including epidermal growth factor, platelet-derived growth factor, various antigens, integrins, and cytokines (1, 2, 4). Moreover, Cbl and Cbl-b interact with critical signaling molecules in both phosphorylation-dependent and -independent fashions. Their binding partners include Src family tyrosine kinases, Zap-70/Syk family tyrosine kinases, the p85 subunit of PI 3-kinase, Vav, Crk, and the Slp-76/BLNK family of linker proteins (5–9). Recent studies have also shown that Cbl family proteins negatively regulate protein-tyrosine kinase signaling. This effect may be dependent, at least in part, on the activity of Cbl as an E3 ubiquitin-protein ligase (10–14). However, evidence has also emerged for positive roles of Cbl proteins in cellular signaling processes. For example, c-Cbl facilitates Met-induced activation of c-Jun N-terminal kinase and ERK in HeLa cells via two separate mechanisms. Although binding of tyrosine-phosphorylated c-Cbl to c-Crk is crucial for c-Jun N-terminal kinase activation, the activation of ERK in response to Met is Crk-independent (15). A recent study has also shown that Cbl-b positively regulates the activation of phospholipase C-γ2 by Btk in B cells (16).

Our previous studies demonstrated that c-Cbl plays an important role in insulin action. This function is regulated by two additional adapter proteins, APS (for adapter containing PH and SH2 domains) and CAP (for Cbl-associated protein). Insulin stimulates the tyrosine phosphorylation of c-Cbl in 3T3-L1 adipocytes, inducing its association with Crk (9). The phosphorylation of c-Cbl by the insulin receptor kinase is facilitated by APS. Upon stimulation, the insulin receptor catalyzes the tyrosine phosphorylation of APS on tyrosine 618. Once phosphorylated, APS recruits c-Cbl to the insulin receptor for subsequent phosphorylation of tyrosines 700 and 774 (17). CAP contains three SH3 domains in its C terminus and a region of homology to the gut peptide sorbin (SoHo domain) in its N terminus. CAP constitutively interacts with Cbl via its C-terminal SH3 domain (18). Upon Cbl phosphorylation, the CAP/Cbl complex migrates to caveolin-enriched lipid rafts, as a result of the interaction of the SoHo domain on CAP with the lipid raft-associated protein flotillin (19, 20). This leads to the recruitment of the Crk/C3G complex to this microdomain of the plasma membrane, where C3G, a guanyl nucleotide exchange factor, activates the small G protein TC10. The activation of TC10 has been shown to occur independently of the PI 3-kinase pathway and, more importantly, to be crucial to insulin-stimulated GLUT4 translocation (21).

c-Cbl and Cbl-b are ubiquitously expressed in a variety of mammalian cells. They share an evolutionarily conserved N-terminal region, and both possess multiple tyrosine phosphorylation sites at the C-terminal region (1, 2, 4). In 3T3-L1 adipocytes, overexpression of the Y618F APS mutant inhibited insulin-stimulated tyrosine phosphorylation and subsequent Crk binding of c-Cbl, as well as the membrane translocation of GLUT4 (17). In separate studies, overexpression of a CAP mutant in which either the SH3 domains or the SoHo domain were deleted blocked the recruitment of Cbl to lipid rafts and GLUT4 translocation in response to insulin (19, 20).

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Although these results strongly suggest an essential signaling role of c-Cbl in insulin-stimulated glucose transport, it remains to be elucidated whether Cbl-b becomes tyrosine-phosphorylated and functions as an adapter protein in this pathway, and how these proteins interact with the insulin receptor. We report here that both c-Cbl and Cbl-b undergo tyrosine phosphorylation and membrane translocation in response to insulin and, further, that these events require Cbl dimerization.

MATERIALS AND METHODS
Antibodies—The HA (F-7), Myc (9E10), c-Cbl (C-15), Cbl-b (G-1 and C-20), and phospho-ERK antibodies were purchased from Santa Cruz Inc. The FLAG (M2) monoclonal antibody was obtained from Stratagene. The anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology, Inc. The Crl monoclonal antibody was purchased from Transduction Laboratories. Horseradish peroxidase-linked secondary antibodies were from Pierce. The Alexa Fluor secondary antibodies were from Molecular Probes.

