Requirements for pYXXM Motifs in Cbl for Binding to the p85 Subunit of Phosphatidylinositol 3-Kinase and Crk, and Activation of Atypical Protein Kinase C and Glucose Transport during Insulin Action in 3T3/L1 Adipocytes†

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ABSTRACT: Cbl is phosphorylated by the insulin receptor and reportedly functions within the flotillin/CAP/Cbl/Crk/C3G/TC10 complex during insulin-stimulated glucose transport in 3T3/L1 adipocytes. Cbl, via pYXXM motifs at tyrosine-371 and tyrosine-731, also activates phosphatidylinositol (PI) 3-kinase, which is required to activate atypical protein kinase C (aPKC) and glucose transport during thiazolidinedione action in 3T3/L1 and human adipocytes [Miura et al. (2003) *Biochemistry 42,* 14335−14341]. Presently, we have examined the importance of Cbl in activating PI 3-kinase and aPKC during insulin action in 3T3/L1 adipocytes by expressing Y371F and Y731F Cbl mutants, which nullify pYXXM binding of Cbl to SH2 domains of downstream effectors. Interestingly, these mutants inhibited insulin-induced increases in (a) binding of Cbl to both Crk and the p85 subunit of PI 3-kinase, (b) activation of Cbl-dependent PI 3-kinase, (c) activation and translocation of aPKC to the plasma membrane, (d) translocation of Glut4 to the plasma membrane, (e) and glucose transport. Importantly, coexpression of wild-type Cbl reversed the inhibitory effects of Cbl mutants. In contrast to Cbl-dependent PI 3-kinase, Cbl mutants did not significantly inhibit the activation of PI 3-kinase by IRS-1, which is also required during insulin action. Our findings suggest that (a) Cbl uses pYXXM motifs to simultaneously activate PI 3-kinase and Crk/C3G/TC10 pathways and (b) Cbl, along with IRS-1, functions upstream of PI 3-kinase and aPKCs during insulin-stimulated glucose transport in 3T3/L1 adipocytes.

Upon binding of insulin to its receptor in 3T3/L1 adipocytes, the signaling protein Cbl, in association with Cbl-associated protein (CAP)[1] (*1*, *2*), and APS (*3*), an adapter protein that contains pleckstrin homology (PH) and SH2 domains, are rapidly recruited to the insulin receptor, which phosphorylates tyrosine residues of both Cbl (*1*) and APS (*3*). The tyrosine-phosphorylated CAP/Cbl complex, via a C-terminal SH3 domain in CAP, then binds to flotillin in caveolin-rich invaginations/lipid raft microdomains of the Triton-insoluble fraction of the plasma membrane (*4*). Tyrosine-phosphorylated Cbl also binds to the SH3/SH2/SH3 adapter protein Crk (*5*, *6*), which then recruits C3G (*6*), a GTP/GDP exchange factor for the small G-protein Rho family member TC10α (*6*, *7*), along with other proteins, e.g., CIP/2 (*8*), thus forming a highly integrated signaling complex within specific microdomains of the plasma membrane, as required for insulin-stimulated translocation of the Glut4 glucose transporter to the plasma membrane and subsequent increases in glucose transport in 3T3/L1 adipocytes.

The flotillin/CAP/Cbl/Crk/C3G/TC10 signaling complex forms independently of phosphatidylinositol (PI) 3-kinase (*1*−*8*). Of further note, inhibition of this flotillin/CAP/Cbl/Crk/C3G/TC10 signaling complex does not compromise the PI 3-kinase-dependent phosphorylation/activation of protein kinase B (PKB/Akt) (*4*). However, there is relatively little information on whether inhibition of this complex alters other PI 3-kinase-dependent factors, e.g., aPKCs, required for insulin-stimulated glucose transport.

As alluded to, although PI 3-kinase is not required for formation of the flotillin/CAP/Cbl/Crk/C3G/TC10 signaling complex, PI 3-kinase is simultaneously activated by insulin and is corequired for insulin-stimulated Glut4 translocation/glucose transport. In this regard, atypical protein kinase C (aPKC) isoforms, ζ and/or λ (*9*−*13*), and PKB/Akt (*14*−*17*), which are activated by PI 3-kinase-dependent increases in PI-3,4,5-$(PO_4)_3$ (PIP_3) and enhanced action of 3-phosphoinositide-dependent protein kinase-1 (PDK-1), are thought to function downstream of PI 3-kinase during insulin-stimulated glucose transport. Of further note, it has recently been reported that aPKCs function within the aforesaid lipid rafts, downstream of the flotillin/CAP/Cbl/Crk/C3G/TC10 signaling complex (*18*). It therefore seems likely that aPKCs

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[1] Abbreviations: CAP, Cbl-associated protein; APS, adapter protein containing pleckstrin homology and SH2 domains; PKC, protein kinase C; aPKC, atypical protein kinase C; PKB/Akt, protein kinase B; DOG, 2-deoxyglucose; PDK1, 3-phosphoinositide-dependent protein kinase-1; IRS, insulin receptor substrate; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; KRP, Krebs Ringer phosphate buffer.

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are activated within these specific lipid raft/microdomains of the plasma membrane. On the other hand, there is remaining uncertainty as to how aPKCs are activated by PI 3-kinase by this signaling complex within these lipid raft microdomains.

With respect to PI 3-kinase activation, it is generally assumed that IRS family members, in particular IRS-1 and IRS-2, are largely responsible for activating one or more PI 3-kinase pools that are used to activate aPKCs during insulin-stimulated glucose transport. Indeed, IRS-1 knockout mice have defects in insulin-induced activation of aPKCs in skeletal muscle and white adipocytes (*19*), and as reported in the following paper in this issue, aPKC activation is impaired in immortalized brown adipocytes in which either IRS-1 or IRS-2 has been knocked out. However, the precise role of IRS family members is still not clear. First, for example, there is no evidence that IRS family members are exclusive activators of PI 3-kinase during insulin-stimulated glucose transport. Second, it has been reported that insulin stimulates the translocation of IRS-1 and IRS-2 to the plasma membrane by a PI 3-kinase-dependent mechanism, suggesting that PI 3-kinase functions upstream as well as downstream of IRS-1/2 (*20*). Third, some findings have suggested that IRS family members are not sufficient for activation of downstream signaling factors during insulin-stimulated glucose transport (*21*−*23*). Finally, IRS-1/2 may have functions other than, or in addition to, PI 3-kinase activation during insulin-stimulated glucose transport.

