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The Insulin Receptor Catalyzes the Tyrosine Phosphorylation of Caveolin-1*

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Our previous studies revealed that insulin stimulates the tyrosine phosphorylation of caveolin in 3T3L1 adipocytes. To explore the mechanisms involved in this event, we evaluated the association of the insulin receptor with caveolin. The receptor was detected in a Triton-insoluble low density fraction, co-sedimenting with caveolin and flotillin on sucrose density gradients. We also detected the receptor in caveolin-enriched rosette structures by immunohistochemical analysis of plasma membrane sheets from 3T3L1 adipocytes. Insulin stimulated the phosphorylation of caveolin-1 on Tyr^{14}. This effect of the hormone was not blocked by overexpression of mutant forms of the Cbl-associated protein that block the translocation of phospho-Cbl to the caveolin-enriched, lipid raft microdomains. Moreover, this phosphorylation event was also unaffected by inhibitors of the MAPK and phosphatidylinositol 3-kinase pathways. Although previous studies demonstrated that the Src family kinase Fyn was highly enriched in caveolae, an inhibitor of this kinase had no effect on insulin-stimulated caveolin phosphorylation. Interestingly, overexpression of a mutant form of caveolin that failed to interact with the insulin receptor did not undergo phosphorylation. Taken together, these data indicate that the insulin receptor directly catalyzes the tyrosine phosphorylation of caveolin.

The insulin receptor is a tyrosine kinase that undergoes ligand-stimulated autophosphorylation and activation of its intrinsic substrate kinase activity (1). Once activated, the receptor phosphorylates intracellular substrates on tyrosine, including members of the insulin receptor substrate family (IRS1/2/3/4),[1] Shc, SIRP, Gab-1, Cbl, and APS (2–4). Tyrosine phosphorylation of these proteins creates recognition sites for effector molecules containing Src homology 2 (SH2) and phosphotyrosine binding domains. These include the small adapter proteins Grb2, CrkII, and Nck, the SHP2 protein-tyrosine phosphatase and the regulatory subunit of the type 1A phosphatidylinositol 3-kinase (PI3K) (4). The insulin receptor-dependent tyrosine phosphorylation of both IRS1 and IRS2 are critical in maintaining proper glucose homeostasis through its interaction with the PI3K (5–9). This interaction appears to serve a dual function by stimulating PI3K activity and targeting the enzyme to a critical intracellular site (10). Studies using various pharmacological inhibitors, microinjection of blocking antibodies, expression of dominant-interfering, and constitutively active mutants have consistently demonstrated a necessary role for PI3K activity in most of the metabolic effects of insulin (11, 12). Furthermore, inhibition of $PI(3,4,5)P_3$ formation (a product of PI3K) by expression of the 3′-phosphatase phosphatase and tensin homolog or the 5′-phosphatase SH2 inositol 5-phosphatase prevents insulin-stimulated glucose uptake and Glut4 translocation (13, 14).

Despite the general agreement that PI3K activity is necessary for insulin action, several studies have demonstrated the requirement for additional signals (15–18). For example, activation of PI3K by platelet-derived growth factor and interleukin-4 or through engagement of integrin receptors does not induce Glut4 translocation (15, 16, 19). Furthermore, two naturally occurring insulin receptor mutations were unable to induce Glut4 translocation and glucose uptake, yet were fully capable of activating PI3K (20). Additionally, a cell-permeable analog of $PI(3,4,5)P_3$ had no effect on glucose uptake (17). These data suggest that, although the PI3K pathway is necessary, there is at least one additional pathway required.

Recent data suggest that the second requisite pathway might involve tyrosine phosphorylation events that are restricted to subdomains of the plasma membrane. The insulin-stimulated tyrosine phosphorylation of Cbl results in its appearance in caveolin-enriched, lipid raft subdomains of the plasma membrane in 3T3L1 adipocytes (21–23). Cbl is recruited to the insulin receptor by the adapter protein APS, which is necessary for its insulin-stimulated tyrosine phosphorylation (24). The Cbl and APS proteins form a trimeric complex with the adapter protein CAP, a member of the SoHo family of proteins that contains a flotillin-binding SoHo domain in it amino terminus, and three adjacent SH3 domains in its carboxyl terminus (22, 23, 25). Once phosphorylated, the Cbl·CAP complex is recruited to lipid rafts through the interaction of CAP with flotillin. Expression of dominant-interfering CAP mutants that lack either the SH3 or SoHo domains prevented the localization of this complex to plasma membrane microdomains and inhibited the stimulation of glucose uptake and GLUT4 translocation by insulin (25).