Plasmids and Mutagenesis—The Myc-APS (pRK5-Myc-APS) and HA-Cbl-b (pCEFL-Cbl-b-HA) constructs were kindly provided by Dr. David Ginty and Dr. Stanley Lipkowitz, respectively. c-Cbl full-length cDNA was derived from pSX-HA-Cbl by PCR. HA-tagged c-Cbl was made as previously described (17). FLAG-tagged Cbl was generated by placing c-Cbl cDNA with FLAG tag fused at the C terminus in frame in the BamHI and EcoRI sites of pRK7 vector. CAP full-length cDNA was derived from FLAG-tagged CAP constructs made as described previously (19, 20, 22). Myc-CAP was constructed by cloning CAP cDNA in the BamHI and EcoRI sites of pRK7-Myc vector. All mutated forms of CAP and Cbl were generated by using the Stratagene Quick Change mutagenesis kit, according to the protocol from the manufacturer. The mutations were confirmed by automated DNA sequencing.

Cell Culture and Transfection—CHO-IR cells were maintained in α-minimal essential medium containing 10% fetal bovine serum. COS-1 cells were grown in DMEM containing 10% fetal bovine serum. 3T3-L1 fibroblasts. Individual clones were maintained in DMEM supplemented with 10% calf serum, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. Differentiation to adipocytes was induced as described previously (23). The cells were then cultured in DMEM containing 10% fetal bovine serum. Before insulin treatment, CHO-IR cells were serum-deprived for 3 h in F-12 Ham’s medium. 3T3-L1 adipocytes were routinely serum-starved for 3 h in low glucose DMEM with 0.5% bovine serum albumin. Both CHO-IR cells and 3T3-L1 adipocytes were transfected by electroporation as described previously (19, 24). COS-1 cells in 60-mm dishes were transfected by using FuGENE 6 reagent (Roche Diagnostics) as described previously (25).

Immunoprecipitation and Immunoblotting—Cells in 60-mm dishes were washed twice with ice-cold phosphate-buffered saline, and lysed for 30 min at 4 °C with buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1.0 mM EDTA, 1.0 mM sodium pyrophosphate, 1.0 mM sodium orthovanadate, 10 mM NaF, and protease inhibitors (1 tablet/7 ml of buffer) (Roche Diagnostics). The clarified lysates were incubated with the indicated antibodies for 2 h at 4 °C. The immune complexes were precipitated with protein A/G-agarose (Santa Cruz, Inc.) for 1 h at 4 °C and were washed extensively with lysis buffer before solubilization in SDS sample buffer. For anti-Cbl immunoprecipitation, Cbl antibodies conjugated on agarose beads were incubated with 3 μg of lystate protein for 2 h to overnight at 4 °C. Bound proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Individual proteins were detected with the specific antibodies and visualized by blotting with horseradish peroxidase-conjugated secondary antibodies.

Subcellular Fractionation—Subcellular fractions including cytosol, plasma membrane, high density membranes, and low density membranes were isolated from 3T3-L1 adipocytes cell homogenates using a combination of differential and equilibrium centrifugation as described by Fisher and Frost (26). Briefly, cells plated on 150-mm plates were incubated in DMEM containing 0.5% fetal bovine serum for 3 h before treating cells with or without 100 nM insulin for various times. Cells were washed three times on ice with ice-cold PBS before collecting the cells in 10 ml of TES buffer (20 mM Tris, 1 mM EDTA, and 250 mM sucrose) containing protease inhibitor mixture (Roche Diagnostics), 100 μM phenylmethylsulfonylfluoride, 100 μM sodium vanadate, 100 μM sodium pyrophosphate, and 1 mM sodium fluoride. Cells were homogenized in a 10-m1 Potter-Elvehjem homogenizing flask using 20 strokes. The cell homogenates were fractionated into various fractions, and the final pellets were resuspended in TES. Protein concentration was determined using a Bio-Rad protein assay kit. Fifty micrograms of each sample was separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblot analysis performed using appropriate antibodies.

Fluorescence Microscopy—Electroporated 3T3-L1 adipocytes were grown on glass cover slips in 6-well dishes. After insulin treatment, cells were fixed with 10% formalin for 15 min, permeabilized with 0.5% Triton X-100 for 5 min, and then blocked with 1% bovine serum albumin and 1% ovalbumin for 1 h. Primary and Alexa Fluor secondary antibodies were used at 2 μg/ml in blocking solution, and samples were mounted on glass slides with Vectashield (Vector Laboratories). Cells were imaged using confocal fluorescence microscopy.

