Cbl PYXXM Motifs Activate the P85 Subunit of Phosphatidylinositol 3-Kinase, Crk, Atypical Protein Kinase C, and Glucose Transport during Thiazolidinedione Action in 3T3/L1 and Human Adipocytes†

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ABSTRACT: The thiazolidinedione (TZD), rosiglitazone, has previously been found to tyrosine-phosphorylate Cbl and activate Cbl-dependent phosphatidylinositol (PI) 3-kinase and atypical protein kinase Cs (aPKCs) while stimulating glucose transport in 3T3/L1 adipocytes. Presently, the role of Cbl in rosiglitazone action was further assessed in both 3T3/L1 and human adipocytes by expressing Y371F and/or Y731F mutant forms of Cbl that nullified the functionality of canonical pYXXM motifs in Cbl. These mutants diminished the interaction of Cbl with the p85 subunit of PI 3-kinase and inhibited subsequent increases in Cbl-dependent PI 3-kinase activity, aPKC activity, and glucose transport. These mutants also inhibited the interaction of Cbl with Crk, which has been implicated in the activation of other PI 3-kinase-independent signaling factors that have been found to be required during activation of glucose transport by insulin and other agonists. We conclude that pYXXM motifs in Cbl serve to activate PI 3-kinase-dependent and possibly PI 3-kinase-independent pathways that are required for TZD-dependent glucose transport in adipocytes.

Thiazolidinediones (TZDs),[1] as insulin-sensitizing agents, have, as one of their major actions, the ability to increase basal and insulin-stimulated glucose transport in adipocytes and skeletal muscle cells without necessarily increasing glucose transporter levels (*1*−*3*). The mechanisms whereby TZDs activate glucose transport, however, are only partly understood.

In stimulating glucose transport, insulin provokes increases in Glut4 glucose transporter translocation to the plasma membrane by a mechanism that is thought to be dependent on the activation of insulin receptor substrate (IRS)-dependent phosphatidylinositol (PI) 3-kinase, which, in conjunction with 3-phosphoinositide-dependent protein kinase-1 (PDK1), activates apparent downstream effectors, viz., atypical protein kinase C (aPKC) isoforms (*4*−*7*) and protein kinase B (PKB) (*8*−*11*). In this respect, and pertinent to present studies in both 3T3/L1 adipocytes (which contain PKC-λ as its major aPKC) and human adipocytes (which contain PKC-ζ as its major aPKC), both PDK1 and aPKCs, as well as PI 3-kinase, appear to be required for insulin-stimulated glucose transport in these adipocytes (*4*, *7*, *12*).

With respect to TZDs, rosiglitazone was found to enhance insulin-stimulated Glut4 translocation and glucose transport by a mechanism involving increases in levels of IRS-1 and IRS-2 and increases in insulin-stimulated activation of IRS-1/2-dependent PI 3-kinase, PKC-λ, and PKB n 3T3/L1 adipocytes (*2*). In addition to enhancing insulin actions, and more germane to the presently described studies, rosiglitazone, in the absence of insulin, and, in the absence of activation of IRS-1/2-dependent PI 3-kinase, provokes sizable increases in basal (i.e., noninsulin-stimulated) Glut4 translocation and glucose transport in 3T3/L1 adipocytes (*2*). Importantly, these effects of rosiglitazone on basal glucose transport, like those of insulin, are dependent on PI 3-kinase, PDK1, and PKC-λ, and moreover, are associated with increases in tyrosine phosphorylation of Cbl and increases in activity of Cbl-dependent PI 3-kinase (*2*). It was therefore postulated that rosiglitazone provokes increases in Cbl-dependent PI 3-kinase, thereby increasing membrane levels of PI-3,4,5-$(PO_4)_3$ (PIP_3), which in conjunction with PDK1, activates aPKCs and glucose transport during rosiglitazone action in 3T3/L1 adipocytes (*2*).

In support of an apparent relationship between Cbl-dependent PI 3-kinase and aPKC activation, plasmid-mediated expression of deleted forms of Cbl that lacked middle or C-terminal regions, or contained phenylalanine substitutions in all eight C-terminal tyrosine residues, inhibit the activation

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[1] Abbreviations: TZD, thiazolidinedione; RSGZ, rosiglitazone; IRS, insulin receptor substrate; PI, phosphatidylinositol; PI3P, phosphatidylinositol-3-PO_4; PIP_3, phosphatidylinositol-3,4,5-$(PO_4)_3$; PKC, protein kinase C; aPKC, atypical protein kinase C; PKB, protein kinase B; CAP, Cbl-associated protein; DOG, deoxyglucose; PDK1, 3-phosphoinositide-dependent protein kinase-1; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; KRP, Krebs Ringer phosphate buffer.

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of cotransfected aPKCs by rosiglitazone in 3T3/L1 adipocytes (*2*). However, cotransfection studies with these mutants did not provide conclusive evidence that Cbl-dependent PI 3-kinase as such was involved in endogenous aPKC activation, and moreover, could not be used to establish a link between Cbl-dependent PI 3-kinase and glucose transport during rosiglitazone action.