With respect to other activators of PI 3-kinase, we have reported that there is a relatively small (i.e., approximately 15−20% relative to IRS-1-dependent PI 3-kinase activity) pool of Cbl-dependent PI 3-kinase that is rapidly activated during insulin action in 3T3/L1 adipocytes (*24*). Although it seems clear that this Cbl-dependent PI 3-kinase activity is not required for insulin-induced formation of the flotillin/CAP/Cbl/Crk/C3G/TC10 signaling complex (*1*−*8*), the mechanism of activation and functional role of this relatively small pool of Cbl-dependent PI 3-kinase during insulin action is uncertain. Indeed, despite the apparently relatively small size of the Cbl-dependent PI 3-kinase pool, its potential importance cannot be discounted, since thiazolidinedione-induced activation of a similarly sized pool of Cbl-dependent PI 3-kinase is capable of activating PKC-λ and Glut4 translocation/glucose transport nearly as effectively as insulin in 3T3/L1 adipocytes, even in the complete absence of activation of IRS-1/2-dependent PI 3-kinase and PKB (*24*, *25*).

Presumably, Cbl activates PI 3-kinase through two pYXXM motifs that start at tyrosine (Y) residues 371 and 731 and are known to bind to the SH2 domain of the p85 regulatory subunit of PI 3-kinase and thereby activate its p110 catalytic domain during the action of certain noninsulin agonists (e.g., see refs *26* and *27*). Therefore, to gain insight into the questions of how and whether Cbl-dependent PI 3-kinase could be involved in insulin-stimulated glucose transport, we used adenoviruses to express Y371F and Y731F Cbl mutants in 3T3/L1 adipocytes. As now reported, expression of Y371F Cbl or Y731F Cbl inhibited insulin-induced binding of Cbl to the p85 subunit of PI 3-kinase and activation of Cbl-dependent PI 3-kinase, aPKCs, and Glut4 translocation/glucose transport. Thus, in addition to IRS-1/2, Cbl may be required for insulin-induced activation of PI 3-kinase, aPKCs, and glucose transport in 3T3/L1 adipocytes. Accordingly, it is entirely possible that Cbl, in conjunction with IRS-1/2, serves to localize and/or activate aPKCs within strategic sites in the plasma membrane that are essential for Glut4 translocation.

In addition to inhibiting Cbl-dependent PI 3-kinase and PKC activation, these Cbl mutants inhibited the binding of Cbl to Crk, suggesting that pYXXM motifs in Cbl mediate its binding to both the p85 subunit of PI 3-kinase and Crk. Accordingly, it appears that Cbl plays an important role in activating both PI 3-kinase-dependent and -independent pathways required for insulin-stimulated glucose transport in 3T3L1 adipocytes.

EXPERIMENTAL PROCEDURES

Cell Culture, Incubations, and Treatments. 3T3/L1 adipocytes were cultured and differentiated as described previously (*9*, *13*, *24*, *25*). Insulin, dexamethasone, and isobutylmethylxanthine were withdrawn, and fully differentiated adipocytes were incubated in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (Sigma) for 48 h without or with the indicated concentrations of adenovirus alone or adenovirus encoding wild-type or mutated forms of Cbl. The cells were then incubated in serum-free DMEM containing 2% bovine serum albumin (BSA) for 3 h, and finally incubated for 30 min in glucose-free Krebs Ringer phosphate (KRP) medium containing 1% BSA prior to acute treatment with insulin for the indicated times.

PKC-λ Activation. PKC-λ activity was measured as described previously (*9*, *10*, *13*, *24*, *25*). In brief, PKC-λ was immunoprecipitated from salt/detergent-treated cell lysates with a rabbit polyclonal antiserum (Santa Cruz Biotechnologies, Inc.) that recognizes the C-termini of both PKC-λ and PKC-ζ (note that 3T3/L1 adipocytes contain only PKC-λ), collected on Sepharose-AG beads (Santa Cruz Biotechnologies), and incubated for 8 min at 30 °C in 100 μL of buffer containing 50 mM Tris/HCl (pH 7.5), 100 μM Na_3VO_4, 100 μM $Na_4P_2O_7$, 1 mM NaF, 100 μM PMSF, 4 μg of phosphatidylserine (Sigma), 50 μM [γ-^{32}P]ATP (NEN/Life Science Products), 5 mM $MgCl_2$, and, as substrate, 40 μM serine analogue of the PKC-ϵ pseudosubstrate (BioSource). After incubation, ^{32}P-labeled substrate was trapped on P-81 filter paper and counted.

PI 3-Kinase Activation. Immunoprecipitable Cbl-dependent (using rabbit polyclonal anti-Cbl antiserum from Santa Cruz Biotechnologies) and IRS-1-dependent (using rabbit polyclonal anti-IRS-1 antiserum from UBI) PI 3-kinase activities were determined as described (*24*, *25*). In these assays, the product of the reaction of PI 3-kinase with PI as a substrate, viz., ^{32}PI-3-PO_4, was purified by thin-layer chromatography and quantified in a BioRad PhosphorImager with a Molecular Analyst program. Levels of IRS-1 and the p85 subunit of PI 3-kinase were not altered by adenoviral treatments.

2-Deoxyglucose Uptake and Glut4 Translocation. Adipocytes were incubated in glucose-free KRP medium for 30 min with or without insulin, prior to measurement of uptake of [^{3}H]-2-deoxyglucose (50 μM) over 5 min, as described previously (*9*, *13*, *24*, *25*). The plasma membrane content of Glut4 (and aPKC) was determined by discontinuous sucrose gradient ultracentrifugation as described (*9*, *24*).

