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Atypical protein kinase C (PKCζ/λ) is a convergent downstream target of the insulin-stimulated phosphatidylinositol 3-kinase and TC10 signaling pathways

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Insulin stimulation of adipocytes resulted in the recruitment of atypical PKC (PKCζ/λ) to plasma membrane lipid raft microdomains. This redistribution of PKCζ/λ was prevented by *Clostridium difficile* toxin B and by cholesterol depletion, but was unaffected by inhibition of phosphatidylinositol (PI) 3-kinase activity. Expression of the constitutively active GTP-bound form of TC10 (TC10Q/75L), but not the inactive GDP-bound mutant (TC10/T31N), targeted PKCζ/λ to the plasma membrane through an indirect association with the Par6–Par3 protein complex. In parallel, insulin stimulation as well as TC10/Q75L resulted in the activation loop phosphorylation of PKCζ. Although PI 3-kinase activation also resulted in PKCζ/λ phosphorylation, it was not recruited to the plasma membrane. Furthermore, insulin-induced GSK-3β phosphorylation was mediated by both PI 3-kinase–PKB and the TC10–Par6–atypical PKC signaling pathways. Together, these data demonstrate that PKCζ/λ can serve as a convergent downstream target for both the PI 3-kinase and TC10 signaling pathways, but only the TC10 pathway induces a spatially restricted targeting to the plasma membrane.

Introduction

TC10 is a member of the Rho family of small GTP-binding proteins and has been recently identified as an important proximal protein in the control of insulin action in adipocytes (Chiang et al., 2001; Watson et al., 2001). This signaling pathway appears to involve the insulin receptor–dependent tyrosine phosphorylation of Cbl and its recruitment to lipid raft microdomains through the adaptor proteins APS, Cbl-associated protein (CAP), and flotillin (Baumann et al., 2000; Liu et al., 2002). The tyrosine-phosphorylated Cbl can form a ternary complex with the guanylnucleotide exchange factor C3G through the small adaptor protein CrkII. Because TC10 is constitutively localized to plasma membrane lipid raft microdomains, the recruitment of C3G places it in close proximity and results in the conversion of TC10 from the inactive GDP-bound state to the active GTP-bound state (Baumann et al., 2000; Chiang et al., 2001).

In vitro binding assays have indicated that active GTP-bound TC10 can directly interact with a number of potential effectors that were originally identified as binding proteins for Cdc42 and/or Rac (Neudauer et al., 1998; Murphy et al., 1999; Joberty et al., 2000; Lin et al., 2000). In mammalian cells, over 20 potential effector-binding proteins have been identified; however, the functional role of these in specific cellular events appears to be highly dependent upon cell context. For example, although expression of Cdc42 in fibroblasts has marked effects on actin organization, there is no significant change in the adipocyte actin cytoskeleton. In contrast, TC10 expression results in a marked disruption of adipocyte cortical actin organization, leading to an inhibition of insulin-stimulated GLUT4 translocation (Chiang et al., 2001; Kanzaki and Pessin, 2002; Kanzaki et al., 2002). Part of this difference can be accounted for by the distinct spatial compartmentalization of TC10 compared with Cdc42 and other Rho family members. Unlike other Rho family proteins

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Abbreviations used in this paper: CAP, Cbl-associated protein; CRIB, Cdc42/Rac-interacting binding; MβCD, methyl-β-cyclodextrin; PDK, phosphoinositide-dependent protein kinase; PH, pleckstrin homology; PI, phosphatidylinositol.

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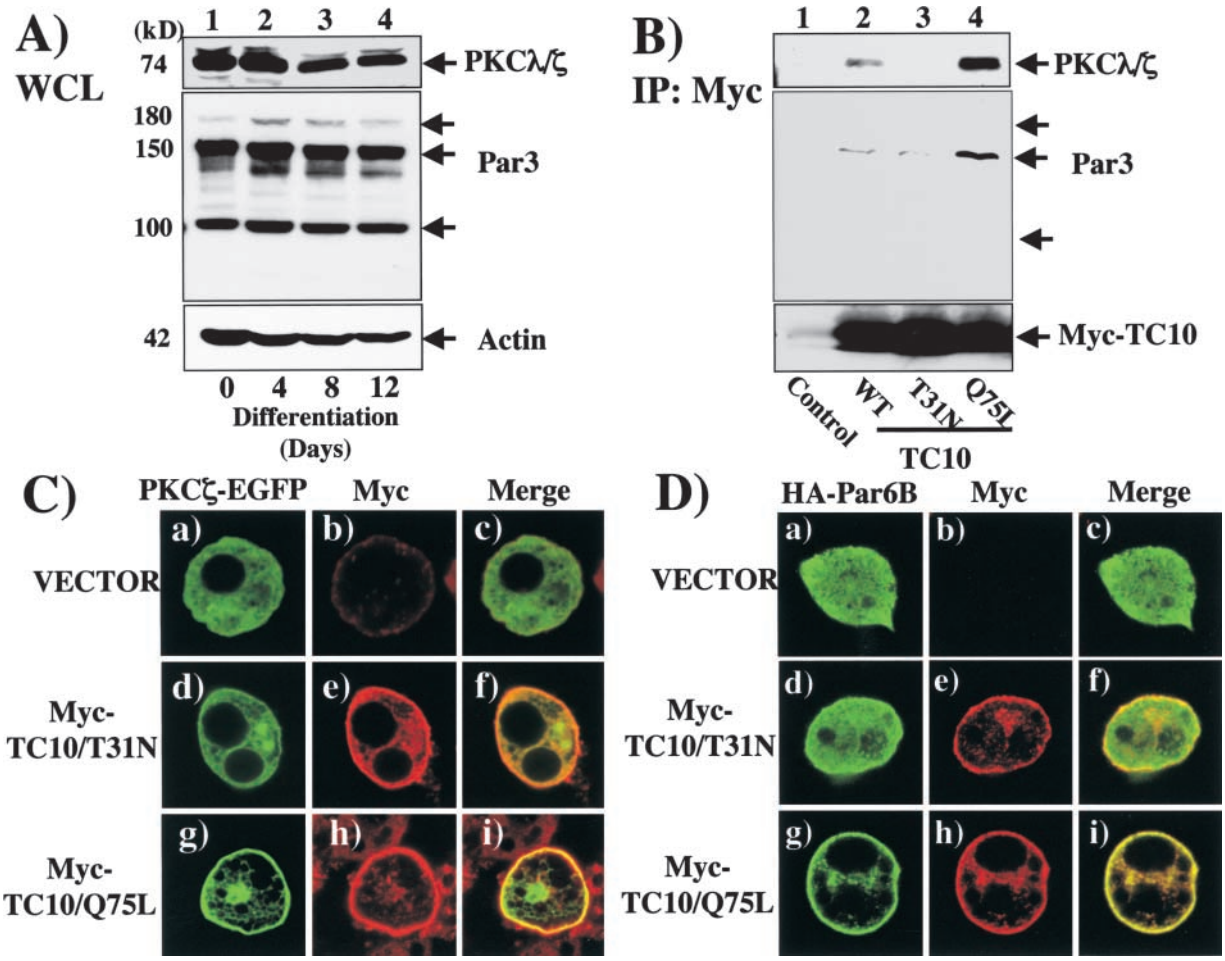

Figure 1. **TC10 binds to PKCζ/λ through the Par6–Par3 complex in 3T3L1 adipocytes.** (A) 3T3L1 cells were allowed to reached confluency (d 0, lane 1) and were subjected to the adipocyte differentiation protocol for 4 (lane 2), 8 (lane 3), and 12 d (lane 4). Whole-cell detergent extracts were subjected to SDS-PAGE and immunoblotted with the indicated antibodies. (B) Differentiated adipocytes were electroporated with the cDNAs encoding for the empty vector (lane 1), the human Myc epitope-tagged wild-type TC10 protein (TC10/WT, lane 2), dominant-negative TC10 (TC10/T31N, lane 3), and constitutively active TC10 (TC10/Q75L, lane 4) as described in the Materials and methods. 18 h later, whole-cell detergent extracts were prepared and immunoprecipitated with a Myc antibody and immunoblotted with indicated antibodies. These are representative immunoblots obtained in three independent experiments. (C) Adipocytes were electroporated with cDNAs encoding for PKCζ-EGFP plus empty vector (a–c) or the cDNA for the human Myc epitope-tagged TC10/T31N (d–f) or TC10/Q75L (g–i). 18 h later, the cells were serum starved, fixed, and subjected to confocal fluorescent microscopy. (D) Adipocytes were electroporated with cDNAs encoding HA-Par6B plus empty vector (a–c) or the cDNA for Myc-TC10/T31N (d–f) or Myc-TC10/Q75L (g–i). 18 h later, the cells were serum starved, fixed, and subjected to confocal fluorescent microscopy. Images are representative from three independent experiments.

that undergo carboxyl-terminal geranylgeranylation, TC10 contains a CAAX sequence that specifies farnesylation and dual palmitoylation responsible for targeting to plasma membrane lipid raft microdomains (Watson et al., 2001). Thus, the compartmentalization of these proteins implies that functionally relevant downstream effectors must also be spatially restricted to their appropriate sites of action.