With respect to Cbl, this scaffolding/adapter protein is involved in a number of cellular functions, including serving as a ubiquitin ligase for protein degradation, and more pertinent to the present studies, Cbl serves as a substrate for protein tyrosine kinases, and via its two canonical pYXXM motifs that start at tyrosine residues 371 and 731 or via its multiple proline-rich sequences, can bind to SH2 or SH3 domains, respectively, in the p85 subunit of PI 3-kinase and thereby activate the p110 catalytic unit of PI 3-kinase (*13*).

Cbl is also recognized to be important in mediating insulin effects on Glut4 translocation and glucose transport that are independent of PI 3-kinase. Thus, upon binding of insulin to its receptor, Cbl, in association with CAP (*14*, *15*), which is induced by TZDs via PPAR-γ receptors (*16*) and APS (*17*), is rapidly recruited to the insulin receptor, which thereupon phosphorylates tyrosine residues of Cbl (*14*) and APS (*17*). The phosphorylated CAP/Cbl complex then dissociates from the insulin receptor and binds to flotillin in caveolin-rich lipid raft microdomains of the Triton-insoluble fraction of the plasma membrane (*18*). Cbl thereupon binds to the SH3/SH3 adapter protein Crk, which in turn recruits C3G, a GTP/GDP exchange factor for the small G-protein Rho family member TC10 (*19*, *20*), along with other proteins, to form a highly integrated signaling complex that is required for insulin-stimulated Glut4 translocation/glucose transport. Although this flotillin/CAP/Cbl/Crk/C3G/TC10 signaling complex forms independently of PI 3-kinase, PI 3-kinase is simultaneously activated by insulin and is corequired for insulin-stimulated glucose transport (*14*−*20*). Whether or not this complex or elements thereof are activated during TZD-induced activation of glucose transport is uncertain.

Presently, we used forms of Cbl in which tyrosine residues 371 and 731 were mutated to phenylalanine to test the hypothesis that Cbl-dependent PI 3-kinase functions upstream of aPKCs and glucose transport during TZD action in both 3T3/L1 and human adipocytes. Indeed, we found that expression of these mutated forms of Cbl markedly inhibited rosiglitazone-induced activation of Cbl-dependent PI 3-kinase, aPKCs, and glucose transport in both 3T3/L1 and human adipocytes. Moreover, we found that, in response to rosiglitazone treatment in 3T3/L1 adipocytes, Cbl binds to both the p85 subunit of PI 3-kinase and Crk, and binding to both of these SH2-containing factors is diminished or abrogated by the expression of Cbl mutants that lack functional pYXXM motifs. Our findings therefore provided clear evidence that pYXXM motifs of Cbl are required for TZD-induced activation of the signaling pathway of Cbl-dependent PI 3-kinase, PDK1, and aPKCs that is required for TDZ-stimulated glucose transport in adipocytes. In addition, the finding that pYXXM motifs in Cbl participate in binding to SH2 domains of Crk raises the possibility that the flotillin/CAP/Cbl/Crk/C3G/TC10 pathway may be activated during TZD action in adipocytes, and this pathway may participate in conjunction with the Cbl/PI 3-kinase/PDK1/aPKC pathway in promoting glucose transport.

EXPERIMENTAL PROCEDURES

Cell Culture, Incubations, and Treatments. 3T3/L1 adipocytes (*2*, *4*) and human adipocytes (*12*) were cultured and differentiated from precursor cells (i.e., pre-adipocyte/fibroblasts, as described. Insulin, isobutyrylmethyxanthine, and dexamethasone were withdrawn from fully differentiated adipocytes 48 h prior to experimentation. Where indicated, adipocytes were incubated in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (Sigma) for 48 h with indicated concentrations of adenovirus alone or adenovirus encoding wild type or mutated forms of Cbl. During this 48 h period, cells were treated with indicated concentrations of rosiglitazone (kindly supplied by Smith-Kline Beecham), as described previously (*2*). The cells were then incubated in serum-free DMEM for 3−4 h with or without rosiglitazone and finally incubated in glucose-free Krebs Ringer phosphate (KRP) medium containing 1% bovine serum albumin (BSA) (Sigma).

aPKC Activation. aPKC activity was measured as described (*1*, *2*, *4*−*7*, *12*). In brief, aPKCs were immunoprecipitated from salt/detergent-treated cell lysates with a rabbit polyclonal antiserum (Santa Cruz Biotechnologies) that recognizes the C-termini of both PKC-λ and PKC-ζ (note that mouse-derived 3T3/L1 adipocytes contain only PKC-λ, and human adipocytes contain only PKC-ζ), 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, a 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 (rabbit polyclonal antiserum from Santa Cruz Biotechnologies) PI 3-kinase activity was determined as described (*2*).

2-Deoxyglucose Uptake. Cells were incubated in glucose-free KRP medium for 30 min, prior to measurement of uptake of [^{3}H]-2-deoxyglucose (50 μM) over 5 min, as described (*2*, *4*, *12*).