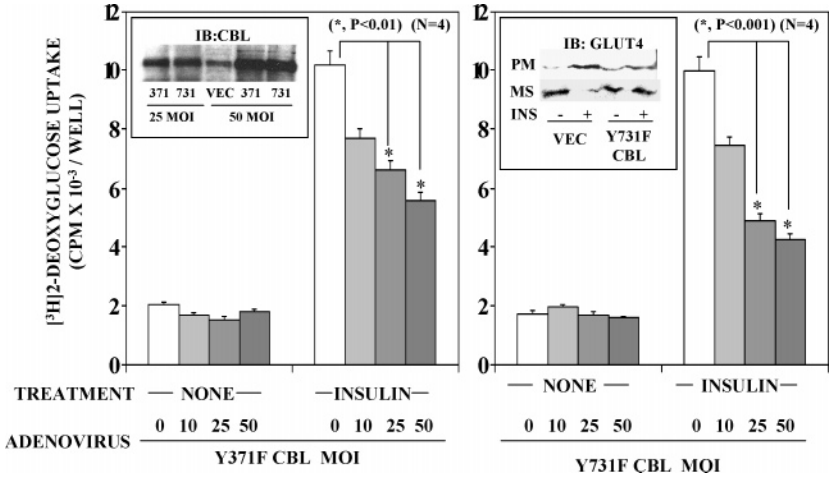

FIGURE 1: Effects of expression of Y371F and Y731F Cbl mutants on insulin-stimulated ^{3}[H]-2-deoxyglucose uptake and Glut4 translocation from microsomes to the plasma membrane in 3T3/L1 adipocytes. Cells in 24-well (for 2DOG uptake) or 100 mm diameter (for Glut4 translocation) plates were incubated for 48 h with increasing amounts (MOI, multiplicity of infection) of adenovirus encoding indicated Cbl mutants or adenovirus alone (the level of total adenovirus was kept constant at 50 MOI by varying the amount of adenovirus vector), and finally incubated for 30 min with or without 100 nM insulin in glucose-free KRP medium containing 1% BSA before the uptake of ^{3}[H]-2-deoxyglucose over 5 min was measured. Shown in the left inset are increases in total content of Cbl in cells infected with 25 and 50 MOI of the indicated adenovirus. Although not shown, adenovirus alone did not alter Cbl content or [^{3}H]-2-deoxyglucose uptake. Shown in the right inset are representative blots depicting plasma membrane (PM) and microsomal (MS) Glut4 contents in control and insulin-stimulated adipocytes infected with 50 MOI of adenoviral vector or adenovirus containing cDNA encoding the Y731F Cbl mutant. Values in the bar graphs are the mean ± SE of four determinations. Asterisks indicate P values of <0.01 in the left panel and <0.001 in the right panel, in comparing insulin-stimulated values in vector-treated versus Cbl mutant-treated cells.

Immunoblotting. Western analyses were conducted as described previously (*24*, *25*) and blotted with (a) rabbit polyclonal anti-PKC-ζ/λ C-terminal antiserum (Santa Cruz Biotechnologies), (b) rabbit polyclonal anti-p85 subunit of PI 3-kinase antiserum (UBI), (c) rabbit polyclonal anti-Cbl antiserum (Santa Cruz Biotechnologies), (f) mouse monoclonal anti-Crk antibodies (Transduction Labs), (d) rabbit polyclonal anti-phosphotyrosine-731-Cbl antiserum (Cell Signaling), (e) mouse monoclonal anti-Glut4 antibodies (Biogenesis), (f) rabbit polyclonal anti-PDK1 antiserum (UBI), and (g) rabbit polyclonal anti-IRS-1 antiserum (UBI). Blots were quantified by measurement of extended chemiluminescence (ECL) in a BioRad PhosphorImager/chemiluminescence imaging system using a Molecular Analyst program, or by scanning laser densitometry.

Cbl Immunoprecipitation. As described (*25*), Cbl was immunoprecipitated from cell lysates with rabbit polyclonal anti-c-Cbl and anti-Cbl-b antisera using directions supplied by the supplier (Santa Cruz Biotechnologies).

Adenoviral Constructs. As described (*24*, *25*), pCMV2 plasmid encoding wild-type Cbl was used to generate Y371F and Y731F single and Y371F/Y731F double mutants of Cbl using a site-directed mutagenesis Gene Editor kit obtained from Promega. All final constructs were sequenced to ensure that the mutations were maintained through the preparative methods. Adenoviruses encoding wild-type, Y371F, Y731F, and Y371F/Y731F forms of Cbl were constructed using plasmids encoding these forms and an Adeno-X Expression kit obtained from Clontech.

Statistical Methods. P values were determined by one-way ANOVA and the least significant multiple comparison method.

RESULTS

Effects of Cbl Mutants on Insulin-Stimulated Glucose Transport. As seen in Figure 1, insulin-stimulated increases in 2-deoxyglucose uptake were progressively inhibited by increasing levels of viral-expressed Y371F or Y731F Cbl mutant. In contrast, basal 2-deoxyglucose uptake was not altered by expression of these mutants (Figure 1). Although not shown, adenovirus vector alone had no effect on either basal or insulin-stimulated 2-deoxyglucose uptake.

In addition to inhibiting insulin-stimulated 2-deoxyglucose uptake, the expression of the Y731F Cbl mutant (at 50 MOI of adenovirus) markedly inhibited insulin-stimulated translocation of the Glut4 glucose transporter from the microsomal fraction to the plasma membrane (right inset of Figure 1).

It may be noted that, for both Cbl mutants, at viral MOIs of 25 and 50, there was approximately a doubling and quadrupling, respectively, of total cellular Cbl content (left inset of Figure 1). Thus, in evaluating the inhibitory effects of these Cbl mutants, if it is assumed that, except for specific effects of the mutations, endogenous Cbl and mutant Cbl are equally effective in coupling to other components of the relevant signaling pathway, the expected inhibition of Cbl-dependent processes would be approximately 50% and 80% at 25 and 50 viral MOI, respectively, i.e., roughly commensurate with the inhibition of insulin-stimulated glucose transport observed with the Y731F mutant (Figures 1 and 2). In this regard, the slightly, but not significantly, lesser inhibitory effects of the Y371F Cbl mutant, relative to those of the Y731F Cbl mutant (Figures 1 and 2) may reflect the fact that the pYXXM motif at Y371 is thought to be less exposed than that initiated by Y731, and thus less able to interact with SH2 domains of other proteins (*26*, *27*). Nevertheless, the Y371F mutant was clearly very effective in inhibiting insulin-stimulated glucose transport.