Images were then imported into Adobe Photoshop (Adobe Systems, Inc.) for processing.

RESULTS
Insulin Stimulates the Tyrosine Phosphorylation of Cbl-b in 3T3-L1 Adipocytes—To address a possible role of Cbl-b in insulin action, we examined the tyrosine phosphorylation of this protein in 3T3-L1 adipocytes. Lysates were prepared from cells treated with or without insulin, and incubated with anti-Cbl-b antibodies. The resultant immunoprecipitates were separated by SDS-PAGE, and tyrosine phosphorylation was analyzed by immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 1A, Cbl-b was not tyrosine-phosphorylated in unstimulated cells. The addition of insulin caused a marked tyrosine phosphorylation of a 120-kDa protein, representing Cbl-b. Intense phosphorylation of Cbl-b was detected within 2 min of insulin stimulation, reached a maximum by 5 min, and declined thereafter. Anti-Cbl-b immunoblotting confirmed that equal amounts of Cbl-b protein were immunoprecipitated at each time point. A similar time course of c-Cbl tyrosine phosphorylation in response to insulin was observed. Thus, insulin stimulation of 3T3-L1 adipocytes induces a prominent, rapid, and transient tyrosine phosphorylation of both Cbl-b and Cbl-C. To further characterize insulin-induced Cbl-b tyrosine phosphorylation, we pretreated cells with inhibitors of different kinase pathways downstream of the insulin receptor (Fig. 1B). Treatment of cells with the mitogen-activated protein kinase/ERK kinase inhibitor PD098059 to block the mitogen-activated protein kinase pathway or the PI 3-kinase inhibitor wortmannin to block the PKB pathway did not change the stimulation of Cbl-b tyrosine phosphorylation observed in response to insulin. The effects of PD098059 and wortmannin on the activation of mitogen-activated protein kinase and PKB were demonstrated by immunoblotting of cell lysates with respective phosphospecific antibodies (Fig. 1B). In adipocytes, c-Cbl phosphorylation seems to be catalyzed by the insulin receptor rather than Src family tyrosine kinases such as Fyn (17). In this regard, pretreatment of cells with the selective Src kinase inhibitor PP2 did not affect the tyrosine phosphorylation of Cbl-b in response to insulin. These results suggest that the tyrosine phosphorylation of Cbl-b and Cbl-C is directly catalyzed by the insulin receptor.

Cbl-b and c-Cbl Move to the Plasma Membrane in Response to Insulin—In 3T3-L1 adipocytes, c-Cbl is translocated to the plasma membrane in response to insulin (19). Hence, we wanted to test whether the Cbl-b protein also changes its intracellular localization in response to insulin. To this end, serum-starved 3T3-L1 adipocytes were treated with insulin, and membranes were prepared by differential centrifugation. Equal amounts of total protein from low density microsome and plasma membrane fractions were separated by SDS-PAGE and probed with anti-c-Cbl and anti-Cbl-b antibodies (Fig. 2). Under basal conditions both c-Cbl and Cbl-b were found only at low levels in the plasma membrane fraction. However, both proteins were significantly increased in the plasma membrane fraction upon treatment of cells with insulin for 5 min. Plotillin, a lipid raft protein, did not change its membrane localization in...
The results indicate an insulin-triggered movement of Cbl-b to the plasma membrane similar to that observed for c-Cbl.

**In Vivo Interactions of Cbl-b with Crk and CAP**—Because tyrosine-phosphorylated c-Cbl binds to the SH2 domain of Crk (9), we evaluated whether the insulin-stimulated tyrosine phosphorylation of Cbl-b might result in a similar association. Lysates derived from unstimulated or insulin-stimulated 3T3-L1 adipocytes were immunoprecipitated with anti-Crk antibodies. As shown by anti-Cbl-b immunoblotting of the immunoprecipitates, insulin rapidly stimulated the association of Cbl-b with endogenous Crk (Fig. 3A). Anti-Crk immunoblotting revealed that equal amounts of Crk were immunoprecipitated from unstimulated and insulin-stimulated samples.