Immunoblotting. Western analyses were conducted as described (*1*, *2*, *4*−*7*, *12*) and blotted with (a) rabbit polyclonal anti-PKC-ζ/λ C-terminal antiserum (Santa Cruz Biotechnologies); (b) mouse monoclonal anti-GLUT 4 antibodies (Biogenesis); (c) rabbit polyclonal anti-p85 subunit of PI 3-kinase antiserum (UBI); (d) rabbit polyclonal anti-PDK-1 antiserum (UBI); (e) rabbit polyclonal anti-IRS-1 antiserum (UBI; (f) rabbit polyclonal anti-IRS-2 antiserum (kindly supplied by Dr. Morris White); (g) rabbit polyclonal anti-Cbl antiserum (Santa Cruz Biotechnologies); and (h) mouse monoclonal anti-Crk antibodies (Transduction Labs). Blots were quantified by measurement of extended chemiluminescence (ECL) in a BioRad PhosphorImager/Chemiluminescence Imaging System using a Molecular Analyst Program.

Adenoviral Constructs. pCMV2 plasmid encoding wild type Cbl (*2*) 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.

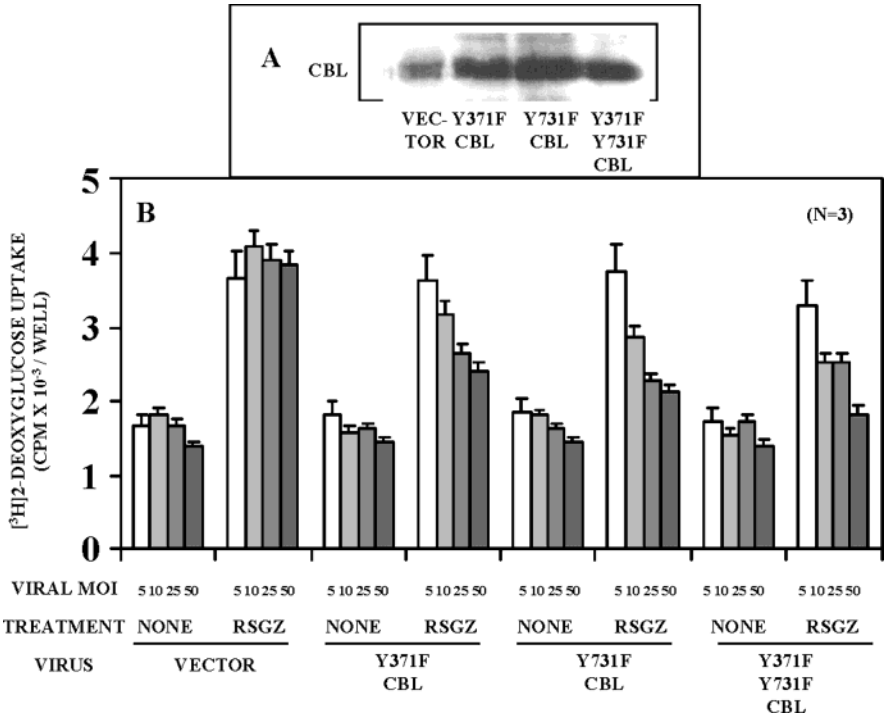

FIGURE 1: Effects of expression of Cbl mutants, Y371F, Y731F, and Y371F/Y731F, on rosiglitazone-stimulated [^{3}H]2-deoxyglucose uptake in 3T3/L1 adipocytes. Cells in 24-well plates were incubated for 48 h with or without 1 μM rosiglitazone (RSGZ) and increasing amounts (MOI, or multiplicity of infection) of adenovirus alone (vector) or adenovirus encoding indicated Cbl mutants and finally incubated for 30 min in glucose-free KRP medium containing 1% BSA before measuring the uptake of [^{3}H[2-deoxyglucose over 5 min. Values are mean $\pm$ SE of three determinations. Shown in the inset are increases in total content of Cbl in cells incubated with 25 MOI of indicated adenovirus (note that vector alone did not alter Cbl content).

Adenoviruses encoding wild type, Y371F, Y731F, and Y371F/Y731F forms of Cbl were constructed using plasmids encoding these forms of Cbl and an Adeno-X Expression kit obtained from Clontech. All final constructs were sequenced to ensure that the mutations were maintained through the preparative measures.

RESULTS

Studies in 3T3/L1 Adipocytes. It was previously established that 1 μM rosiglitazone provokes maximal stimulatory effects on Glut4 translocation/glucose transport and activation of Cbl, PI 3-kinase, and PKC-λ in 3T3/L1 adipocytes (*2*), and this concentration was therefore used throughout the present studies of these cells. Note that rosiglitazone alone does not activate IRS-1, IRS-2, IRS-1/2-dependent PI 3-kinase, or PKB in 3T3/L1 adipocytes (*2*); consequently, these parameters were not presently studied.