In this regard, several reports have implicated atypical PKCs (PKCζ/λ) as direct substrates for the phosphoinositide-dependent protein kinase 1 (PDK1). Insulin activates PDK1 through the generation of phosphatidylinositol (PI)-3,4,5P3 by the stimulation of the type 1A PI 3-kinase (Standaert et al., 1997; Kotani et al., 1998; Bandyopadhyay et al., 1999a). On the other hand, PKCζ/λ has also been reported to form a quaternary complex with Par6, Par3/ASIP, and activated Cdc42 in various cell types (Joberty et al., 2000; Lin et al., 2000; Noda et al., 2001). The Par proteins were originally identified as proteins involved in asymmetric cell division and polarized growth in the *Caenorhabditis elegans* development (Etemad-Moghadam et al., 1995; Watts et al., 1996). Par6 is composed of a PDZ (PSD-95/Dlg/ZO-1) domain downstream of a motif that is similar to a Cdc42/Rac-interacting binding (CRIB) domain, and both are apparently required for the association of Par6 with Cdc42. In addition, Par6 and atypical PKCs both contain PB1 (Phox and Bem1) domains that are required for forming heterodimeric complexes (Ponting et al., 2002). Par3, also termed ASIP, contains three PDZ domains and specifically binds to both Par6 and atypical PKCs at cell–cell contact sites in fibroblasts and epithelial cells (Izumi et al., 1998; Suzuki et al., 2001). Thus, Par6 and Par3 proteins appear to provide scaffolding functions, linking atypical PKCs and the Rho family small GTPases Cdc42 and Rac. Although it is not known whether TC10 can form a similar signaling complex in vivo,

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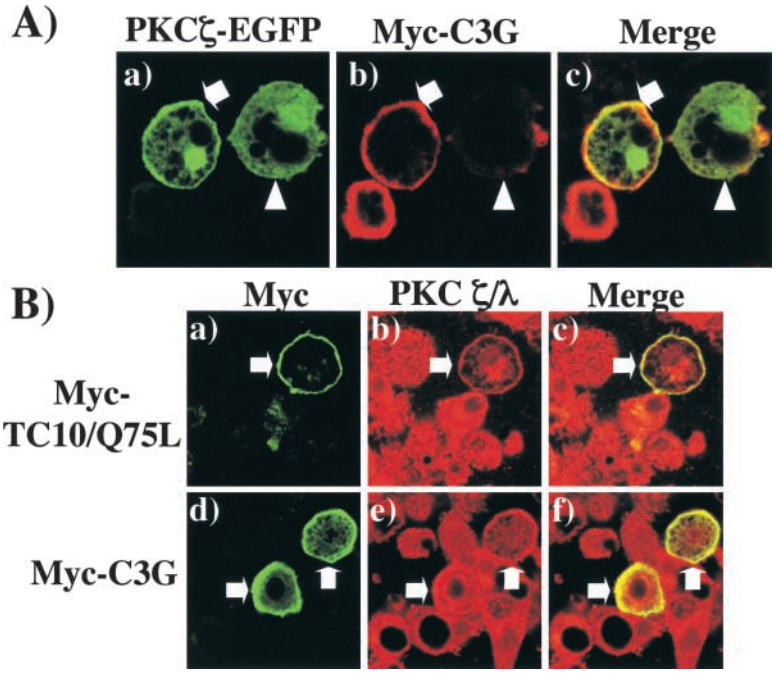

Figure 2. **Expression of C3G or the constitutively active TC10 induces the recruitment of PKCζ to the plasma membrane.** (A) 3T3L1 adipocytes were electroporated with the cDNAs encoding PKCζ-EGFP and Myc-C3G. 18 h later, the cells were serum starved, fixed, and subjected to confocal fluorescent microscopy for EGFP (a) and the Myc epitope-tagged TC10 proteins (b). PKCζ-EGFP was recruited to the plasma membrane in the C3G-expressing cell (arrow), but not in the C3G-negative cell (arrowhead). This is a representative field of cells from 3–4 independent experiments. (B) 3T3L1 adipocytes were electroporated with cDNA encoding for Myc-TC10/Q75L (a–c) or Myc-C3G (d–f). 18 h later, the cells were serum starved, fixed, and subjected to confocal fluorescent microscopy for the expressed Myc-TC10/Q75L (a), Myc-C3G (d), and endogenous PKCζ/λ (b and e) proteins. This is a representative field of cells from 3–4 independent experiments.

GTP-bound active TC10 has been reported to bind the CRIB domain of Par6 using in vitro binding assays (Joberty et al., 2000). Furthermore, it has been reported that overexpression of Par3 in adipocytes inhibits insulin-induced glucose uptake and GLUT4 translocation (Kotani et al., 2000).

To reconcile the apparent role of PI 3-kinase signaling with the scaffolding function of Par6–Par3, we have examined the intracellular compartmentalization, Par6–Par3 interaction, and phosphorylation of PKCζ/λ in adipocytes. Our data demonstrate that TC10 stimulates PKCζ/λ phosphorylation and recruitment to plasma membrane lipid raft microdomains in adipocytes through the Par6–Par3 complex. In contrast, activation of PI 3-kinase signaling results in PKCζ/λ phosphorylation without detectable recruitment to the plasma membrane. Importantly, insulin stimulation of adipocytes results in an identical PKCζ/λ localization as TC10 activation that is completely distinct from PI 3-kinase activation. Furthermore, insulin-induced phosphorylation of GSK-3β appears to be mediated not only by the PI 3-kinase–PKB pathway, but also by the TC10–Par6–atypical PKC signaling pathway. Thus, PKCζ/λ appears to function as a convergent downstream target that can differentiate these two pathways through restricted spatial compartmentalization.

Results

Activation of TC10 recruits PKCζ/λ to the plasma membrane through the Par6–Par3 complex in adipocytes

To determine whether TC10 can interact with the Par6–Par3 complex and PKCζ/λ in adipocytes, we initially examined the protein expression levels of Par3/ASIP and PKCζ/λ during adipocyte differentiation (Fig. 1 A). As previously reported in several other cell types (Lin et al., 2000), three different isoforms of Par3 (180, 150, and 100 kD) were detected in both 3T3L1 fibroblasts and fully differentiated adipocytes (Fig. 1 A, lanes 1–4). There was no significant change in either Par3 or PKCζ/λ protein expression during adipocyte differentiation, as there was a small but similar parallel decrease in the β-actin loading control.

Immunoprecipitation of an expressed myc epitope-tagged wild-type TC10 protein (TC10/WT) resulted in the specific coimmunoprecipitation of the 150-kD Par3 isoform as well as PKCζ/λ (Fig. 1 B, lanes 1 and 2). Consistent with this finding, expression of a constitutively active TC10 mutant (TC10/Q75L) resulted in a greater extent of Par3 and PKCζ/λ coimmunoprecipitation (Fig. 1 B, lane 4). In contrast, expression of an inactive TC10 mutant (TC10/T31N) was unable to coimmunoprecipitate Par3 or PKCζ/λ (Fig. 1 B, lane 3).

To confirm this observation in vivo, we coexpressed PKCζ-EGFP with either empty vector, TC10/T31N, or TC10/Q75L in 3T3L1 adipocytes (Fig. 1 C). The expressed PKCζ-EGFP was primarily localized to the cytoplasm with no evidence for any membrane association (Fig. 1 C, a–c). As previously reported (Kanzaki and Pessin, 2001), the expressed TC10/T31N protein was predominantly localized to the plasma membrane (Fig. 1 C, d–f). However, there was no significant redistribution of PKCζ-EGFP, which remained predominantly cytosolic. TC10/Q75L was also primarily concentrated at the plasma membrane, but in this case there was a marked colocalization and recruitment of PKCζ-EGFP to the plasma membrane (Fig. 1 C, g–i). In parallel, expression of Par6 resulted in a diffuse cytosolic distribution that was not significantly different when coexpressed with TC10/T31N (Fig. 1 D, a–f). In contrast, Par6 was recruited to the plasma membrane when coexpressed with TC10/Q75L (Fig. 1 D, g–i).