We next examined the ability of Cbl-b to form complexes with CAP in 3T3-L1 adipocytes. Previous studies have shown that the third SH3 domain of CAP (SH3C) is responsible for its interaction with c-Cbl (17, 18). We overexpressed Myc-tagged CAP or CAPASH3C along with FLAG-tagged Cbl-b in cells, and evaluated the co-immunoprecipitation of FLAG-Cbl-b with Myc-CAP (Fig. 3B). Immunoblotting of the cell lysates indicated that FLAG-Cbl-b and the two different Myc-CAP proteins were expressed at comparable levels. FLAG-Cbl-b was found to interact specifically with wild type CAP. However, deletion of the third SH3 domain in CAP completely abolished the co-precipitation of FLAG-Cbl-b. Similar results were obtained by co-immunoprecipitation of endogenous Cbl-b with Myc-CAP. These data indicate that Cbl-b and CAP are capable of forming complexes via an interaction between the third SH3 domain of CAP and the proline-rich region(s) of Cbl-b.
APS Mediates the Tyrosine Phosphorylation of Cbl-b by the Insulin Receptor—APS is a Lnk family adaptor protein that is tyrosine-phosphorylated by the insulin receptor (17, 27, 28). Insulin-stimulated phosphorylation of tyrosines 618 in APS is necessary for its association with c-Cbl and the subsequent tyrosine phosphorylation of c-Cbl by the insulin receptor in 3T3-L1 adipocytes (17). To determine whether APS also couples Cbl-b to the insulin receptor for phosphorylation, we expressed HA-tagged Cbl-b alone or with Myc-tagged APS in 3T3-L1 adipocytes (Fig. 4). Following treatment of cells with or without insulin, comparable amounts of HA-Cbl-b were isolated by anti-HA immunoprecipitation. Anti-phospho-tyrosine immunoblotting revealed no insulin-stimulated tyrosine phosphorylation of HA-Cbl-b when expressed alone. Co-expression with Myc-APS dramatically increased the tyrosine phosphorylation of HA-Cbl-b in response to insulin. These results indicate that APS is capable of mediating Cbl-b tyrosine phosphorylation by the insulin receptor.

Previous studies have shown that tyrosines 700 and 774 are the two major residues in c-Cbl phosphorylated in response to insulin. Phosphorylation of these two sites in c-Cbl mediates its interaction with Crk. Cbl-b has tyrosines at 709 and 665 in its C-terminal region with flanking sequences that are homologous to Tyr<sup>700</sup> and Tyr<sup>774</sup> in c-Cbl, respectively (29). To determine whether the insulin receptor phosphorylates these two sites in Cbl-b, we generated a Cbl-b construct with both Tyr<sup>665</sup> and Tyr<sup>709</sup> mutated to phenylalanine. Compared with the wild type Cbl-b protein, the phosphorylation of the Y665F/Y709F mutant was profoundly decreased in response to insulin when Myc-APS was co-expressed (Fig. 4). Thus, tyrosines 665 and 709 are the major sites phosphorylated by the insulin receptor in the presence of APS.

APS Promotes the Movement of Cbl Proteins to the Plasma Membrane in Response to Insulin—APS is constitutively localized at the plasma membrane in 3T3-L1 adipocytes (17). To assess the role of APS in the translocation of Cbl proteins to the plasma membrane upon insulin stimulation, FLAG-c-Cbl or HA-Cbl-b was expressed in 3T3-L1 adipocytes in the presence or absence of Myc-APS, followed by double immunofluorescence staining (Fig. 5). Cells were treated with or without insulin, fixed, and incubated with anti-Myc and anti-FLAG or anti-HA antibodies that were later labeled with complementary fluorescence-conjugated secondary antibodies. Consistent with our previous report, Myc-APS was localized at the plasma membrane independent of insulin treatment. Under basal conditions, FLAG-c-Cbl and HA-Cbl-b were both present throughout the cytoplasm regardless of expression of Myc-APS. Insulin stimulation did not significantly change this diffusional distribution of FLAG-c-Cbl and HA-Cbl-b when Myc-APS was not co-expressed. However, the co-expression of APS was found to cause a robust recruitment of c-Cbl and Cbl-b to the plasma membrane in response to insulin. A single Gly to Glu mutation in the TKB domain of Cbl-b prevented this membrane translocation induced by APS co-expression, whereas Y665F/Y709F double mutations had no effect. These results indicate that the interaction between tyrosine-phosphorylated APS and the TKB domain of Cbl-b is crucial for the membrane translocation of Cbl and, further, that the tyrosine phosphorylation of Cbl requires its translocation to the plasma membrane in response to insulin.