Effects of Cbl Mutants on 2-Deoxyglucose Uptake. Whereas the adenovirus vector had little or no effect on basal or rosiglitazone-stimulated 2-deoxyglucose uptake, the addition of increasing amounts of adenovirus encoding Y371F or the Y731F single, or Y371F/Y731F double, Cbl mutant resulted in graded decreases in rosiglitazone-induced increases in 2-deoxyglucose uptake (Figure 1). The expression of the three Cbl mutants is also shown in the inset of Figure 1. At a viral dose of 25 MOI, the total cellular Cbl content was approximately doubled in cells infected with adenovirus encoding each of the three Cbl mutants, as compared to vector alone. Although not shown, increasing the dose of adenovirus expressing these Cbl mutants to 50 MOI led to proportionate increases in total cellular Cbl content.

Effects of Cbl Mutants on Cbl-Dependent PI 3-Kinase. In addition to inhibiting effects of rosiglitazone on 2-deoxyglucose uptake, the expression of the Y371F, Y731F, and Y371F/Y731F Cbl mutants markedly inhibited rosiglitazone-induced increases in Cbl-dependent PI 3-kinase activity (Figure 2). Note that, in other similar experiments in which Cbl mutants were expressed, basal Cbl-dependent PI 3-kinase activity was not appreciably altered by mutant expression (data not shown); thus, the mild relative increase in basal activity of cells infected with the Y371F mutant shown in Figure 2 is not significant.

In contrast to the expression of mutant forms of Cbl, expression of wild type Cbl did not significantly alter the activation of Cbl-dependent PI 3-kinase (Figure 2). Note that the relative stimulatory effects of rosiglitazone varied from experiment to experiment (see Figure 2), and this is at least partly due to varying basal levels. Also note that, as reported previously, in contrast to increasing Cbl-dependent PI 3-kinase activity, rosiglitazone, in the absence of concurrent insulin treatment, has no effect on IRS-1- or IRS-2-dependent PI 3-kinase activity in 3T3/L1 adipocytes (*2*).

Effects of Cbl Mutants on PKC-λ Activation. In addition to inhibiting increases in 2-deoxyglucose uptake and Cbl-dependent PI 3-kinase activity, the expression of Y371F, Y731F, and Y371F/Y731F Cbl mutants inhibited rosiglitazone-induced increases in PKC-λ activity in 3T3/L1 adipocytes (Figure 3B). As inhibitory effects of the Y731F Cbl single mutant on both rosiglitazone-stimulated 2-deoxyglucose uptake and PKC-λ activation were comparable to those observed with the Y371F/Y731F Cbl double mutant, in most subsequent studies in 3T3/L1 adipocytes, we used only the Y731F Cbl mutant.

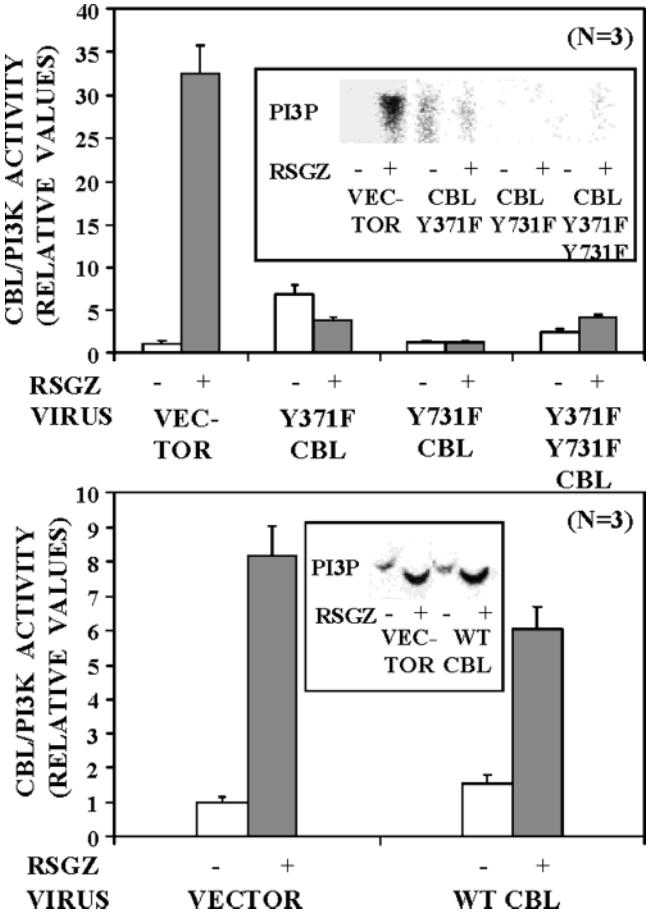

FIGURE 2: Effects of expression of Cbl mutants, Y371F, Y731F, and Y371F/Y731F, and wild type (WT) Cbl on rosiglitazone-induced increases in Cbl-dependent PI 3-kinase activity in 3T3/L1 adipocytes. Cells in 100 mm plates were incubated for 48 h with or without 1 μM rosiglitazone (RSGZ), and 50 MOI (multiplicity of infection) of adenovirus alone (vector) or adenovirus encoding indicated Cbl mutants or wild type Cbl, and finally incubated for 15 min in glucose-free KRP medium containing 1% BSA before measuring Cbl-dependent PI 3-kinase activity in cell lysates. Shown in the insets are representative autoradiograms. Mean relative values $\pm$ SE of three determinations are shown in bargrams. PI3P, phosphatidylinositol-3-PO_4.