In adipocytes, expression of the guanine nucleotide exchange factor C3G activates TC10 and potentiates the insulin stimulation of GLUT4 translocation (Chiang et al., 2001). Therefore, we activated the endogenous TC10 protein by

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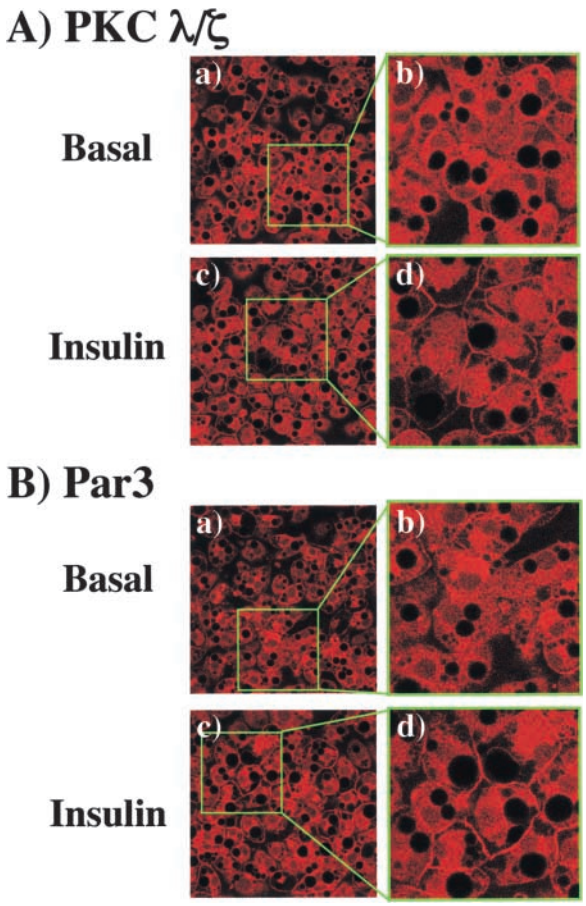

Figure 3. **Insulin stimulates recruitment of both the endogenous PKCζ/λ and Par3 proteins to the plasma membrane.** 3T3L1 adipocytes were serum starved and then incubated in the absence (a and b) or presence (c and d) of 100 nM insulin for 5 min. The cells were fixed and then subjected to immunostaining. (A) The endogenous PKCζ/λ protein was labeled with a PKCζ/λ antibody and Texas red–conjugated anti–rabbit IgG. (B) The endogenous Par3 protein was labeled with a Par3 antibody and Texas red–conjugated anti–rabbit IgG. The images in b and d were magnified 2.4 times compared with a and c. These are representative fields of cells from three independent experiments.

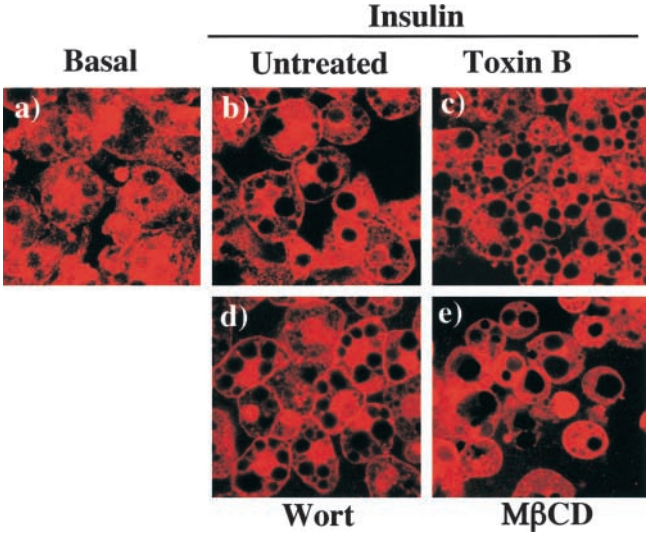

Figure 4. **Insulin-induced PKCζ/λ recruitment to the plasma membrane is PI 3-kinase–independent, but sensitive to toxin B and MβCD.** 3T3L1 adipocytes were serum starved and either left untreated (a and b) or incubated with 0.5 μg/ml *C. difficile* toxin B (c), 100 nM wortmannin (Wort, d), or MβCD (e) as described in the Materials and methods. The cells were then incubated in the absence (a) or presence (b–e) of 100 nM insulin for 5 min. Endogenous PKCζ/λ was detected by confocal fluorescent microscopy using a PKCζ/λ antibody and Texas red–conjugated anti–rabbit IgG. These are representative fields from 3–4 independent experiments.

C3G overexpression and assessed the subsequent recruitment of PKCζ/λ (Fig. 2 A). In intact cells, the expressed PKCζ-EGFP protein was primarily cytosolic (Fig. 2 A, a–c; arrowhead). However, upon coexpression with C3G there was a marked redistribution of PKCζ-EGFP to the plasma membrane (Fig. 2 A, a–c; arrow). In addition, pretreatment of the transfected adipocytes with the Rho-specific toxin *Clostridium difficile* toxin B completely prevented the C3G-stimulated recruitment of PKCζ-EGFP (unpublished data).

To assess whether the endogenous adipocyte PKCζ/λ is recruited to the plasma membrane by TC10 activation, immunofluorescent localization of endogenous PKCζ/λ was performed in TC10/Q75L- and C3G-transfected 3T3L1 adipocytes (Fig. 2 B). Consistent with that observed with overexpressed PKCζ-EGFP, the endogenous PKCζ/λ protein was dispersed throughout the cells without any evidence for membrane association in the nontransfected cells (Fig. 2 B, b and c). In contrast, adipocytes expressing either TC10/Q75L (Fig. 2 B, a–c) or C3G (Fig. 2 B, d–f) exhibited a clear plasma membrane recruitment of the endogenous PKCζ/λ protein. Together, the data presented in Fig. 1 and Fig. 2 demonstrate that TC10 activation (expressed and endogenous) results in plasma membrane recruitment of the expressed or endogenous PKCζ/λ protein in adipocytes. Furthermore, these results establish an in vivo interaction between TC10 and the ternary Par6–Par3–PKCζ/λ protein complex.

Insulin stimulates plasma membrane recruitment of PKCζ/λ and the Par6–Par3 complex through TC10 activation

Previous works have demonstrated that insulin stimulation results in the activation of TC10 (Chiang et al., 2001; Watson et al., 2001). Therefore, to determine whether a physiological agonist can also induce the plasma membrane recruitment of the endogenous Par3 and PKCζ/λ proteins, immunofluorescent localization was performed in basal and insulin-stimulated adipocytes (Fig. 3). As previously observed, PKCζ/λ was distributed throughout the cells with no indication of compartmentalized localization at either low or high magnification (Fig. 3 A, a and b). As expected, insulin stimulation resulted in a distinct PKCζ/λ translocation to the plasma membrane (Fig. 3 A, c and d). In parallel, Par3 was also distributed throughout the cytosol in the basal state and underwent insulin-stimulated translocation to the plasma membrane (Fig. 3 B, a–d).