Effects of Cbl Mutants on Insulin-Induced Activation of Cbl-Dependent PI 3-Kinase and PKC-λ. In addition to inhibiting insulin-stimulated 2-deoxyglucose uptake and Glut4 translocation, expression of Y371F Cbl and Y731F Cbl single mutants, and the Y371F/Y731F Cbl double

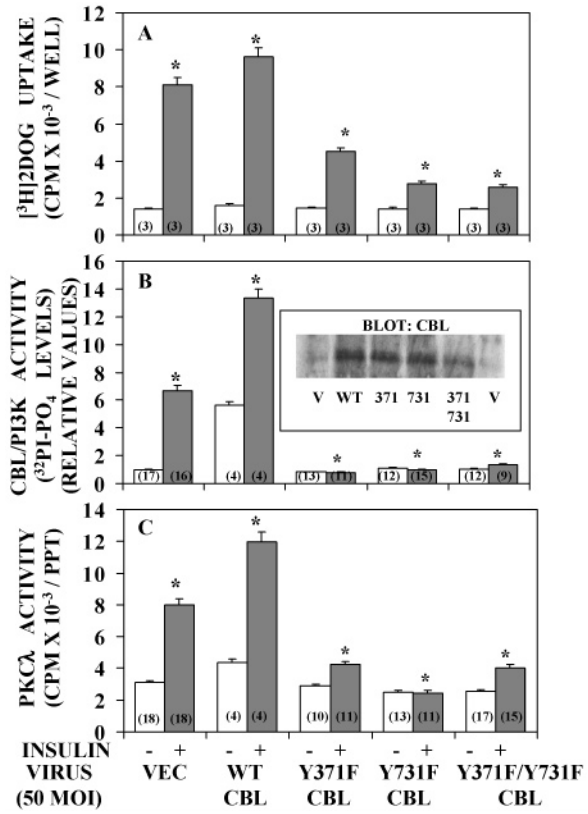

FIGURE 2: Effects of expression of wild-type (WT) and Cbl mutants, Y371F, Y731F, and Y371F/Y731F, on insulin-induced increases in ^{3}[H]-2-deoxyglucose (DOG) uptake (A), Cbl-dependent PI 3-kinase (PI3K) activity (B), and PKC-λ activity (C) in 3T3/L1 adipocytes. Cells in 24-well plates (A) or 100 mm plates (B, C) were incubated for 48 h with 50 MOI of adenovirus alone (vector, VEC) or adenovirus encoding indicated forms of Cbl, and finally incubated with (filled bars) or without (clear bars) 100 nM insulin in glucose-free KRP medium containing 1% BSA for 30 min before ^{3}[H]-2-deoxyglucose uptake over 5 min was measured, or for 15 min before Cbl-dependent PI 3-kinase activity (viz., relative ^{32}PI-3-PO_4 levels following purification by thin-layer chromatography) and PKC-λ activity were measured. The effects of expression of Cbl on total cellular content of Cbl are shown in the inset of panel B. Values in the bar graphs are the mean $\pm$ SE of the number of determinations shown in parentheses. Single asterisks indicate P values of <0.001 in comparing (a) insulin-stimulated versus basal values in vector-treated (VEC) and WT Cbl-treated cells and (b) insulin-treated values in Cbl mutant-treated cells versus those of vector-treated cells.

mutant, inhibited insulin-induced increases in Cbl-dependent PI 3-kinase and PKC-λ activity (Figures 2 and 3). Also, as depicted in Figure 2, inhibitory effects of the Y731F Cbl single mutant on both insulin-stimulated 2-deoxyglucose uptake and activation of Cbl-dependent PI 3-kinase and PKC-λ were virtually the same as those observed with the Y371F/Y731F Cbl double mutant. Thus, in some studies, we used only the Y731F Cbl mutant.

Effects of Wild-Type Cbl on Insulin-Induced Activation of Glucose Transport, Cbl-Dependent PI 3-Kinase, and PKC-λ. In contrast to that of mutant forms of Cbl, expression of wild-type Cbl (which was expressed to approximately the same extent as mutant forms of Cbl; see the inset in Figure 2) had no significant effect on insulin-stimulated 2-deoxyglucose uptake, and either had no effect on or, if anything, enhanced the activity and/or activation of Cbl-dependent PI 3-kinase and PKC-λ (Figure 2). It may therefore be surmised that the inhibitory effects of mutant forms of Cbl are specifically due to the presence of mutated tyrosine residues, rather than to simple overexpression of Cbl, which could theoretically compete with IRS family members or other insulin receptor substrates that bind to upstream and downstream signaling proteins that contain SH2 or SH3 domains. This conclusion is further supported by the fact that (a) overexpression of wild-type Cbl reversed (or prevented) the inhibitory effects of the Y731F Cbl and (b) Cbl mutants did not inhibit IRS-1-dependent PI 3-kinase activation (see below).

It may be noted that the stimulation of basal Cbl-dependent PI 3-kinase and PKC-λ elicited by expression of wild-type Cbl did not result in significant increases in 2-deoxyglucose transport (Figure 2). This suggested that additional insulin-dependent factors were required to increase glucose transport, perhaps to both localize aPKCs and other factors needed for Glut4 translocation and to activate translocated Glut4 transporters.

Effects of Mutant and Wild-Type Cbl on Insulin-Induced Activation of IRS-1-Dependent PI 3-Kinase. In contrast to the Cbl mutant-dependent inhibition of insulin effects on 2-deoxyglucose uptake and activation of Cbl-dependent PI 3-kinase and PKC-λ, the expression of the Y731F Cbl mutant did not have a significant effect on insulin-induced increases in IRS-1-dependent PI 3-kinase activity (Figure 3). Similarly, expression of wild-type Cbl did not inhibit insulin-stimulated IRS-1-dependent PI 3-kinase activity (Figure 3). It may therefore be surmised that expression of Cbl in its various forms did not indiscriminately inhibit other substrates of the insulin receptor that signal to relevant downstream factors that contain SH2 and SH3 domains.

Rescue of Y731F Cbl-Induced Inhibition of Insulin Effects on Glucose Transport, Cbl-Dependent PI 3-Kinase, and PKC-λ by Expression of Wild-Type Cbl. It was important to find that the inhibitory effects of the Y731F Cbl mutant on insulin-induced increases in 2-deoxyglucose uptake and activation of Cbl-dependent PI 3-kinase and PKC-λ were reversed or diminished by overexpression of wild-type Cbl (Figure 4). This ability of wild-type Cbl to rescue these processes provided further support for the contention that inhibitory effects of Cbl mutants on insulin-stimulated increases in 2-deoxyglucose uptake and activation of Cbl-dependent PI 3-kinase and PKC-λ were specifically due to the presence of the mutated tyrosine residues, rather than to simple overexpression of Cbl and nonspecific inhibition of other insulin receptor substrates that are coupled to downstream effectors that contain SH2 or SH3 domains.