Effects of wild type Cbl on 2-Deoxyglucose Uptake and PKC-λ Activation. In contrast to mutant forms of Cbl, expression of wild type Cbl did not diminish the stimulatory effects of rosiglitazone on 2-deoxyglucose uptake (Figure 3A) and PKC-λ activation (Figure 3B) in 3T3/L1 adipocytes. Note that wild type Cbl was expressed to the same degree as mutant forms of Cbl (inset, Figure 3B). The inhibitory effects of expression of mutant forms of Cbl can therefore be specifically attributed to the mutated tyrosine residues rather than to simple overexpression of Cbl, which theoretically could cause nonspecific inhibitory effects by excessively binding and thereby incapacitating other signaling factors. In further support of this conclusion is the finding that that the overexpression of wild type Cbl completely reversed the inhibitory effects of the Y731F Cbl mutant on insulin-induced increases in 2-deoxyglucose uptake (Figure 4A) and PKC-λ activity (Figure 4B). Finally, in other studies (unpublished), we found that expression of the presently used Cbl mutants did not significantly inhibit insulin-induced activation of IRS-1-dependent PI 3-kinase.

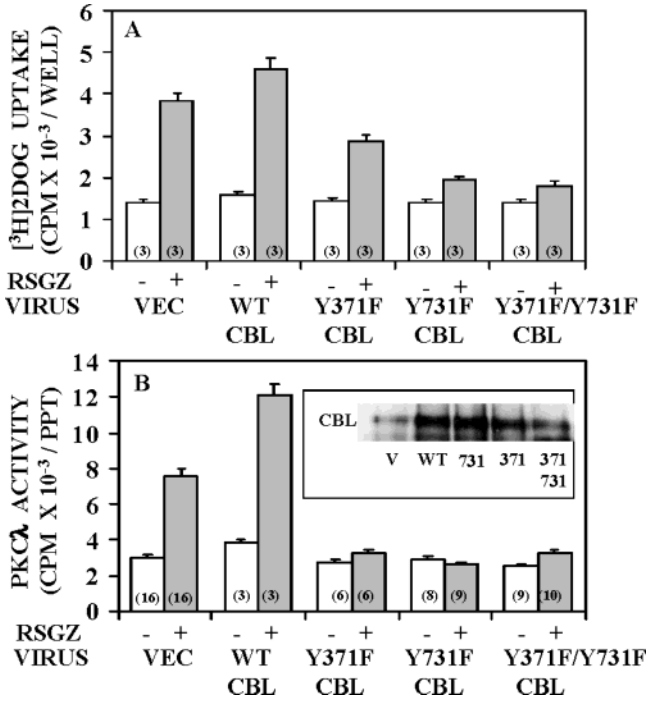

FIGURE 3: Effects of expression of Cbl mutants, Y371F, Y731F, and Y371F/Y731F, and wild type (WT) Cbl on rosiglitazone-induced increases in [^{3}H]2-deoxyglucose (DOG) uptake (A) and PKC-λ activity (B) in 3T3/L1 adipocytes. Cells in 100 mm plates were incubated for 48 h with or without 1 μM rosiglitazone (RSGZ), and with 50 MOI (multiplicity of infection) of adenovirus alone (vector) or adenovirus encoding indicated WT Cbl or Cbl mutants, and finally incubated for 30 and 15 min in glucose-free KRP medium containing 1% BSA before measuring [^{3}H]2-deoxyglucose uptake and lysate PKC-λ activity, respectively. Values are mean $\pm$ SE of the number of determinations shown in parentheses. Levels of immunoreactive Cbl in viral-infected cells (50 MOI of Vector, V; WT, WT Cbl; 731, Y731F Cbl; 371, Y371F Cbl; and 371/731, Y371F/Y731F Cbl) are shown in the representative experiment depicted in the inset.

Binding of Cbl to the p85 Subunit of PI 3-Kinase and Crk. In addition to activating Cbl-dependent PI 3-kinase, rosiglitazone provoked increases in the recovery of the p85 subunit of PI 3-kinase in Cbl immunoprecipitates (Figure 5A). This binding of the p85 subunit to Cbl was, moreover, diminished upon expression of the Y731F Cbl mutant (Figure 5A). Similarly, rosiglitazone provoked increases in the recovery of Crk in Cbl immunoprecipitates, and this binding was markedly inhibited by expression of the Y731F Cbl mutant (Figure 5B). It may therefore be surmised that pYXXM motifs in Cbl bind to SH2 domains in both the p85 subunit of PI 3-kinase and Crk.