In addition to the recently identified insulin-stimulated CAP–Cbl–TC10 pathway, insulin is well established to activate PI 3-kinase–dependent signaling, resulting in the formation of PI3,4,5P3 and subsequent activation of PDK1 (Toker and Newton, 2000; Cantley, 2002). Because PDK1 phosphorylates and activates PKCζ/λ, we next compared the relative contribution of TC10 and PI 3-kinase signaling in the plasma

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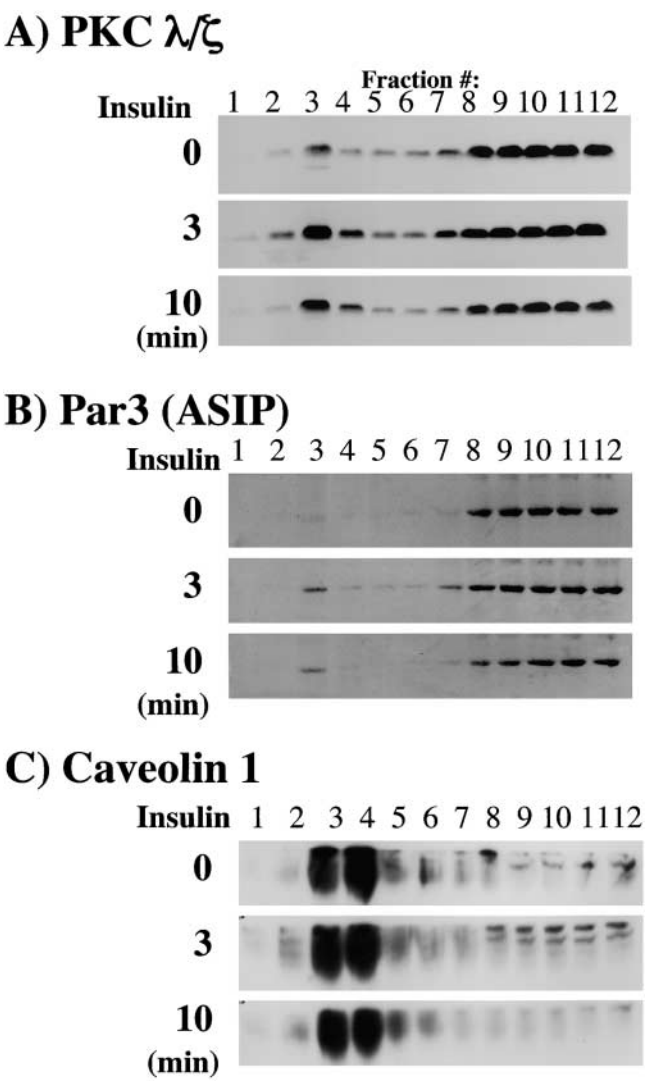

Figure 5. **Insulin stimulates the redistribution of PKCζ/λ and Par3 by sucrose gradient fractionation.** 3T3L1 adipocytes were serum starved and then incubated in the absence (0 min) or presence (3 and 10 min) of 100 nM insulin. The cells were nondetergent homogenized, sonicated, and subjected to continuous sucrose gradient fractionation as described in the Materials and methods. Fractions were then immunoblotted for the presence of PKCζ/λ (A), Par3 (B), or caveolin 1 (C). These are representative immunoblots from three independent experiments. Fraction 1 is the top of the gradient (~5% sucrose) and fraction 12 is the bottom gradient (~35% sucrose).

membrane recruitment of PKCζ/λ (Fig. 4). As typically observed, insulin stimulation resulted in the translocation of endogenous PKCζ/λ from the cytoplasm to the plasma membrane (Fig. 4, a and b). Pretreatment of the adipocytes with the Rho family *C. difficile* toxin B resulted in a complete inhibition of the insulin-induced PKCζ/λ recruitment (Fig. 4 c). In contrast, pretreatment with the PI 3-kinase inhibitor wortmannin had no significant effect on the insulin-induced plasma membrane PKCζ/λ recruitment (Fig. 4 d). In addition, cholesterol depletion with methyl-β-cyclodextrin (MβCD) disperses proteins associated with plasma membrane lipid raft microdomains and prevents insulin-stimulated TC10 activation without affecting PI 3-kinase activation or PI 3-kinase–dependent downstream signaling (Watson et al., 2001). Under these conditions, MβCD also prevented the insulin-stimulated plasma membrane recruitment of PKCζ/λ (Fig. 4 e). Together, these data strongly suggest that the insulin-induced recruitment of PKCζ/λ to the plasma membrane is primarily regulated by TC10 activation.

Insulin stimulates recruitment of PKCζ/λ to caveolin-containing lipid raft microdomains

In adipocytes, TC10 is primarily localized to the large clustered caveolin-containing cholesterol-enriched plasma membrane lipid raft microdomains, and this spatial compartmentalization is necessary for TC10 activation and modulation of GLUT4 translocation (Chiang et al., 2001; Watson et al., 2001). Therefore, we analyzed the compartmentalization of PKCζ/λ and Par3 by nondetergent homogenization and sucrose gradient fractionation (Fig. 5). As previously reported (Baumann et al., 2000), caveolin was primarily found in the low density regions of these gradients and was not affected by insulin stimulation (Fig. 5 C, fractions 3 and 4). Although a small amount of PKCζ/λ was detected in the low density fractions in the basal state, the majority of PKCζ/λ was confined to the denser regions of the gradient (Fig. 5 A, fractions 8–12). Insulin stimulation for 3 or 10 min resulted in a significant increase in the amount of PKCζ/λ that fractionated in the low density fractions. Similarly, Par3 was exclusively found in the high density fractions isolated from cells in the basal state, whereas after insulin stimulation Par3 was recruited into the low density fractions (Fig. 5 B).

The recruitment of PKCζ/λ to caveolin-enriched lipid raft microdomains was further assessed by confocal immunofluorescence microscopy of plasma membrane sheets (Fig. 6 A). In adipocytes, caveolae are clustered into large 0.5–1.0-μm aggregates that can be readily visualized as ringlike structures. TC10 appeared to be persistently localized to these structures (Parpal et al., 2001; Watson et al., 2001; Kanzaki and Pessin, 2002). Immunofluorescence microscopy of isolated plasma membrane sheets demonstrated the presence of these caveolin-containing structures in both the basal and insulin-stimulated adipocytes (Fig. 6 A, a and d). In the basal state, there was a relatively low level of PKCζ/λ associated with the isolated plasma membrane sheet with no apparent colocalization with caveolin (Fig. 6 A, b and c). In contrast, insulin stimulation resulted in an increased amount of immunoreactive PKCζ/λ at the plasma membrane that was specifically colocalized with the caveolin-positive ringlike structures (Fig. 6 A, e and f). Moreover, exogenously expressed Par6 was also recruited to the Triton X-100 resistant membrane raft microdomains in response to insulin stimulation (Fig. 6 B). We interpret these data to indicate that a portion of PKCζ/λ and Par6 that undergoes insulin-stimulated plasma membrane recruitment is specifically targeted to the large organized caveolin-positive plasma membrane microdomains that are also the sites of TC10 localization.

Activation of the PI 3-kinase signaling pathway does not recruit PKCζ/λ to the plasma membrane

It has been established that PKCζ/λ is an important downstream effector for the PI 3-kinase in various cell types because the enzymatic activity of PKCζ/λ is dependent on the phosphorylation by PDK1, a target of the PI 3-kinase signal-

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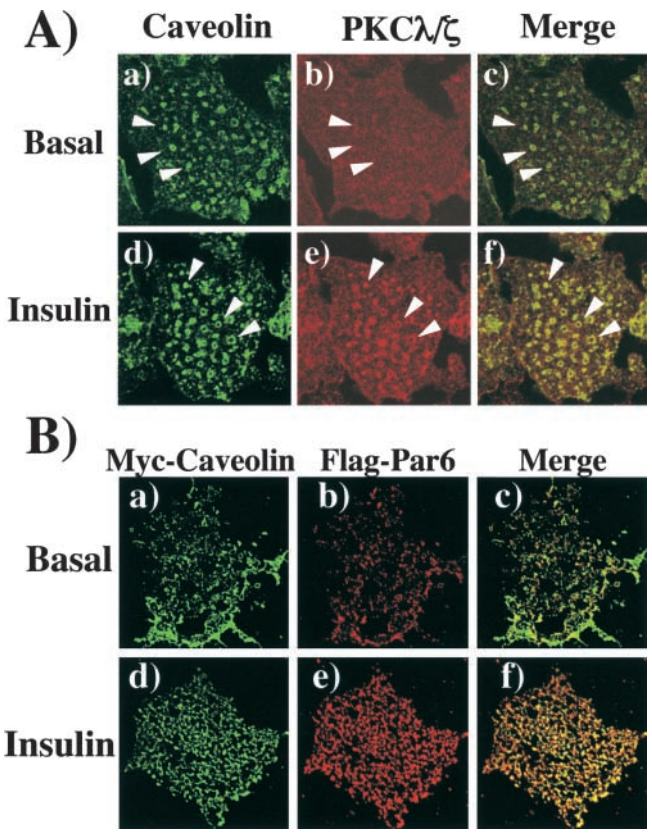