Homo- and Hetero-dimerization of Cbl-b and c-Cbl—It has been reported previously that c-Cbl forms homodimers through its C-terminal leucine zipper domain, and deletion of this domain decreases Cbl tyrosine phosphorylation and its association with the epidermal growth factor receptor (30). Because the leucine zipper domain is highly conserved between Cbl-b and c-Cbl, we tested whether Cbl-b would form heterodimers with c-Cbl in 3T3-L1 adipocytes. Cells were transfected with
vector alone or HA-tagged c-Cbl. HA-c-Cbl was then immunoprecipitated from cell lysates, and the immune complexes were immunoblotted for the presence of endogenous Cbl-b. As can be seen in Fig. 6A, a protein of ∼120 kDa, which corresponds to the correct size of Cbl-b, was detected in anti-HA immunoprecipitates from cells expressing HA-c-Cbl, whereas no Cbl-b could be detected in immunoprecipitates from vector-transfected cells. HA-c-Cbl was isolated only from cells expressing HA-c-Cbl. To examine whether endogenous Cbl proteins also heterodimerize, we immunoprecipitated c-Cbl and Cbl-b from lysates of 3T3-L1 adipocytes with the appropriate antibodies (Fig. 6B). As negative controls, immunoprecipitations were performed by using nonspecific immunoglobulins. Cbl-b was specifically co-immunoprecipitated with c-Cbl. Stimulation of cells with insulin did not influence this interaction. Reciprocally, c-Cbl was found to co-precipitate with Cbl-b when an antibody recognizing the N-terminal region of Cbl-b (G1), or an antibody recognizing C-terminal region of Cbl-b (C20), c-Cbl and Cbl-b present in the immunoprecipitates were detected by immunoblotting with appropriate antibodies.

To examine the role of the leucine zipper domain in the heterodimerization of Cbl proteins, we generated a mutant of c-Cbl in which the leucine zipper domain was deleted from the full-length TAGG-tagged c-Cbl (FLAG-c-CblΔLZ). COS-1 cells were transfected with vector alone, FLAG-c-Cbl/wild type, FLAG-c-CblΔLZ, or FLAG-c-Cbl/1–700 in the presence of either HA-c-Cbl or HA-Cbl-b. As shown in Fig. 7A, all FLAG-c-Cbl proteins were expressed at comparable levels. Although FLAG-c-Cbl/wild type dimerized with both HA-tagged Cbl proteins, neither c-CblΔLZ nor c-Cbl/1–700 associated with c-Cbl or Cbl-b. This result suggests that the leucine zipper domain is absolutely required for the homodimerization and heterodimerization to occur. Because the region containing only the acidic and leucine zipper domains is sufficient to mediate homodimerization of Cbl, we finally tested the interaction of HA-Cbl-AcLZ, which contains only the acidic and leucine zipper domains, with endogenous c-Cbl and Cbl-b proteins in 3T3-L1 adipocytes (Fig. 7B). HA-c-Cbl-AcLZ was found to bind c-Cbl but not Cbl-b. In contrast, HA-Cbl-b-AcLZ bound Cbl-b but not c-Cbl. Therefore, the acidic and leucine zipper domains are sufficient to mediate homodimerization of Cbl proteins. However, additional upstream sequences may be involved in the
heterodimerization between c-Cbl and Cbl-b.

**Dimerization Is Required for Efficient Binding of c-Cbl to APS and Subsequent Tyrosine Phosphorylation of c-Cbl following Insulin Stimulation**—The stimulation of the insulin receptor induces the rapid association of c-Cbl with APS and the recruitment of c-Cbl to insulin receptor for tyrosine phosphorylation (17). To test whether these events are influenced by the dimerization of Cbl, CHO-IR cells were transiently transfected with FLAG-c-Cbl/wild type or FLAG-c-CblΔLZ along with Myc-APS. Following treatment of cells with or without insulin, comparable amounts of Myc-APS were isolated by anti-Myc immunoprecipitation (Fig. 8A). Consistent with our previous findings, insulin stimulated the rapid association of FLAG-c-Cbl with Myc-APS. Deletion of the leucine zipper domain in c-Cbl completely abolished its interaction with Myc-APS in response to insulin. Immunoblotting of cell lysates showed that equivalent amounts of wild type c-Cbl and c-CblΔLZ were expressed in all samples.