Studies in Human Adipocytes. We have previously reported that insulin provokes increases in 2-deoxyglucose uptake and PKC-ζ activity in human adipocytes that are derived from pre-adipocytes harvested during cosmetic liposuction procedures in nondiabetic women (*12*). We have also previously reported that insulin-induced increases in 2-deoxyglucose uptake in human adipocytes, like those in 3T3/L1 adipocytes, are inhibited by kinase-inactive forms of PDK1 and PKC-ζ (*12*).

Effects of Cbl Mutants on 2-Deoxyglucose Uptake. As in 3T3/L1 adipocytes (*2*), rosiglitazone provoked increases in basal (noninsulin-stimulated) glucose transport that were nearly as great as those provoked by insulin treatment (Figure

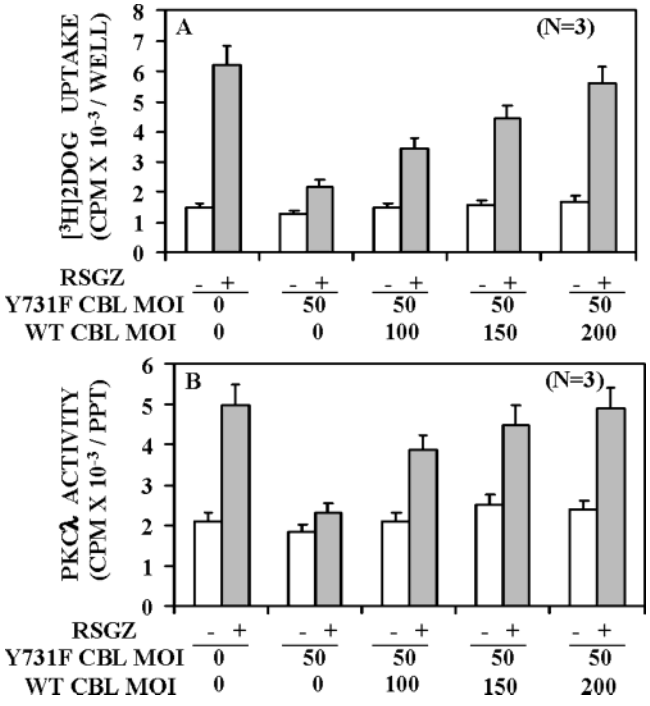

FIGURE 4: Rescue of inhibitory effects of the Y731F-Cbl on rosiglitazone-induced increases in [^{3}H]2-deoxyglucose (DOG) uptake (A) and PKC-λ activity (B) by expression of wild type (WT) Cbl in 3T3/L1 adipocytes. Cells in 100 mm plates were incubated for 48 h with or without 1 μM rosiglitazone, and with 50 MOI (multiplicity of infection) of adenovirus encoding Y731F-Cbl mutant and indicated MOI of adenovirus encoding WT Cbl, and finally incubated for 30 or 15 min in glucose-free KRP medium containing 1% BSA before measuring [^{3}H]2-deoxyglucose uptake and lysate PKC-λ activity, respectively. Values are mean $\pm$ SE of three determinations.

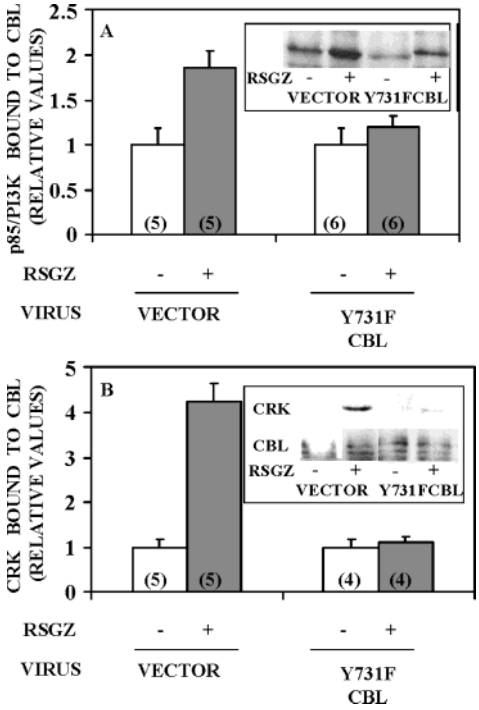

FIGURE 5: Effects of expression of the Y731F Cbl mutant on rosiglitazone-induced increases in recovery of the p85 subunit of PI 3-kinase (A) and Crk (B) in Cbl immunoprecipitates. Cells in 100 mm plates were incubated for 48 h with or without 1 μM rosiglitazone (RSGZ), and with 50 MOI (multiplicity of infection) of adenovirus alone (vector) or adenovirus encoding the Y731F Cbl mutant, and finally incubated for 15 min in glucose-free KRP medium containing 1% BSA. Lysates were then subjected to immunoprecipitation with anti-Cbl antiserum (Santa Cruz Biotechnologies), and precipitates were resolved by SDS−PAGE and blotted for the p85 subunit of PI 3-kinase, Crk and Cbl. Representative blots are shown in the insets, and results of multiple (see number in parentheses) determinations are depicted by bargrams.