Effects of Cbl Mutants on Insulin-Induced Increases in the Binding of Cbl to the p85 Subunit of PI 3-Kinase and Crk. In conjunction with increases in Cbl-dependent PI 3-kinase activity, insulin provoked sizable increases in the binding of Cbl to the p85 subunit of PI 3-kinase (Figure 5). Of further note, expression of the Y731F Cbl mutant markedly inhibited insulin effects on Cbl/p85 binding (Figure 5). Although not shown, neither insulin nor expression of Y731F Cbl altered the recovery of Cbl in Cbl immunoprecipitates. Also not portrayed, as with 2-deoxyglucose uptake and aPKC activation, the Y731F Cbl single mutant was as effective as the Y371F/Y731F Cbl double mutant in inhibiting the binding of Cbl to the p85 subunit of PI 3-kinase.

As with the p85 subunit of PI 3-kinase, the binding of Cbl to Crk was increased by insulin, and this binding of Cbl

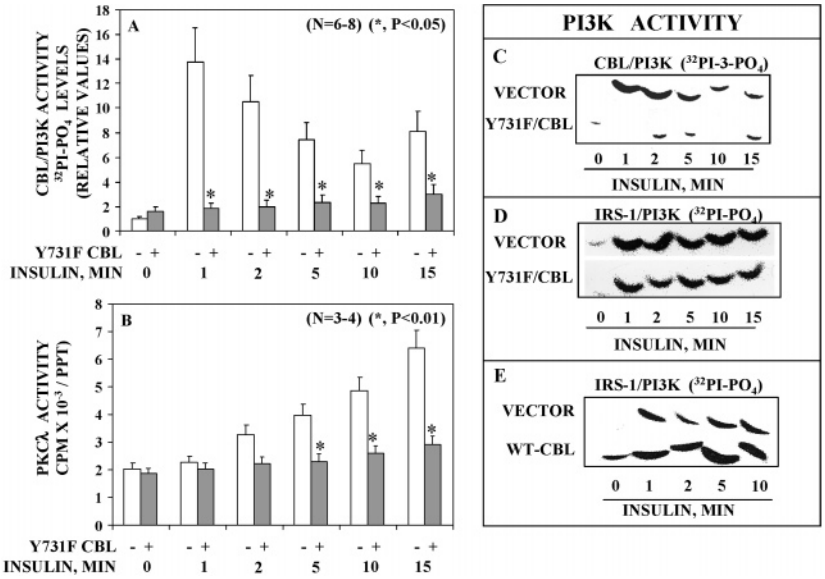

FIGURE 3: Effects of expression of the Y731F Cbl mutant (A−D) and WT Cbl (E) on time-dependent insulin-induced activation of Cbl-dependent PI3K (A, C), PKCλ (B), and IRS-1-dependent PI 3-kinase (D, E) in 3T3/L1 adipocytes. Cells in 100 mm plates were incubated for 48 h with 50 MOI of adenovirus alone (vector or clear bars) or adenovirus encoding Y731F Cbl mutant (A−D), or 200 MOI of adenovirus alone (vector) or adenovirus encoding WT Cbl (E), and finally incubated in glucose-free KRP medium containing 1% BSA without or with 100 nM insulin for the indicated times, before the activities of Cbl- or IRS-1-dependent PI 3-kinase and PKCλ were measured. Shown at the right are representative autoradiograms of ^{32}PI-3-PO$_4$, the reaction product of the PI 3-kinase assays, following purification by thin-layer chromatography. Data for PI 3-kinase activity (^{32}PI-3-PO$_4$ levels) are expressed relative to the level of basal unstimulated PI 3-kinase activity (as determined in a BioRad PhosphorImager with a Molecular Analyst program) observed in samples infected with adenovirus vector alone and analyzed on the same thin-layer chromatography plate/autoradiogram. Bar graph values in panels A and B are the mean ± SE of the number of determinations shown in parentheses. Asterisks indicate P values of <0.05 in panel A and <0.01 in panel B, in comparing insulin-stimulated values in vector-treated versus Y731F Cbl mutant-treated cells.

to Crk was inhibited by expression of the Y731F Cbl mutant (Figure 5). These results suggested that insulin increased binding of Cbl to both the p85 subunit of PI 3-kinase and Crk through pYXXM motifs in Cbl that bind to SH2 domains of the p85 subunit of PI 3-kinase and Crk.

It may be noted that binding of Cbl to the p85 subunit of PI 3-kinase and Crk in basal unstimulated samples also appeared to be diminished slightly by expression of the Y731F Cbl mutant, perhaps reflecting mild degrees of basal activation of Cbl-dependent pathways.

In contrast to the p85 subunit and Crk, Cbl immunoprecipitates did not contain immunoreactive aPKC, PKB, IRS-1, or IRS-2, either basally or in response to insulin (data not shown).

Insulin Increases Phosphorylation of Y731 in Cbl. Previous studies have shown that the mutation of the tyrosine-371 residue of Cbl substantially diminishes insulin-dependent phosphorylation of Cbl, suggesting that insulin increases the phosphorylation of the tyrosine-371 residue (*3*). Presently, using a phospho-specific antiserum, we found that insulin increased the phosphorylation of the tyrosine-731 residue of Cbl (Figure 6A). This effect appeared to be greatest at 1−2 min of insulin action, i.e., coinciding with the initial peak of insulin-stimulated Cbl-dependent PI 3-kinase activity (Figure 3).

Effects of Expression of Y731F Cbl on Insulin-Induced Increases in aPKC Translocation to the Plasma Membrane. Insulin provokes increases in aPKC activity in the plasma membrane of rat adipocytes (*28*), and aPKCs have been suggested to function downstream of the flotillin/CAP/Cbl/Crk/CG3/TC10 complex (*18*), which is present in lipid raft microdomains of the plasma membrane. It was therefore of considerable interest to find that expression of Y731F Cbl blocked insulin-induced increases in aPKC levels recovered in the plasma membrane of 3T3/L1 adipocytes (Figure 6B). In contrast to findings in plasma membranes, neither insulin nor expression of Y731F Cbl altered the levels of aPKC in crude microsomal membranes (Figure 6B). Note, however, although we presently observed no increase in aPKC levels in microsomes, insulin has been found to increase (a) microsomal aPKC activity and (b) aPKC content of purified microsomal Glut4 vesicles in rat adipocytes (*28*).