To analyze the role of Cbl dimerization in its insulin-stimulated tyrosine phosphorylation, FLAG-c-Cbl proteins were immunoprecipitated by anti-FLAG antibodies, and the immune complexes were blotted with antibodies against phosphotyrosine. As shown in Fig. 8B, insulin stimulation resulted in a dramatic increase in tyrosine phosphorylation of FLAG-c-Cbl/wt when APS was co-expressed. However, FLAG-c-CblΔLZ showed no detectable phosphorylation under the same conditions. Thus, the leucine zipper-mediated dimerization of c-Cbl appears to be required for the insulin-stimulated association of c-Cbl with APS and for the efficient tyrosine phosphorylation of c-Cbl. Finally, the effect of dimerization on the subcellular localization of c-Cbl was examined. 3T3-L1 adipocytes were transfected withFLAG-c-Cbl/wt or FLAG-c-CblΔLZ (Fig. 8C). After fixation, cells were incubated with anti-FLAG antibodies followed by fluorescence-conjugated secondary antibodies. Although wild type c-Cbl localized diffusely throughout the cytoplasm, c-CblΔLZ was found to be confined predominantly to the plasma membrane. Insulin treatment of cells did not change the distribution of either protein (data not shown). This result suggests that Cbl dimerization is required for its cytoplasmic retention.

Expression of a C-terminal Truncated Form of c-Cbl Attenuates Insulin-stimulated GLUT4 Translocation—A critical role of Cbl in insulin-stimulated GLUT4 translocation has been implicated by several studies, using approaches in which c-Cbl tyrosine phosphorylation was blocked by expressing mutated forms of Cbl-binding proteins. For example, overexpression in 3T3-L1 adipocytes of either a CAP mutant (CAPΔSH3) or an APS mutant (APS/Y618F) deficient in Cbl binding inhibits insulin-stimulated tyrosine phosphorylation of c-Cbl and membrane translocation of GLUT4 (17, 19). To provide additional evidence that Cbl phosphorylation is an essential step in insulin-stimulated glucose transport, we tested the dominant negative effect of ectopic expression of a C-terminal deletion mutant of c-Cbl (c-Cbl/1-700) in 3T3-L1 adipocytes. Although it lacks the C-terminal tyrosine phosphorylation sites for Crk binding and sequences for mediating dimerization, c-Cbl/1-700 possesses the intact proline-rich region for CAP association. To examine whether c-Cbl/1-700 would still form a stable complex with CAP, we co-expressed vector alone, FLAG-tagged c-Cbl or c-Cbl/1-700 along with Myc-CAP in 3T3-L1 adipocytes (Fig. 9). Immunoblotting of cell lysates revealed that Myc-CAP and FLAG-c-Cbl were expressed at comparable levels. Anti-FLAG immunoprecipitation followed by anti-Myc immunoblotting showed that c-Cbl/1-700 bound CAP with an affinity similar to that observed with the wild type c-Cbl. The result indicated that the deletion of the C-terminal 206 amino acids did not affect the ability of c-Cbl to form complexes with CAP.

![Fig. 8. Deletion of the leucine zipper domain abolishes the interaction of Cbl with APS as well as the APS-mediated tyrosine phosphorylation of Cbl in response to insulin.](http://www.jbc.org/)

A.

|          | FLAG-c-Cbl/ΔLZ | FLAG-c-Cbl/ΔLZ |
|----------|----------------|----------------|
| pY       |                |                |
| anti-FLAG i.p. |                |                |
| FLAG-c-Cbl |                |                |
| Myc-APS   |                |                |

B.

|          | FLAG-c-CblΔLZ |
|----------|---------------|
| Insulin  |               |
| -       |               |
| +       |               |
| -       |               |
| +       |               |
| FLG-c-Cbl |               |
| Myc-APS   |               |
| anti-Myc i.p. |           |
| FLAG-c-Cbl |               |
| pMAPK     |               |

C.

![c-Cbl/ΔLZ](http://www.jbc.org/)