6A). Further, combined treatment with rosiglitazone and insulin provoked increases in glucose transport that were less than additive (Figure 6A). (It may be noted that human adipocytes are more sensitive to rosiglitazone than 3T3L1 adipocytes, with maximal effects observed at 30−50 nM rosiglitazone in human adipocytes.) As seen in Figure 7E, rosiglitazone-induced increases in 2-deoxyglucose uptake were inhibited by expression of the Y371F/Y731F Cbl double mutant but not by expression of wild type Cbl (also see Figure 7C for expression of wild type and mutant forms of Cbl in human adipocytes).

Effects of Cbl Mutants on PKC-ζ Activation. As in 3T3/L1 adipocytes (*2*), rosiglitazone and insulin provoked increases in PKC-ζ activity that were less than additive (Figure 6B) but roughly commensurate with increases in 2-deoxyglucose uptake (Figures 6B and 7D,E). Further, as seen in 3T3/L1 adipocytes, rosiglitazone-induced increases in PKC-ζ activity were inhibited by the expression of the Y371F/Y731F Cbl double mutant but not by expression of wild type Cbl (Figure 7D).

Effects of Rosiglitazone and Insulin on PI 3-Kinase Activation. As in 3T3/L1 adipocytes (*2*), rosiglitazone provoked increases in Cbl-dependent PI 3-kinase activity in human adipocytes (Figure 7A,B). Also, as in 3T3/L1 adipocytes (*2*), insulin, but not rosiglitazone, increased IRS-1-dependent PI 3-kinase activity in human adipocytes (data not shown).

DISCUSSION

The present findings provide clear evidence that tyrosine residues 371 and 731 that initiate pYXXM motifs in Cbl are required for rosiglitazone-induced activation of aPKCs and glucose transport in 3T3/L1 and human adipocytes. As these tyrosine residues were also required for rosiglitazone-induced increases in binding of Cbl to the p85 subunit of PI 3-kinase and Cbl-dependent PI 3-kinase activity, and since it has previously been shown that independently of IRS-1 and IRS-2, PI 3-kinase and PDK1 are required for rosiglitazone-stimulated glucose transport in 3T3/L1 adipocytes (*2*), it is reasonable to postulate that pYXXM motifs initiated by tyrosine residues 371 and 731 in Cbl are required for rosiglitazone-induced increases in Cbl-dependent PI 3-kinase, which in turn is required for the PDK1-dependent activation of aPKCs and glucose transport in both 3T3/L1 and human adipocytes. As discussed further, this postulation does not preclude the possibility that Cbl may also serve in other capacities during rosiglitazone-induced increases in noninsulin-stimulated glucose transport.

Although our findings with Y371F and Y731F Cbl mutants, coupled with previous findings (*2*), provided strong evidence that Cbl-dependent PI 3-kinase is required for rosiglitazone-induced activation of aPKCs and glucose transport, we cannot disregard the possibility that these

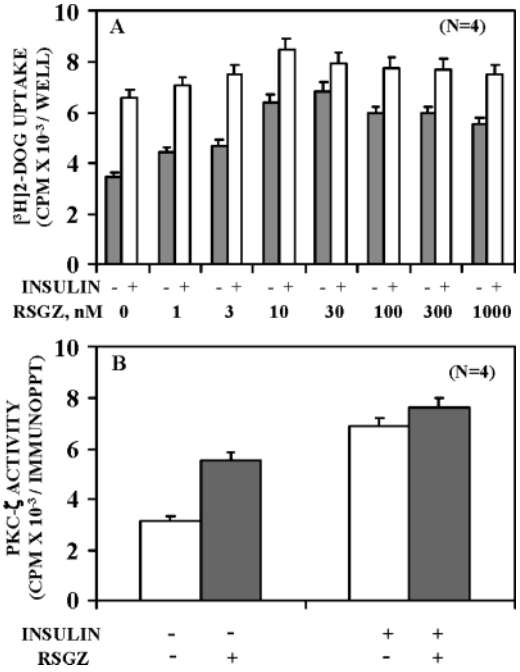

FIGURE 6: Effects of rosiglitazone (RSGZ) and insulin on [^{3}H]2-deoxyglucose (DOG) uptake (A) and PKC-ζ activity (B) in human adipocytes. Cells were incubated for 48 h with indicated concentrations of rosiglitazone in panel A, and with or without 50nM rosiglitazone in panel B, and finally incubated in glucose-free KRP medium containing 1% BSA and where indicated 100 nM insulin. [^{3}H]2-Deoxyglucose uptake and lysate PKC-ζ activity were measured after 30 and 15 min, respectively, of insulin treatment. Values are mean ± SE of four determinations.