Figure 6. **Insulin stimulates the recruitment of endogenous PKCζ/λ to the large organized caveolae-rosette structures in 3T3L1 adipocytes.** (A) 3T3L1 adipocytes were serum starved and then incubated in the absence (a–c) or presence (d–f) of 100 nM insulin for 5 min. Plasma membrane sheets were prepared as described in the Materials and methods. The plasma membrane sheets were then subjected to confocal fluorescent microscopy using a caveolin 2 antibody (a and d) and a PKCζ/λ antibody (b and e). Arrowheads indicate caveolin-rosette structures, and the merged images are shown in c and f. These are representative fields of the cells from three independent experiments. (B) 3T3L1 adipocytes were electroporated with cDNA encoding for Myc-caveolin and Flag-Par6. 18 h later, the cells were serum starved and then incubated in the absence (a–c) or presence (d–f) of 100 nM insulin for 5 min. The cells were then treated for 5 min with ice-cold PBS containing 0.2% Triton X-100 before fixation, and were imaged using the indicated antibodies.

ing pathway (Chou et al., 1998; Le Good et al., 1998). To examine a possible role of PI 3-kinase in the recruitment of PKCζ/λ in adipocytes, we took advantage of the membrane-targeted catalytic subunit of PI 3-kinase (p110-CAAX) to selectively induce PI 3-kinase–dependent responses (Fig. 7). Previous analyses have demonstrated that the pleckstrin homology (PH) domain of Grp1 has a high affinity and selectivity for PI3,4,5P3, and when expressed as a GFP fusion protein, can be readily used to detect the production of this lipid product (Kavran et al., 1998; Kanzaki et al., 2000). Expression of p110-CAAX resulted in the constitutive formation of PI3,4,5P3 as observed by the plasma membrane localization of the coexpressed Grp1/PH-EGFP reporter fusion construct (Fig. 7 a). This was specific to p110-CAAX activity, as the localization of Grp1/PH-EGFP was completely abolished by wortmannin, but not by toxin B (Fig. 7, b and c). Although expression of p110-CAAX resulted in PI3,4,5P3 formation and PKB phosphorylation (unpublished data), there was no significant recruitment of coexpressed PKCζ-EGFP to the plasma membrane (Fig. 7, d–f).

PKCζ/λ is a convergent downstream target substrate for both TC10 and PI 3-kinase–dependent phosphorylation

It is well established that an enzymatic activity of PKCζ/λ can be stimulated by phosphorylation of the activation loop consensus threonine residue (Thr410 in PKCζ or Thr402 in PKCλ) by PDK1 (Chou et al., 1998; Le Good et al., 1998). Furthermore, several works have reported that insulin induces the phosphorylation and activation of PKCζ/λ in a PI 3-kinase–dependent manner in adipocytes (Kotani et al., 1998; Bandyopadhyay et al., 1999b; Sajan et al., 1999; Standaert et al., 1999, 2001). To investigate the relationship between PKCζ/λ recruitment by TC10 and that of PDK1-dependent phosphorylation, we examined the phosphorylation state of the consensus threonine residue (Thr410) of PKCζ-EGFP in 3T3L1 adipocytes coexpressing empty vector, TC10/Q75L, or p110CAAX (Fig. 8 A). As previously reported (Kotani et al., 1998; Standaert et al., 2001), insulin stimulation resulted in an approximate twofold increase in the phosphorylation of PKCζ-EGFP (Fig. 8 A, lanes 1 and 2). Expression of p110CAAX resulted in a marked phosphorylation of PKCζ-EGFP in the absence of insulin stimulation (Fig. 8 A, lane 3), with no additional effect of insulin (Fig. 8 A, lane 4). Expression of TC10/Q75L, which caused the recruitment of PKCζ-EGFP to the plasma membrane, also resulted in the spontaneous phosphorylation of PKCζ-EGFP in the basal state (Fig. 8 A, lane 5). The TC10/Q75L-induced phosphorylation was not further augmented by insulin stimulation (Fig. 8 A, lane 6). Furthermore, the PKCζ phosphorylation induced by TC10/Q75L expression was not inhibited by 100 nM wortmannin (Fig. 8 B, lanes 9 and 10), suggesting that basal activity of PDK1 and/or the presence of another PKCζ/λ kinase is sufficient to phosphorylate PKCζ as a consequence of membrane recruitment. A similar phenomenon has also been observed for the plasma membrane targeting of PKB by N-myristoylation (Kohn et al., 1996). In any case, these data demonstrate that PKCζ can be phosphorylated by both activation of PI 3-kinase signaling and by TC10 recruitment to lipid raft microdomains.

To examine participation of the Par protein complex in the TC10-mediated phosphorylation of PKCζ, wild-type Par6B (WT), amino terminus (aa 1–154) deleted form (ΔN), carboxy terminus (aa 154–370) deleted form (ΔC), CRIB domain (131–140) deleted form (ΔCRIB), or PB1 domain (D64A/D68A) points mutant form (DD/AA) of Par6B were coexpressed with TC10/Q75L plus PKCζ-EGFP, and the phosphorylation state of PKCζ-EGFP was determined (Fig. 8 B). In control cells, there was a low basal level of PKCζ phosphorylation that was reduced in cells expressing the dominant-interfering TC10 mutant TC10/T31N (Fig. 8 B, lanes 1 and 2). Expression of TC10/Q75L increased PKCζ phosphorylation, which was partially reduced by expression of Par6-WT, Par6-ΔN, and Par6-ΔC (Fig. 8 B, lanes 3–6). Because Par6 functions as a scaffolding protein, that small degree of inhibition probably reflects a partial disruption of the appropriate stoichiometry of the TC10–Par6–Par3–PKC complex. Importantly, the TC10/

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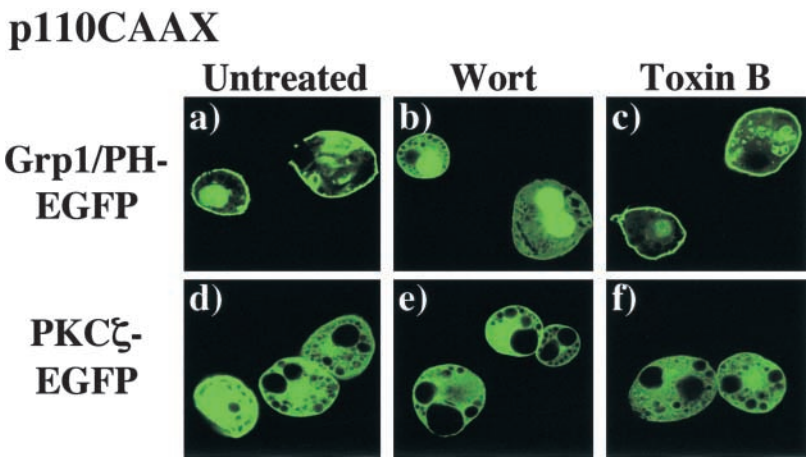

Figure 7. **Expression of p110-CAAX does not induce the recruitment of PKCζ/λ to the plasma membrane.** 3T3L1 adipocytes were electroporated with the cDNA encoding for p110-CAAX plus the cDNAs encoding either Grp1/PH-EGFP or PKCζ-EGFP as described in the Materials and methods. 18 h later, the cells were serum starved and were either untreated (a and d) or treated with 500 nM wortmannin (Wort, b and e) or 0.5 μg/ml toxin B for 2 h (c and f). The subcellular localization of Grp1/PH-EGFP (a–c) and PKCζ-EGFP (d–f) was determined by confocal fluorescent microscopy. These are representative fields of the cell from 3–4 independent experiments.