DISCUSSION

From the present findings, it seems clear that tyrosine residues that initiate one or both of the two canonical pYXXM motifs in Cbl, viz., tyrosine-731 and/or tyrosine-371, is/are required for insulin-stimulated activation of aPKCs and glucose transport in 3T3/L1 adipocytes. As these tyrosine residues were also required for insulin-induced increases in Cbl/p85 subunit binding and activation of Cbl-dependent PI 3-kinase and since PI 3-kinase is required for insulin-induced activation of aPKCs and subsequent increases in glucose transport, it may be argued that Cbl-dependent PI 3-kinase activity per se is required for the activation of aPKCs during insulin-stimulated glucose transport. However, since expression of Y731F and Y371F Cbl mutants also inhibited the binding of Cbl to Crk, it is possible that IRS-1/2-dependent PI 3-kinase may function downstream of Crk to activate aPKC in 3T3/L1 adipocytes. In either case, Cbl would be required for activation of PI 3-kinase-dependent aPKCs that are required for insulin-stimulated glucose transport in 3T3/L1 adipocytes.

In support of the postulation that pYXXM motifs in Cbl are critical for insulin-stimulated activation of PI 3-kinase,

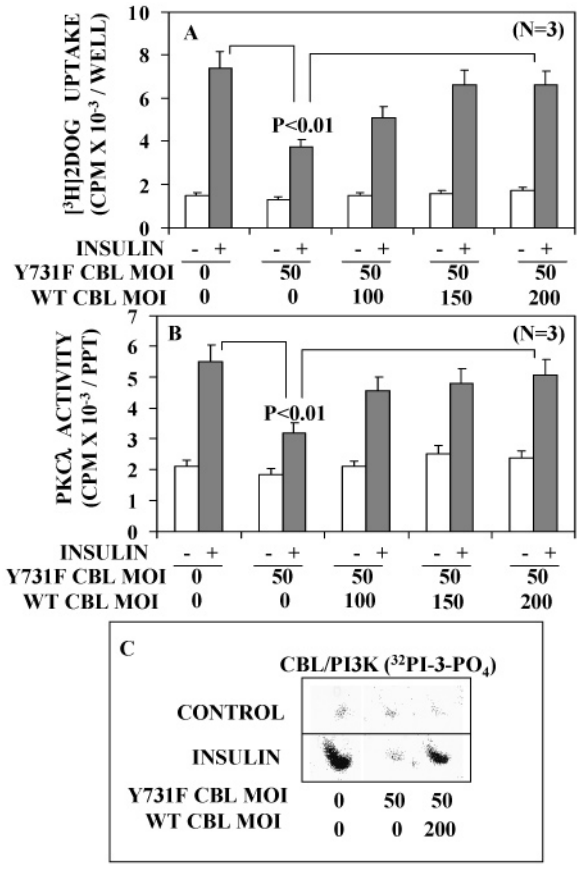

FIGURE 4: Rescue of inhibitory effects of the Y731F Cbl mutant on insulin-induced increases in ^{3}[H]-2-deoxyglucose uptake (A), PKC-λ activity (B), and Cbl-dependent PI3K activity (C) by expression of WT Cbl in 3T3/L1 adipocytes. Cells were incubated for 48 h without or with 50 MOI of adenovirus encoding Y731F Cbl mutant and the indicated MOI of adenovirus encoding WT Cbl (varying amounts of adenovirus vector were also added to keep the total virus at 250 MOI in all groups), and finally incubated without (clear bars) or with (filled bars) 100 nM insulin in glucose-free KRP medium containing 1% BSA for 30 min before ^{3}[H]-2-deoxyglucose uptake over 5 min was measured, and for 15 min before PKC-λ activity and Cbl-dependent PI 3-kinase activity (viz., relative ^{32}PI-3-PO$_4$ levels following purification by thin-layer chromatography) were measured. Bar graph values in panels A and B are the mean ± SE of the number of determinations shown in parentheses. *P* values of <0.01 in panels A and B reflect comparisons of insulin-stimulated values in Y731F Cbl-treated cells versus vector-treated and WT Cbl-treated cells. The autoradiogram shown in panel C is representative of three determinations.

aPKCs, and glucose transport is the finding that inhibitory effects of Y731F and Y371F Cbl mutants on insulin-stimulated increases in these parameters could not be explained by a nonspecific inhibition of IRS-1-dependent PI 3-kinase, which was not significantly inhibited by expression of Cbl mutants. Thus, expression of these Cbl mutants did not appear to indiscriminately inhibit the activation or function of non-Cbl insulin receptor substrates that, via pYXXM motifs, bind to SH2 domains in the p85 regulatory subunit of PI 3-kinase. This conclusion is further strengthened by the fact that overexpression of wild-type Cbl, which should theoretically be just as inhibitory as mutant Cbl in causing nonspecific inhibition of other insulin receptor substrates, either alone or in conjunction with Cbl mutants in rescue experiments, did not inhibit insulin effects on aPKC activity and glucose transport.

It should be emphasized that the suggestion that a Cbl-dependent PI 3-kinase is required for insulin-induced activation of aPKCs and glucose transport does not imply that IRS-1/2 and/or IRS-1/2-dependent PI 3-kinase is/are not required for these effects of insulin. Indeed, we have recently found in studies of IRS-1 knockout mice (*19*) and immortalized brown adipocytes in which IRS-1 or IRS-2 has been knocked out (see the following paper in this issue) that IRS-1 is required for activation of aPKCs in both skeletal muscle and adipocytes, and both IRS-1 and IRS-2 are required for aPKC activation in brown adipocytes. In addition, insulin-stimulated glucose transport is diminished in (a) both white adipocytes (*29*) and muscles (*30*) of IRS-1 knockout mice and (b) immortalized brown adipocytes deficient in either IRS-1 or IRS-2 (see the following paper in this issue). It therefore seems likely that there are corequirements for both Cbl and IRS-1/2, and possibly PI 3-kinase activities dependent on both Cbl and IRS-1/2, during insulin-induced activation of aPKCs and glucose transport in 3T3/L1 and other adipocytes.

Similarly, our findings suggesting that Cbl-dependent PI 3-kinase is required for insulin-stimulated activation of aPKCs and glucose transport should not be interpreted to suggest that other actions of Cbl, in particular that involving the formation and propagation of the flotillin/CAP/Cbl/Crk/C3G/TC10 complex, are dependent upon the activation of PI 3-kinase by either Cbl or IRS-1/2. Indeed, it seems clear from previous findings (*1*−*8*) that this complex is formed independently of PI 3-kinase. Moreover, in further support of the notion that Cbl is activated independently of PI 3-kinase, we have found that inhibition of PI 3-kinase by wortmannin does not diminish insulin-induced increases in binding of Cbl to either the p85 subunit of PI 3-kinase or Crk in brown adipocytes (see the following paper in this issue).