Following insulin-stimulated tyrosine phosphorylation, Cbl recruits the SH2-containing adapter protein CrkII to lipid rafts; along with the guanine nucleotide exchange factor C3G (21, 23, 26). Upon its translocation, C3G activates TC10, a

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[1] The abbreviations used are: IRS1–4, insulin receptor substrates 1–4; SH2, Src homology 2 domain; PI3K, phosphatidylinositol 3-kinase; CAP, Cbl-associated protein; MES, 4-morpholineethanesulfonic acid; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5 Tris phosphate; Glut4, glucose transporter 4.

member of the Rho family protein of small GTP binding proteins expressed in muscle and adipose tissue (27). TC10 is specifically activated by insulin in 3T3L1 adipocytes in a CAP-dependent but PI3K-independent manner (26). Although the physiologically relevant effectors that interact with TC10 are unknown, disruption of its activation blocks insulin-stimulated glucose transport and Glut4 translocation. Moreover, mistargeting of TC10 to a non-lipid raft domain by production of a TC10/K-Ras chimera or by disruption of lipid raft microdomains via expression of a dominant-interfering mutant form of caveolin-3 also completely prevented the activation of TC10 by insulin (28).

Although the precise function of lipid raft subdomains in assembling this signaling complex remains uncertain, resident structural proteins may play an important role. One such protein is caveolin-1, which is highly expressed in mature adipocytes (29) and is known to undergo tyrosine phosphorylation in various cells (30–33). Caveolin is tyrosine-phosphorylated in response to insulin in 3T3L1 adipocytes but not fibroblasts (30). Although this phosphorylation event correlates well with insulin responsiveness, the significance of caveolin phosphorylation remains uncertain. Moreover, it is unclear whether this phosphorylation event is a direct result of the tyrosine kinase activity of the insulin receptor or perhaps catalyzed by the Src family kinase Fyn, which is also highly enriched in caveolae of adipocytes (30). We demonstrate here that insulin receptor physically associates with caveolin in 3T3L1 adipocytes and, further, that caveolin is a direct substrate of the insulin receptor.

EXPERIMENTAL PROCEDURES

Materials—The anti-caveolin-2, insulin receptor β subunit and Myc antibodies were purchased from Santa Cruz Biotechnology. The FLAG (M2) antibody was obtained from Stratagene. The anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology Inc. The phospho-p42/44 MAPK and phospho-Akt (4E2) antibodies were from Cell Signaling Technology. The anti-insulin receptor β subunit antibodies for immunofluorescence as well as the anti-caveolin-1, paxillin, and phospho-caveolin (Tyr^{14}) antibodies were from Transduction Laboratories. We used horseradish peroxidase-linked secondary antibodies (Bio-Rad) and enhanced chemiluminescence (Amersham Biosciences) for immunoblot detection. The Alexa Fluor secondary antibodies were from Molecular Probes. PD98059 and PP2 were from Calbiochem. Wortmannin was from Sigma Chemical Co. Octylthioglucoside was purchased from Roche Molecular Biochemicals.

Plasmids and Mutagenesis—The cDNA of mouse caveolin-1 was amplified by PCR from a 3T3L1 adipocyte cDNA library as the template. The 5′ primer was designed to have a *Bam*HI restriction site followed by the amino acids 1–27. The 3′ primer was designed to have an *Eco*RI restriction site followed by the amino acids 515–537 according to GenBank™ U07645. These PCR products were cloned into pKMyc vector. FLAG-tagged CAP and CAP mutants were made as described previously (23, 25). All mutated forms of caveolin-1 were generated using the Stratagene QuikChange mutagenesis kit, according to the manufacturer's protocol. The mutations were confirmed by automated DNA sequencing.