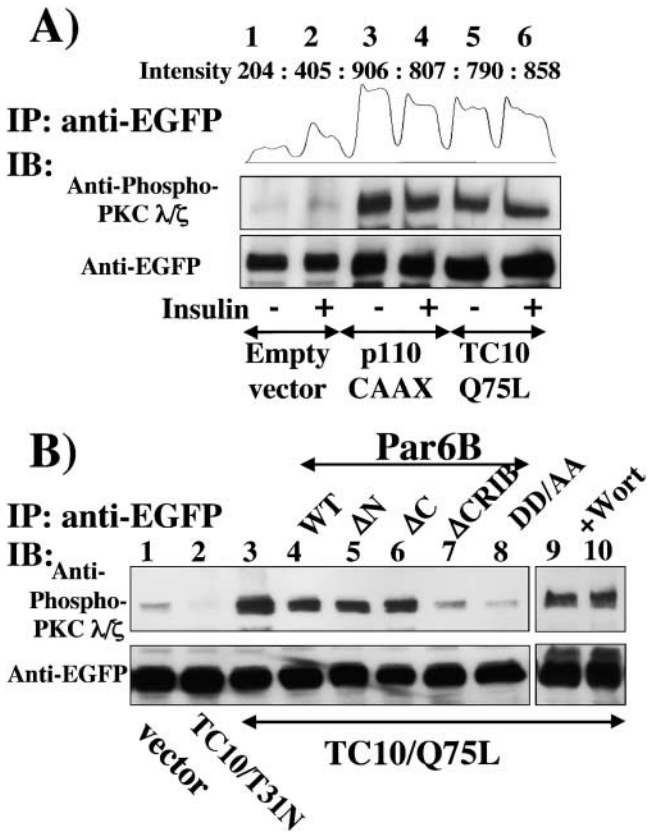

Figure 8. **TC10/Q75L, p110CAAX, and insulin stimulate the phosphorylation of PKCζ-EGFP on threonine 410 in 3T3L1 adipocytes.** (A) 3T3L1 adipocytes were electroporated with the cDNA encoding for PKCζ-EGFP plus the cDNA encoding either the empty vector (lanes 1 and 2), p110-CAAX (lanes 3 and 4), or TC10/Q75L (lanes 5 and 6). 18 h later, the cells were serum starved and then incubated in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 100 nM insulin for 5 min. Cells were lysed in a buffer containing 0.5% saponin plus 1% Triton X-100 as described in the Materials and methods. Whole-cell detergent extracts were immunoprecipitated with an EGFP antibody and immunoblotted with either antibodies against EGFP (bottom panels) or phospho-PKCζ/λ (top panels). The intensity of each band detected by a phospho-PKCζ/λ–specific antibody was calculated by using NIH Image software. (B) 3T3L1 adipocytes were electroporated with the cDNA encoding for PKCζ-EGFP plus the cDNA encoding either the empty vector (lane 1), TC10/T31N (lane 2), or TC10/Q75L (lanes 3–10) plus either Par6B-WT (lane 4), Par6-ΔN (lane 5), Par6-ΔC (lane 6), Par6-ΔCRIB (lane 7), or Par6-DD/AA (lane 8). 18 h later, the cells were serum starved and were either left untreated (lanes 1–9) or incubated with 100 nM wortmannin (lane 10) for 30 min. PKCζ-EGFP was immunoprecipitated as described above, and the samples were immunoblotted with either antibodies against EGFP (bottom) or phospho-PKCζ/λ (top). This is a representative blot from 3–4 independent experiments.

Q75L-mediated PKCζ phosphorylation was markedly reduced by coexpression of Par6-ΔCRIB or Par6-DD/AA (Fig. 8 B, lanes 7 and 8).

To confirm the functional role of Par6 in the insulin-induced plasma membrane translocation of endogenous PKCζ/λ, we next examined the effect of Par6-ΔCRIB and Par6-DD/AA by confocal fluorescent microscopy (Fig. 9). As previously observed, insulin stimulation resulted in the translocation of PKCλ/ζ in control nontransfected cells (Fig. 9, b, c, e, and f; arrowheads). In contrast, cells expressing either Par6-ΔCRIB or Par6-DD/AA failed to undergo an insulin-induced translocation of endogenous PKCζ/λ (Fig. 9, a–f; arrows). Similarly, endogenous PKCλ/ζ failed to undergo insulin-stimulated plasma membrane translocation in cells expressing TC10/T31N (Fig. 9, g–i).

To examine the relative effect of these pathways on a downstream target, we examined the phosphorylation of GSK-3β (Fig. 10). Although serine 9 of GSK-3β is a well-established substrate for PKB, several works have also reported that this site is also a substrate for PKC isoforms (Goode et al., 1992; Cook et al., 1996; Isagawa et al., 2000). As expected, insulin treatment and expression of p110CAAX resulted in the phosphorylation of GSK-3β (Fig. 10 A, lanes 1–4). Similarly, serine 9 phosphorylation of GSK-3β also occurred by expression of TC10/Q75L (Fig. 10 A, lanes 5 and 6). As previously reported (Watson et al., 2001), neither phosphorylation of PKB nor PI3,4,5P3 formation was observed in the cells expressing TC10/Q75L (unpublished data).

Inhibition of PI 3-kinase activity with wortmannin completely abolished insulin-stimulated PKB phosphorylation, but only partially reduced GSK-3β phosphorylation (Fig. 10 B, lanes 3 and 4). Although toxin B itself slightly increased basal GSK-3β phosphorylation, there was also a partial inhibition of insulin-stimulated phosphorylation (Fig. 10 B,

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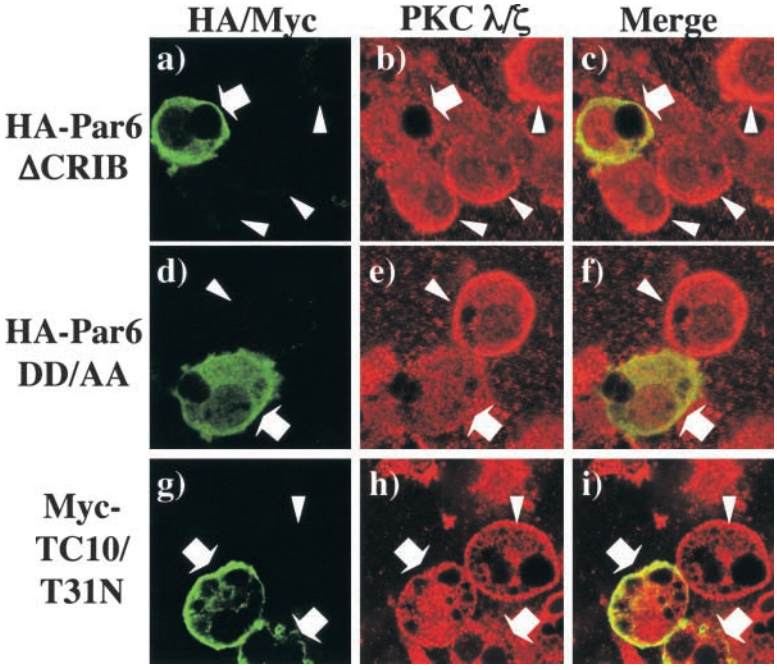

Figure 9. **Dominant-interfering Par6 mutants and TC10/T31N inhibit insulin-induced plasma membrane translocation of endogenous PKCζ/λ.** 3T3L1 adipocytes were electroporated with cDNA encoding for HA-Par6-ΔCRIB (a–c), HA-Par6-DD/AA (d–f), or Myc-TC10/T31N (g–i). 18 h later, cells were serum starved and then incubated in the presence of 100 nM insulin for 5 min. Expressed HA-Par6 mutants and endogenous PKCζ/λ were immunostained with antibodies against HA (a and d) and PKCζ/λ (b and e). The cells expressing Myc-TC10/T31N were detected using the myc antibody. Arrows depict cells expressing the HA-Par6 and Myc-TC10/T31N mutants, and arrowheads indicate the cells not expressing HA-Par6 or myc-TC10. These are representative fields of cells from three independent experiments.

lanes 5 and 6). More importantly, combined treatment with both wortmannin and toxin B completely blocked insulin-stimulated GSK-3β phosphorylation (Fig. 10 B, lanes 9 and 10). Furthermore, the inhibitory PKCζ pseudosubstrate peptide also inhibited insulin-stimulated GSK-3β phosphorylation (Fig. 10 B, lanes 7 and 8). A combination of the inhibitory PKCζ pseudosubstrate peptide and wortmannin completely prevented insulin-stimulated GSK-3β phosphorylation (Fig. 10 B, lanes 11 and 12). These results demonstrate that in adipocytes, insulin stimulates serine 9 phosphorylation of GSK-3β through the activation of both PKCζ/λ and PKB.

Finally, we also examined the effect of dominant-interfering Par6 mutants and TC10/T31N on GSK-3β phosphorylation (Fig. 10 C). The insulin stimulation of GSK-3β phosphorylation was inhibited by expression of Par6-ΔCRIB and Par6-DD/AA (Fig. 10 C, lanes 1–6), but not by expression of wild-type Par6 (Fig. 10 C, lanes 7 and 8). Similar to the dominant-interfering Par6 mutants, expression of TC10/T31N also inhibited insulin-stimulated GSK-3β phosphorylation (Fig. 10 C, lanes 9 and 10). Together, these data demonstrate that at least one pool of GSK-3β is phosphorylated by PKCζ/λ through a mechanism requiring TC10 and interactions with the Par6 protein complex.