**Role of Cbl in the APS/CAP/Cbl Pathway**

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EGFP). The cells were treated with or without insulin, and the localization of GLUT4-EGFP was examined by fluorescence microscopy (Fig. 10A). The co-expressed Cbl proteins were visualized by indirect immunostaining. Although wild type c-Cbl localized diffusely throughout the cytoplasm, c-Cbl/1–700, like c-CblΔLZ, was found to be constitutively at the plasma membrane. As expected, insulin stimulated the translocation of GLUT4-EGFP to the plasma membrane in empty vector-transfected cells. Cotransfection with either wild type c-Cbl or wild type Cbl-b did not have any effect on the insulin-stimulated translocation of GLUT4-EGFP. In contrast, co-expression of c-Cbl/1–700 resulted in marked inhibition of insulin-stimulated translocation of GLUT4-EGFP. Quantitation of these data demonstrated that the number of the cells displaying rim green fluorescence in response to insulin decreased by 64.7% with the coexpression of Cbl/1–700 in comparison with control cells (Fig. 10B). As controls, phosphorylated PKB and caveolin were stained with respective antibodies (Fig. 10C). Expression of Cbl/1–700 had no effect on the constitutive membrane localization of caveolin, nor did it attenuate the insulin-stimulated appearance of phospho-PKB at the plasma membrane, indicative of activation of the PI 3-kinase pathway.

DISCUSSION

Tyrosine phosphorylation-dependent interactions between adapter and effector proteins play important roles in tyrosine kinase signaling pathways. Upon insulin stimulation of differentiated adipocytes, c-Cbl becomes phosphorylated on tyrosines 700 and 774, producing its interaction with SH2-containing signaling molecules such as Crk (9, 17). Tyrosine phosphorylation of c-Cbl by the insulin receptor is mediated by the adapter protein APS. APS is an insulin receptor substrate and targets c-Cbl to the insulin receptor upon phosphorylation on tyrosine 618 (17). Moreover, insulin induces translocation of the c-Cbl/CAP complex to lipid raft subdomains of the plasma membrane, where CAP directly binds to the hydrophobic protein flotillin (19, 20). Overexpression of dominant negative mutant forms of CAP blocks both translocation of phospho-c-Cbl to lipid rafts, as well as the membrane movement of GLUT4 in response to insulin, indicating a critical role of c-Cbl in insulin-stimulated glucose transport (19, 20).

Cbl-b is another mammalian Cbl gene product with multiple proline-rich motifs and tyrosine phosphorylation sites. Although less is known about Cbl-b tyrosine phosphorylation, it is clear that Cbl-b is tyrosine-phosphorylated in immune and hematopoietic cells in response to stimuli such as interleukin-7, FL, and T cell receptor/CD3 ligation (31, 32). We demonstrate here that Cbl-b becomes tyrosine-phosphorylated in response to insulin in 3T3-L1 adipocytes. This transient phosphorylation of Cbl-b is independent of classical PI 3-kinase and mitogen-activated protein kinase pathways, and is also insensitive to the inhibition of Src kinase activity, indicating that the insulin receptor directly catalyzes this phosphorylation. Moreover, we also demonstrate that tyrosine-phosphorylated APS is the adapter that couples Cbl-b to the insulin receptor. As a result, Cbl-b is phosphorylated on tyrosines 665 and 709. Tyrosines 665 and 709 have been determined as CrkL binding sites (29), although the precise tyrosine phosphorylation sites of Cbl-b had not been identified prior to this study.

Although numerous studies show that c-Cbl exerts many of its functions by recruiting various proteins to physiologically relevant complexes, little is known about the biological role of Cbl-b as an adapter protein. Experiments described here reveal that Cbl-b interacts with CAP and Crk in 3T3-L1 adipocytes. Like Cbl-b, Cbl-b appears to form a stable complex with CAP through an SH3 proline-rich motif interaction. The interaction of Cbl-b with Crk is sensitive to insulin stimulation and thus is dependent on the ability of Cbl-b to undergo tyrosine phosphorylation.

In addition to its tyrosine phosphorylation, Cbl-b traffics to the plasma membrane in a manner similar to that observed with c-Cbl (19). Like c-Cbl, Cbl-b appears to transiently relocate to the plasma membrane upon insulin stimulation through its interaction with phosphorylated APS. This was based on the ability of ectopically expressed APS to drive the membrane localization of both c-Cbl and Cbl-b in response to insulin. Moreover, mutation of either the TKD domain of Cbl proteins abolished this translocation. It should be noted that the amount of endogenous Cbl proteins translocated to the plasma membrane is low relative to their total cytosolic pool, possibly as a result of the limited availability of endogenous APS. For the same reason, no significant translocation of ectopically expressed Cbl proteins occurs in the absence of the co-expression of APS.