Whereas formation of the flotillin/CAP/Cbl/Crk/C3G/TC10 complex is independent of PI 3-kinase, as alluded to above, our findings nevertheless suggested that Cbl operates upstream of PI 3-kinase during insulin-stimulated glucose transport in 3T3/L1 adipocytes. The latter interpretation seems likely in view of the finding that inhibition of pYXXM motifs in Cbl blocked the activation of PKC-λ, which is dependent on PI 3-kinase. However, as discussed further below, there is remaining uncertainty as to whether this PI 3-kinase functions immediately downstream of Cbl, or downstream of the other factors in the Cbl/Crk/C3G/TC10 complex.

Although we did not examine the effects of the Cbl mutants on formation of the flotillin/CAP/Cbl/Crk/C3G/TC10 complex, the presently used Y731F and Y371F Cbl mutants inhibited the binding of Cbl to Crk, and this would be expected to inhibit the subsequent propagation of this complex distal to Cbl. Accordingly, it may be surmised that pYXXM motifs in Cbl bind to SH2 domains in Crk, and expression of the Y731F and Y371F Cbl mutants most likely inhibited the recruitment and subsequent assembly and operation of factors that are distal to Crk, including C3G and TC10. It may therefore be argued that the Crk/C3G/TC10 complex could function upstream of PI 3-kinase and aPKCs during insulin-stimulated glucose transport. However, the factor that could directly activate this PI 3-kinase is not readily apparent. In this regard, despite the fact that inhibition of TC10 by *Clostridium difficile* toxin B has been reported

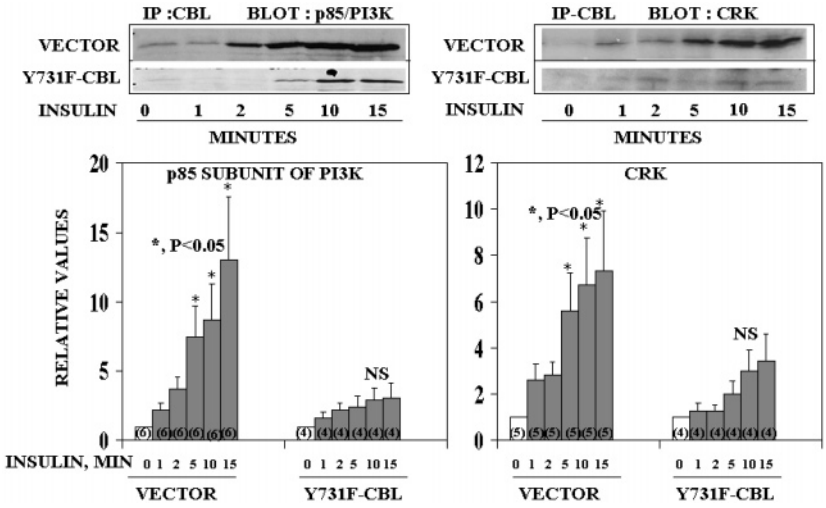

FIGURE 5: Effects of insulin and expression of Y731F Cbl mutant on binding of Cbl to the p85 subunit of PI 3-kinase (left) and Crk (right) in 3T3/L1 adipocytes. Cells in 100 mm plates were incubated for 48 h with 50 MOI of adenovirus vector alone or adenovirus encoding Y731F Cbl mutant, and finally incubated for the indicated times without (clear bars) or with (shaded bars) 100 nM insulin in glucose-free KRP medium containing 1% BSA. Lysates were subjected to immunoprecipitation with anti-Cbl antiserum, resolved by SDS−PAGE, and blotted with anti-p85 and anti-Crk antibodies. Values in the bar graphs are the mean ± SE of the number of determinations shown in parentheses. *P* values reflect comparisons of insulin-stimulated vector-treated and Y731F Cbl-treated cells. NS = not significant.

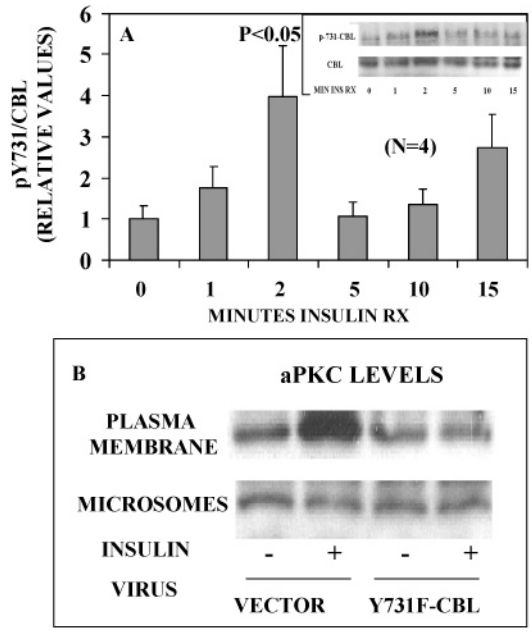

FIGURE 6: Time-dependent phosphorylation/activation of tyrosine-731 in Cbl by insulin (A), and effects of expression of Y731F Cbl on insulin-induced increases in plasma membrane content of aPKC (B) in 3T3/L1 adipocytes. In panel A, adipocytes were treated with 100 nM insulin for the indicated times, following which Cbl was resolved by SDS−PAGE and blotted initially for phosphotyrosine-731-Cbl content (top blot), and then stripped and blotted for total Cbl content (note that the Cbl band was superimposable on the phosphotyrosine-731-Cbl band) (bottom blot). Although not shown, the authenticity of the phosphotyrosine-731-Cbl band was further corroborated by showing that phosphotyrosine-774-Cbl migrated at the identical level on SDS−PAGE. Values in the bar graphs are the mean ± SE of four determinations. *P* values were determined by ANOVA. In panel B, adipocytes were incubated for 48 h with adenovirus vector alone or adenovirus containing cDNA encoding Y731F Cbl, and finally treated with 100 nM insulin for 30 min, before the plasma membranes and microsomal fractions were harvested, which were blotted for aPKC content. Shown here are representative immunoblots.

to inhibit the recruitment of aPKCs to caveolae-associated actin structures that emanate from lipid rafts (*18*), it remains to be seen if TC10 is capable of activating IRS-1/2, or if inhibition of TC10 interferes with the activation of Cbl-dependent PI 3-kinase.