Discussion

The atypical PKCs fall in the general category of the AGC subfamily of protein kinases that includes PKB, serum- and glucocorticoid-induced kinase, and p70S6 kinase (Toker and Newton, 2000). The AGC subfamily of protein kinases possesses two critical regulatory phosphorylation sites, the first of which is a threonine residue that is phosphorylated by PDK1 (Alessi et al., 1997, 1998; Stokoe et al., 1997; Chou et al., 1998; Le Good et al., 1998; Pullen et al., 1998; Frodin et al., 2002). In the case of PKCζ/λ, the first consensus threonine residue (Thr410 in PKCζ and Thr402 in PKCλ) is in the activation loop, whereas the second required phosphorylation threonine/serine site in the carboxyl-terminal tail is replaced by an acidic residue (Glu-579 in PKCζ and Glu-573 in PKCλ; Chou et al., 1998; Le Good et al., 1998; Balendran et al., 2000). Several works have demonstrated that insulin activation of PDK1 (through PI 3-kinase) can directly phosphorylate the first activation loop threonine, resulting in the stimulation of PKCζ/λ catalytic activity (Bandyopadhyay et al., 1999b; Standaert et al., 1999). Furthermore, insulin-stimulated PKCζ/λ activation has been directly implicated in the translocation of GLUT4 and glucose uptake in adipocytes (Standaert et al., 1997; Kotani et al., 1998; Bandyopadhyay et al., 1999b).

In this regard, recent evidence has demonstrated the presence of two distinct insulin signaling pathways that function in concert to mediate GLUT4 translocation and glucose uptake (Baumann et al., 2000; Chiang et al., 2001; Saltiel and Kahn, 2001). One pathway occurs through the insulin stimulation of IRS protein tyrosine phosphorylation, leading to the association and activation of the type 1A PI 3-kinase (Cheatham et al., 1994; Okada et al., 1994; Corvera and Czech, 1998). The subsequent formation of PI3,4,5P3 recruits other downstream signaling molecules such as PKB and PDK1 to nonlipid raft regions of the plasma membrane through their PH domains (Alessi et al., 1997; Stokoe et al., 1997; Stephens et al., 1998). Although PKCζ/λ does not have a PH domain, PKCζ/λ can associate with PDK1 and accounts for the subsequent phosphorylation on the activation loop T410/T402 residues (Chou et al., 1998; Le Good et al., 1998). More recently, a second pathway has been proposed that results from the tyrosine phosphorylation of Cbl and its recruitment to lipid raft microdomains through the adaptor proteins APS and CAP (Ribon and Saltiel, 1997; Baumann et al., 2000; Liu et al., 2002). In turn, tyrosine-phosphorylated Cbl engages the CrkII–C3G complex that

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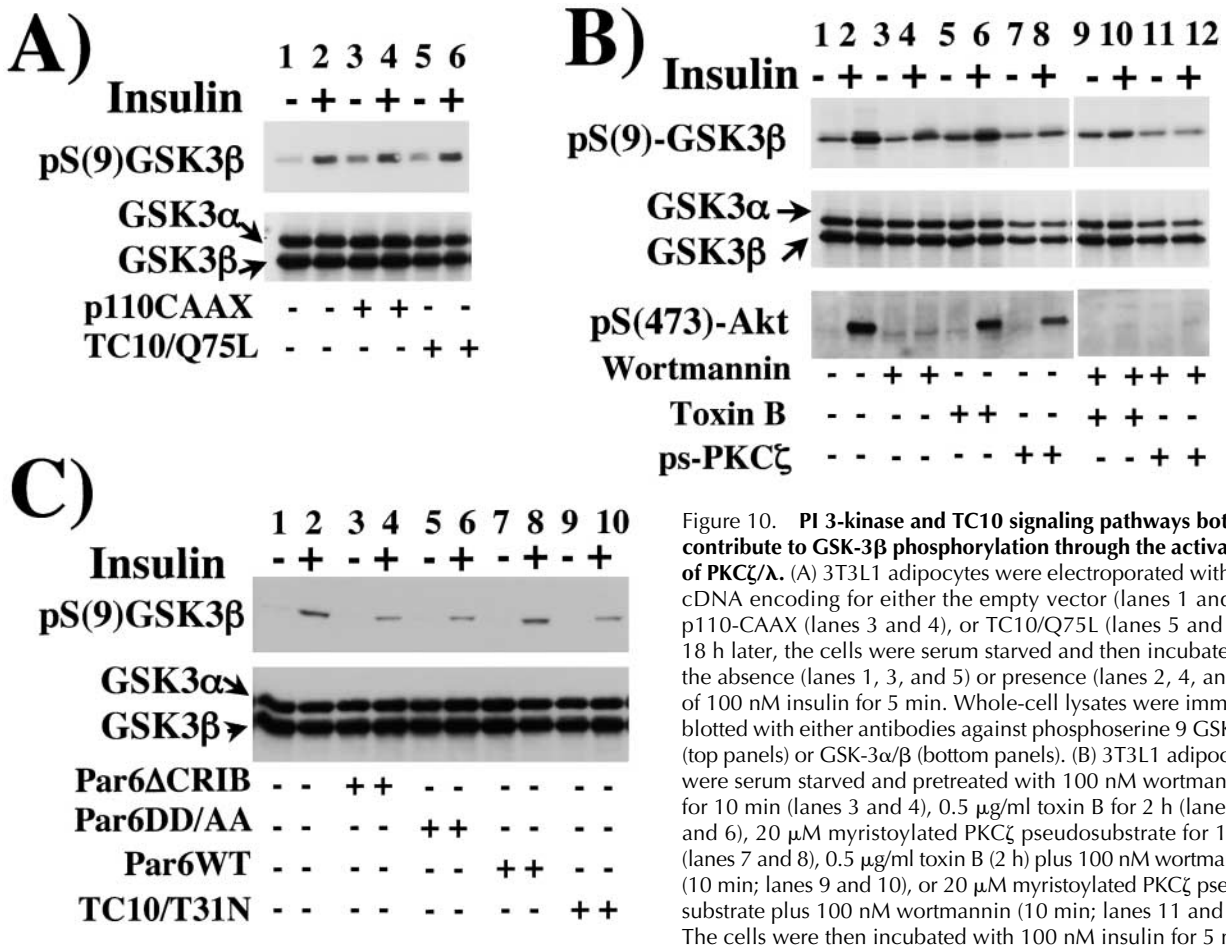

Figure 10. **PI 3-kinase and TC10 signaling pathways both contribute to GSK-3β phosphorylation through the activation of PKCζ/λ.** (A) 3T3L1 adipocytes were electroporated with the cDNA encoding for either the empty vector (lanes 1 and 2), p110-CAAX (lanes 3 and 4), or TC10/Q75L (lanes 5 and 6). 18 h later, the cells were serum starved and then incubated in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 100 nM insulin for 5 min. Whole-cell lysates were immunoblotted with either antibodies against phosphoserine 9 GSK-3β (top panels) or GSK-3α/β (bottom panels). (B) 3T3L1 adipocytes were serum starved and pretreated with 100 nM wortmannin for 10 min (lanes 3 and 4), 0.5 μg/ml toxin B for 2 h (lanes 5 and 6), 20 μM myristoylated PKCζ pseudosubstrate for 1 h (lanes 7 and 8), 0.5 μg/ml toxin B (2 h) plus 100 nM wortmannin (10 min; lanes 9 and 10), or 20 μM myristoylated PKCζ pseudosubstrate plus 100 nM wortmannin (10 min; lanes 11 and 12). The cells were then incubated with 100 nM insulin for 5 min. Whole-cell lysates were immunoblotted with either antibodies against phosphoserine 9 GSK-3β (top panels), GSK-3α/β (middle panels), or phosphoserine 473-Akt (bottom panels). (C) 3T3L1 adipocytes were electroporated with 600 μg cDNA encoding for the empty vector (lanes 1 and 2), Par6-ΔCRIB (lanes 3 and 4), Par6-DD/AA (lanes 5 and 6), Par6-WT (lanes 7 and 8), or TC10/T31N (lanes 9 and 10). The serum-starved cells were incubated without (lanes 1, 3, 5, 7, and 9) or with (lanes 2, 4, 6, 8, and 10) 100 nM insulin for 5 min. Whole-cell lysates were subjected to immunoblotting by using either antibodies against phosphoserine 9 GSK-3β (top panels) or GSK-3α/β (bottom panels). These are representative blots from three independent experiments.

recruits the C3G guanylnucleotide exchange activity to the lipid raft microdomain regions where TC10 is also compartmentalized (Chiang et al., 2001; Watson et al., 2001, 2003).