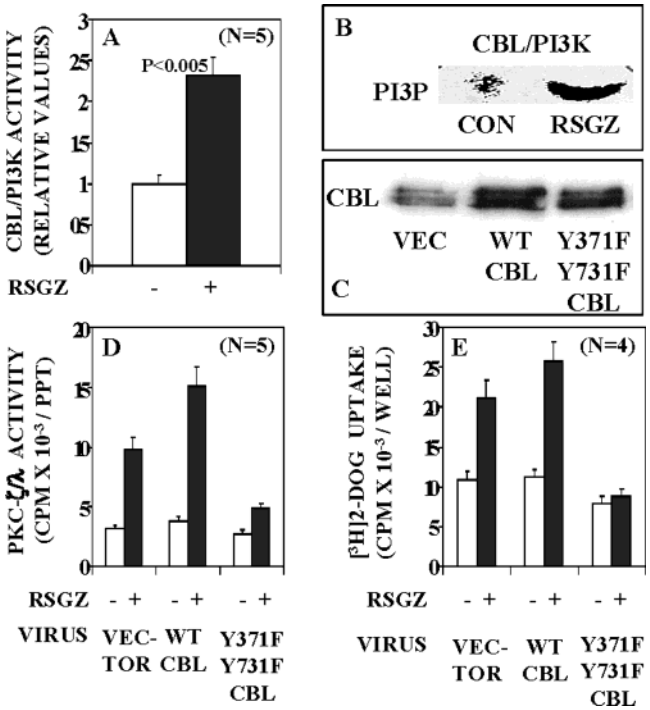

FIGURE 7: Effects of rosiglitazone (RSGZ) on Cbl-dependent PI 3-kinase activity (A) and effects of expression of wild type (WT) Cbl or Y371F/Y731F-Cbl on rosiglitazone-induced increases in [^{3}H]2-deoxyglucose uptake (E) and PKC-ζ activity (D) in human adipocytes. Cells were incubated for 48 h with or without 50 nM rosiglitazone, and with 50 MOI adenovirus alone (vector) or adenovirus encoding WT or mutant Cbl in panels C−E, and finally incubated for 30 or 15 min in glucose-free KRP medium containing 1% BSA prior to measurement of [^{3}H]2-deoxyglucose uptake and lysate PI 3-kinase or PKC-ζ activity, respectively. Panels B and C show representative autoradiograms of Cbl-dependent PI 3-kinase activity (B) and immunoblots of Western analyses of lysates following expression of WT and Y371F/Y731F mutant forms of Cbl (C), respectively. Bargram values are mean ± SE of the number of determinations shown in parentheses. CON, control. PI3P, phosphatidylinositol-3-PO_4.

mutant forms of Cbl may have simultaneously inhibited other relevant actions of Cbl, in particular, the formation of a flotillin/CAP/Cbl/Crk/C3G/TC10 complex on lipid raft microdomains of the plasma membrane, which complex is required during insulin-stimulated Glut4 translocation/glucose transport (see previously). At this point, it is uncertain if this entire complex is assembled on lipid rafts and is required during rosiglitazone-induced increases in Glut4 translocation/glucose transport. However, from the present findings, it is clear that rosiglitazone-induced activation of pYXXM motifs in Cbl results not only in the activation of Cbl-dependent PI 3-kinase but also in the binding of pYXXM motifs in Cbl to SH2 domains of Crk. Further studies are needed to see if C3G and TC10 are recruited by the Cbl/Crk complex and if a flotillin/CAP/Cbl complex forms in response to rosiglitazone-induced activation of an as of yet unidentified tyrosine kinase that is responsible for increases in tyrosine phosphorylation of Cbl (see ref *2*). In this regard, it is known that CAP is increased by TZD treatment (*16*), and CAP may enhance the interaction of Cbl with one or more tyrosine kinases, including the insulin receptor.

It is important to note that, in the action of insulin in 3T3L1 adipocytes, the above-described flotillin/CAP/Cbl/Crk/C3G/TC10 complex is formed independently of PI 3-kinase but nevertheless functions in conjunction with increases in the activities of IRS-1/2-dependent PI 3-kinase, PDK1, aPKCs, and PKB in the activation of glucose transport by insulin (*14*−*19*). Accordingly, it is possible that a flotillin/CAP/Cbl/Crk/C3G/TC10 complex may similarly be formed during rosiglitazone action and may function in conjunction with the Cbl-dependent PI 3-kinase/PDK1/aPKC pathway in stimulating glucose transport during rosiglitazone action. Further studies are needed to test this hypothesis.

To summarize, our findings suggest that pYXXM motifs in Cbl are required for activation of SH2 domains in the p85 subunit of PI 3-kinase and subsequent increases in Cbl-dependent PI 3-kinase activity, which in turn leads to increases in aPKC activity during rosiglitazone-stimulated glucose transport in 3T3/L1 and human adipocytes. pYXXM motifs in Cbl also appear to activate SH2 domains in Crk during rosiglitazone action. Cbl therefore appears to play a pivotal role during rosiglitazone action and may serve to coordinate PI 3-kinase-dependent and PI 3-kinase-independent signaling pathways that are needed to activate glucose transport during thiazolidinedione action.

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