The leucine zipper domain is an α-helical structure formed by several heptad repeats of hydrophobic residues such as leucine and isoleucine. The C-terminal regions of c-Cbl and Cbl-b contain a conserved leucine zipper, which in c-Cbl was reported to mediate homodimerization (30). Our results demonstrate for the first time that Cbl-b is capable of forming a homodimer as well as heterodimers with c-Cbl. Formation of both dimers seems to require the participation of the leucine zipper domain, because deletion of this domain was sufficient to abolish both homo- and heterodimerization. Despite a high degree of sequence similarity in the leucine zipper domains, fusion proteins containing the acidic and leucine zipper domains of c-Cbl and Cbl-b prefer homodimerization. Therefore, the acidic and leucine zipper domains are sufficient for Cbl homodimerization, whereas other upstream sequences may be involved in forming the heterodimeric complexes.

Dimerization of Cbl has functional significance in insulin signaling. In the case of c-Cbl, our data show that the deletion of the leucine zipper and the resultant loss of Cbl dimerization lead to decreased insulin-stimulated Cbl-APS association and tyrosine phosphorylation of c-Cbl. In this regard, APS was also found to have the ability to undergo self-dimerization (33). Thus, it would be interesting to examine whether dimerization of APS is necessary to recruit Cbl dimers to the insulin receptor. Moreover, Cbl dimerization also appears to play an important role in determining its cellular localization. Deletion of the leucine zipper resulted in the constitutive association of c-Cbl with various adapters and effectors.
with the plasma membrane, suggesting that dimerization is indispensable for the intracellular retention of Cbl under basal conditions. One possible mechanism would involve the interaction of Cbl dimers with other cytoplasmic proteins. Insulin might induce the release from these cytoplasmic anchors at the initial stage of membrane translocation of Cbl. Furthermore, deletion of the leucine zipper domain had no effect on the ability of Cbl to interact with CAP, indicating that dimerization, although important for Cbl tyrosine phosphorylation, may not be required for its localization to lipid rafts.

In our previous studies, overexpression of dominant negative mutants of APS or CAP inhibited tyrosine phosphorylation of Cbl and translocation of GLUT4 in response to insulin. The data presented here directly demonstrate a critical role of Cbl as an upstream signaling intermediate in insulin-stimulated glucose transport. The overexpression of a c-Cbl mutant (c-Cbl/1–700) in which a C-terminal portion containing the tyrosine phosphorylation sites and the acidic and leucine zipper domains has been deleted profoundly inhibited insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes. On the other hand, the overexpression of wild type c-Cbl or Cbl-b had no such inhibitory effect. In addition, overexpression of c-Cbl/1–

Role of Cbl in the APS/CAP/Cbl Pathway

FIG. 10. Overexpression of c-Cbl/1–700 blocks insulin-stimulated GLUT4 translocation to the plasma membrane. A. 3T3-L1 adipocytes were electroporated with 100 μg of GLUT4-EGFP plus 200 μg of vector, FLAG-c-Cbl/wt, FLAG-c-Cbl/1–700, or HA-Cbl-b/wt, and allowed to recover for 30 h. The cells were treated with or without 100 nM insulin for 30 min. Cells were fixed and fluorescence visualized by confocal microscopy. GLUT4-EGFP was visualized by direct fluorescence, and Cbl proteins were visualized by indirect immunofluorescence. These are representative images of middle sections of cells obtained from three independent experiments. B, numbers of GLUT4-EGFP transfected cells displaying visually detectable plasma membrane (PM) rim fluorescence were plotted. These data were obtained by blind counting of more than 80 cells from three independent experiments. C, 3T3-L1 adipocytes were electroporated with 200 μg of vector alone or FLAG-c-Cbl/1–700, and allowed to recover for 30 h. FLAG-c-Cbl/1–700, caveolin (Cav), and phospho-Akt were visualized by indirect immunofluorescence. These are representative images of middle sections of cells obtained from three independent experiments.
700 does not impair PKB activation by insulin, another major determinant of GLUT4 translocation. We propose that the apparent dominant negative effect of c-Cbl/1–700 on GLUT4 translocation is the result of the ability of c-Cbl/1–700 to interact with and thus sequester proteins involved in the regulation of insulin signaling through endogenous Cbl. One such candidate protein is CAP. c-Cbl/1–700 binds to CAP with an affinity similar to wild type c-Cbl. Sequestration of endogenous CAP by c-Cbl/1–700 would in turn block the translocation of endogenous Cbl to the flotillin-enriched lipid rafts, a process previously shown to be critical for insulin-stimulated GLUT4 translocation.

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