Cell Culture and Transfection—3T3L1 fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. Differentiation to adipocytes was induced, and cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. 3T3L1 adipocytes were transfected by electroporation as described previously (23, 25).

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

Fractionation of Triton-insoluble Pellet—The Triton-insoluble pellet fraction was generated as described previously (21, 30). Briefly, cells were washed with ice-cold phosphate-buffered saline and lysed in MBS buffer (25 mM MES, pH 6.0; 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 10 mM Na-pyrophosphate, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 1 mM benzamidine) containing protease inhibitors and sodium orthovanadate. Lysed cells were kept at 4 °C to prevent degradation of caveolar complexes. The Triton-insoluble pellet fraction was solubilized in SOL buffer (25 mM Tris, pH 8.0; 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 10 mM Na-pyrophosphate, 1 mM EDTA, 60 mM octylthioglucoside, 1 mM PMSF, 10 μg/ml aprotinin, 1 mM benzamidine) containing 60 mM octylthioglucoside.

Fluorescence Microscopy—3T3L1 adipocytes were grown on glass cover slips in six-well dishes. Cells were fixed with 10% formalin for 10 min, permeabilized with 0.5% Triton X-100 for 5 min, and blocked with 3% bovine serum albumin and 1% ovalbumin for 1 h. Primary and Alexa Fluor secondary antibodies were used at 2 μg/μl in blocking solution, and samples were mounted on glass slides with Vectashield (Vector Laboratories). Cells were imaged using confocal fluorescence microscopy. Fluorescent intensities were quantitated using the Olympus Fluoview software. Images were then imported into Adobe Photoshop (Adobe Systems, Inc.) for processing.

Sucrose Gradient Centrifugation—Isolation of caveolae-enriched fractions was performed as previously described with minor modifications (30). Briefly, differentiated 3T3L1 adipocytes were washed with ice-cold phosphate-buffered saline and rapidly scraped in 0.5 M sodium carbonate buffer (pH 11) and homogenized with 10 strokes in a Dounce glass homogenizer. The homogenates were then sonicated three times for 20 s at the setting of 4.5 in a Fisher 550 sonic dismembrator. The samples were combined with 60% (w/v) sucrose in 25 mM MES, pH 6.5, 150 mM NaCl and well mixed to achieve a final sucrose concentration of 40%. The homogenates were overlaid with 4 ml of 35% (w/v) sucrose and then with 3 ml of 5% (w/v) sucrose in 25 mM MES, pH 6.5, 150 mM NaCl. The samples were spun at 39,000 rpm in an SW 41 rotor at 4 °C for 19 h, and 1-ml fractions were collected for immunoblotting.

RESULTS

The Insulin Receptor Is Localized in Rosette Structures in 3T3L1 Adipocytes—To determine whether the tyrosine phosphorylation of caveolin might be catalyzed directly by the insulin receptor, we explored the localization of the receptor in membranes of 3T3L1 adipocytes. Cells were lysed in buffer containing Triton X-100 and purified on sucrose density gradients (Fig. 1). As previously observed, under these gradient conditions caveolin and flotillin migrate in the low density region (Fig. 1*A*, *fractions 3* and *4*) of the sucrose gradient consistent with lipid raft enrichment. The insulin receptor was found in the more dense regions of the gradient but also co-migrated with caveolin-1 and flotillin in the low density fractions. As expected, insulin stimulation resulted in the recruitment of C3G to the low density fractions, whereas the p85 regulatory subunit of PI3K was not detected in these fractions, irrespective of insulin treatment (Fig. 1*B*). To confirm that a population of the insulin receptor is co-localized with caveolin, plasma membrane sheets were prepared from 3T3-L1 adipocytes (28), and stained with antibodies to caveolin-2 or the insulin receptor beta subunit. As is seen in Fig. 1*C*, both caveolin and insulin receptor reside in characteristic large clusters of caveolae that take on a rosette or ring-like appearance in adipocytes (28). Both caveolin-1 and -2 are localized in these structures (data not shown).