The data presented in this report provide an important connection between these pathways by demonstrating that PKCζ/λ is a convergent downstream target of both the IRS PI 3-kinase and Cbl-TC10 signaling cascades. Because insulin activates PDK1 and induces T410/T402 phosphorylation, it has been assumed that PKCζ/λ is recruited to the plasma membrane by PDK1 (Le Good et al., 1998; Balendran et al., 2000). However, our data demonstrate that TC10-dependent (and not PI 3-kinase–dependent) signals are responsible for PKCζ/λ plasma membrane localization, at least in adipocytes. More precisely, the TC10-dependent recruitment spatially restricts PKCζ/λ to the large caveolin-positive rosette structures in the plasma membrane of adipocytes. However, this interaction directly results from the association of the Par6–Par3–PKCζ/λ complex with activated TC10. This is consistent with the ability of TC10 to bind Par6 in vitro. Similiarly, the highly homologous Rho family member Cdc42 can also form a quaternary complex with Par6, Par3, and atypical PKCs (Joberty et al., 2000). This conclusion is further strengthened by the observation that overexpression of Par3 inhibits insulin-stimulated PKCζ/λ activation and GLUT4 translocation, presumably by disrupting PKCζ/λ phosphorylation and/or localization (Kotani et al., 2000).

In addition to PKCζ/λ, PKB is also a downstream target of the PI 3-kinase pathway and phosphorylates substrates with an RXRXXS consensus motif (Lawlor and Alessi, 2001). Although the substrate sites for PKCζ/λ-dependent phosphorylation are more degenerate (RXS, RXXS, or RXXSXR), they share strong similarity to the PKB substrate recognition motif (Nishikawa et al., 1997). Although serine 9 of GSK-3β is a consensus PKB phosphorylation site, several reports directly demonstrate that this site can also be phosphorylated by atypical PKCs (Ballou et al., 2001; Oriente et al., 2001). More recently, scratch-induced migration in astrocytes resulted in GSK-3β phosphorylation through a Cdc42–Par6–PKCζ signaling cascade independent of PKB (Etienne-Manneville and Hall, 2003). Although the physiological significance of TC10–Par protein–PKCζ regulation of GSK-3β remains to be determined, our data demonstrate that in adipocytes, GSK-3β phosphorylation is controlled not only by PKB, but also by

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PKCζ/λ through both TC10 and PI 3-kinase signals. Importantly, only the TC10 pathway results in the recruitment of PKCζ/λ to plasma membrane lipid raft microdomains.

Consistent with this idea, it is becoming increasingly apparent that lipid raft microdomains play a central importance in insulin action, including insulin-stimulated GLUT4 translocation. For example, multiple papers have demonstrated that disruption of these structures using various pharmacological agents or a dominant-interfering caveolin mutant all perturb insulin-stimulated GLUT4 translocation (Nystrom et al., 1999; Watson et al., 2001). More recently, we have reported that TC10 regulates a unique cortical actin structure (caveolin-associated F-actin) in fully differentiated 3T3L1 adipocytes consisting of F-actin spikes emanating from inside of the clustered caveolin-enriched rosette structures (Kanzaki and Pessin, 2002). Together, the data presented in this paper suggest an intriguing hypothesis that the caveolin-enriched lipid raft microdomains might function as important signaling platforms that orchestrate insulin signaling molecules including PKCζ/λ. This hypothesis is also consistent with several reports showing a functional role of atypical PKCs in actin cytoskeleton regulation in other cell types (Gomez et al., 1995; Coghlan et al., 2000).

In summary, the data presented in this paper demonstrate that PKCζ/λ serves as a convergent downstream target for both the PI 3-kinase and TC10 signals, and can be phosphorylated once either of these pathways is activated. Nevertheless, the spatial compartmentalization of PKCζ/λ is markedly different after activation of these pathways. Moreover, in fully differentiated adipocytes, insulin primarily recruits PKCζ/λ to the lipid raft microdomains and not to ruffling/lamellipodia regions of the plasma membrane despite coactivation of the PI 3-kinase pathway.

Materials and methods

Materials

C. difficile toxin B was obtained from TECHLAB. pcDNA3-C3G, pKH3-TC10/T31N, and TC10/Q75L were prepared as described previously (Chiang et al., 2001). pEGFP-PKCζ cDNA was purchased from CLONTECH Laboratories, Inc. The pCMV-Par6C cDNA and the Par3 antibody were provided by Dr. Ian Macara (University of Virginia, Charlottesville, VA). pKH3-Par6B-ΔN (deletion of aa 1–154), Par6-ΔC (deletion of aa 154–370), Par6-ΔCRIB (deletion of aa 131–140), and Par6-DD/AA (D64A/D68A) were produced by the PCR-based method. The PKCζ/λ antibody was obtained from Santa Cruz Biotechnology, Inc. Antibodies against GSK-3β phosphoserine-9 and PKB phosphothreonine-308 and phosphoserine-473 antibodies were obtained from Cell Signaling Technology. The phospho-specific PKCζ/λ activation loop antibody (T410) was a gift of Dr. Alex Toker (Harvard University, Boston, MA). The caveolin 1 and 2 antibodies and the GSK-3α/β antibody were purchased from Transduction Laboratories, and the HA and Myc epitope tag antibodies were purchased from Santa Cruz Biotechnology, Inc. Fluorescent secondary antibodies were purchased from Jackson ImmunoResearch Laboratories and Molecular Probes, Inc. The myristoylated PKCζ pseudosubstrate was purchased from Biosource International.

Cell culture and transient transfection of 3T3L1 adipocytes

Murine 3T3L1 preadipocytes were maintained, differentiated into adipocytes, and transfected by electroporation as described previously (Thurmond et al., 1998). After electroporation, cells were plated on glass coverslips and allowed to recover in complete medium.

Immunofluorescence and image analysis

Transfected and intact adipocytes were washed in PBS and fixed for 20 min in 4% PFA/PBS. The cells were washed briefly in PBS, permeabilized in PBS containing 0.1% saponin and 0.4% BSA for 10 min, and were then blocked in 5% donkey serum (Sigma-Aldrich) for 1 h at RT. Primary and secondary antibodies were used at 1:100 dilutions (unless otherwise indicated) in 0.4% BSA/PBS, and samples were mounted on glass slides with Vectashield® (Vector Laboratories). Cells were imaged using a confocal fluorescence microscope (model LSM510; Carl Zeiss MicroImaging, Inc.). Images were then imported into Adobe Photoshop® (Adobe Systems, Inc.) for processing, and composite files were generated.

Preparation and processing of plasma membrane sheets

Adipocyte plasma membrane sheets were prepared as described previously (Kanzaki et al., 2000). In brief, cells were incubation with 0.5 mg/ml poly-D-lysine for 1 min and then swollen in a hypotonic buffer (23 mM KCl, 10 mM Hepes, 2 mM $MgCl_2$, and 1 mM EDTA, pH 7.5) by three successive rinses. The swollen cells were sonicated, and the bound plasma membrane sheets were fixed with 2% PFA and blocked with 5% donkey serum. The membrane sheets were then incubated with primary antibodies for 90 min at RT. The primary antibodies were detected with Texas red–conjugated donkey anti–mouse antibody and Alexa® 488–conjugated donkey anti–rabbit antibody for 2 h at RT.

Immunoprecipitation and immunoblotting

After experimental treatments, the cells were solubilized in 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.5% saponin, 150 mM NaCl, 2% glycerol, 5 mM sodium fluoride, 1 mM sodium vanadate, 1 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin A. The extracts were centrifuged at 13,000 *g* for 20 min to remove insoluble material, and 50 μg total protein was resolved by SDS-PAGE followed by immunoblotting and was visualized with the SuperSignal® Chemiluminescence Detection kit (Pierce Chemical Co.). For immunoprecipitation, whole-cell extracts were incubated for 2 h at 4°C with 5 μg monoclonal myc antibody. The samples were then precipitated with protein G PLUS-Sepharose (Santa Cruz Biotechnology, Inc.) and immunoblotted as described above.

Nondetergent sucrose gradient fractionation

3T3L1 adipocytes were either left untreated or were treated with 100 nM insulin for 3, 5, or 10 min. Cells were then washed with ice-cold PBS, rapidly scraped in 0.5 M sodium carbonate buffer (pH 11.0), and homogenized on ice. The homogenates were then sonicated four times for 20 s and combined with a buffer containing 25 mM MES (pH 6.5), 150 mM NaCl, and 250 mM sodium carbonate plus 35% (wt/vol) sucrose. The sample was then loaded on a 5–35% continuous sucrose gradient and centrifuged at 39,000 rpm in a rotor (model SW41; Beckman Coulter) at 4°C for 19 h.

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