With the above considerations in mind, we may speculate further on how IRS-1/2, Cbl, the flotillin/CAP/Cbl/Crk/C3G/TC10 complex, and the PI 3-kinase/PDK1/aPKC pathway may operate during insulin-stimulated glucose transport in 3T3/L1 adipocytes. One possibility discussed above is that Cbl, via Crk/C3G/TC10, could serve to localize IRS-1/2 and thereby activate aPKCs within lipid raft microdomains of the plasma membrane. In this scenario, the presently observed activation of Cbl-dependent PI 3-kinase by insulin would not be important for activating aPKCs. A second possibility is that, in addition to participating in the formation of the flotillin/CAP/Cbl/Crk/C3G/TC10 complex, Cbl, within this complex, activates PI 3-kinase, which, perhaps via local increases in PIP_3 and binding to PH domains of IRS family members, properly localizes and activates one or more pools of IRS-1/2 that is/are responsible for activating the PI 3-kinase that activates aPKCs within specific insulin-sensitive lipid raft microdomains of the plasma membrane. In this regard, note that activated PI 3-kinase is required for binding and translocation of IRS-1 and IRS-2 to membranes or the actin cytoskeleton during insulin action (*20*, *31*). A third possibility is that IRS family members that are recruited to the juxtamembranous region of the β-subunit of the insulin receptor may participate, along with CAP and APS, in the localization and tyrosine phosphorylation/activation of Cbl. In this scenario, the localizing properties of IRS-1/2 may be as or more important than their ability to activate PI 3-kinase. In support of the third possibility that IRS-1 and IRS-2 function upstream of Cbl, we have found that knockout of IRS-1 or IRS-2 in brown adipocytes is accompanied by a marked reduction in the ability of insulin to (a) promote Cbl binding to the p85 subunit of PI 3-kinase and Crk and (b) activate Cbl-dependent PI 3-kinase, aPKCs and glucose transport (see the following paper in this issue). Finally, it is conceivable that possibilities 2 and 3 may both be operative, and Cbl and IRS-1/2 may function in an interde-

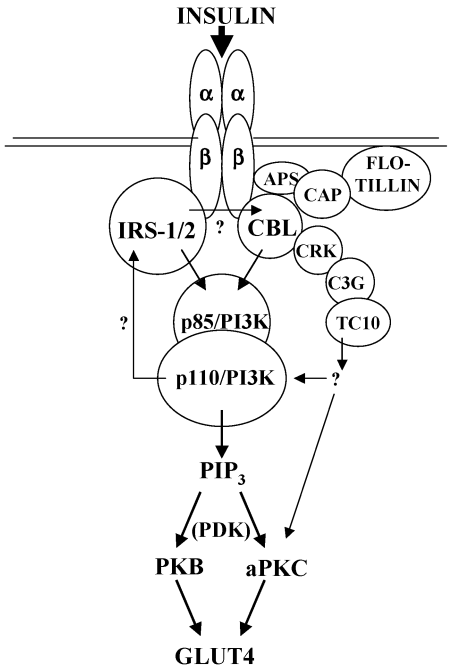

FIGURE 7: Schematic of the signaling network in lipid rafts during insulin stimulation of Glut4 translocation in adipocytes.

pendent "signaling network", instead of separate linear pathways (see Figure 7). Further studies are needed to more thoroughly evaluate these and other possibilities.

It may be noted that recent reports have questioned the relevance of the Cbl/Crk/C3G/TC10 pathway in L6 myotubes (*32*) and rat skeletal muscles (*33*). Similarly, our experience in these cells is also not supportive of an important role for this pathway in muscle. In L6 myotubes, we have not observed an effect of insulin on Cbl-dependent PI 3-kinase, and expression of the Y731F Cbl mutant does not inhibit insulin-induced increases in aPKC activity or glucose transport. Similarly, in rat skeletal muscle, we have not seen effects of either insulin or thiazolidinediones on either Cbl-dependent PI 3-kinase activity or binding of Cbl to either the p85 subunit of PI 3-kinase or Crk. On the other hand, we have observed insulin-induced activation of Cbl-dependent PI 3-kinase in both rat and human adipocytes, and very interestingly, this activation is defective in adipocytes of type 2 diabetic Goto−Kakizaki rats; moreover, Cbl-dependent PI 3-kinase activation is restored by thiazolidinedione treatment (Standaert, M. L., Sajan, M. P., Miura, A., Bandyopadhyay, G., Farese, R. V., unpublished observations). Further studies are needed to see if Cbl-dependent processes are relatively specific for adipocytes, particularly after thiazolidinedione treatment.

Finally, the present findings appear to be at odds with those of a recently published study in which silencing RNA-induced depletion of c-Cbl and Cbl-b did not impair insulin-stimulated glucose transport in 3T3/L1 adipocytes (*34*). On the other hand, a similar use of silencing RNA suggested that insulin-stimulated glucose transport is dependent on Cbl in human adipocytes (*35*), and the reason for these differences in findings is not readily apparent. In any case, Rho family members other than TC10 are known to function upstream of, or in conjunction with, aPKCs, and it may be recalled that the small G-protein Rho A, like TC10, is GTP-loaded/activated independently of PI 3-kinase but, on the other hand, is translocated to the plasma membrane by a PI 3-kinase-dependent mechanism and, moreover, is required for insulin-stimulated glucose transport in isolated rat adipocytes (*36*). Thus, in some circumstances, e.g., with Cbl depletion and loss of TC10 signaling, it is possible that other signaling mechanisms may function in the activation/localization of aPKCs and glucose transport in adipocytes.

In summary, our findings show that tyrosine residues at 371 and 731 and corresponding pYXXM motifs in Cbl are required for (a) binding of Cbl to the p85 subunit of PI 3-kinase and Crk and (b) activation of Cbl-dependent PI 3-kinase and aPKCs during insulin-stimulated glucose transport in 3T3/L1 adipocytes. In view of other findings that underscore the importance of IRS family members, we speculate that Cbl, along with IRS family members, are required for activation of one or more PI 3-kinase activities that are required for activation of aPKCs during insulin stimulation of glucose transport in 3T3/L1 adipocytes.

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