Insulin Stimulates the Phosphorylation of Caveolin-1 on Tyr^{14}—Previous studies suggested that the insulin-stimulated tyrosine phosphorylation of caveolin might be secondary to the phosphorylation of Cbl and its subsequent translocation to lipid raft microdomains (23). This translocation depends on the binding of Cbl to the adapter protein CAP, and the subsequent interaction of the second protein with the raft protein flotillin

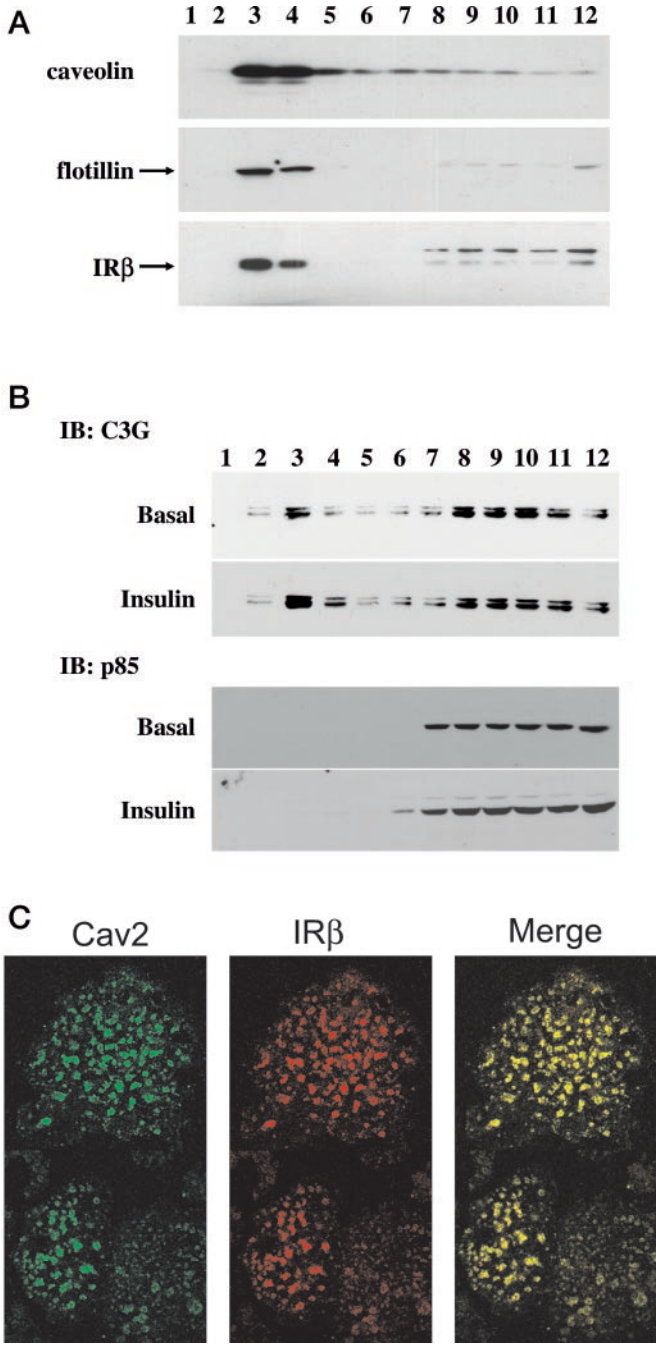

FIG. 1. **Colocalization of insulin receptor and caveolin in 3T3L1 adipocyte plasma membranes.** *A*, fully differentiated 3T3L1 adipocytes were non-detergent-homogenized, sonicated, and subjected to sucrose density gradient fractionation as described under "Experimental Procedures." The gradient fractions from low (*1*) to higher (*12*) density were subjected to SDS-PAGE and immunoblotted with caveolin, flotillin, and insulin receptor β subunit antibodies. *B*, adipocytes were stimulated with (*Insulin*) and without (*Basal*) 100 nM insulin for 5 min prior to sucrose gradient centrifugation. The fractions were immunoblotted with C3G and PI3K p85 regulatory subunit antibodies. *C*, plasma membrane sheets were prepared from 3T3L1 adipocytes, as detailed in "Experimental Procedures." Sheets were probed with antibodies to caveolin or the β subunit of the insulin receptor, as indicated. These are representative experiments that were repeated five times.

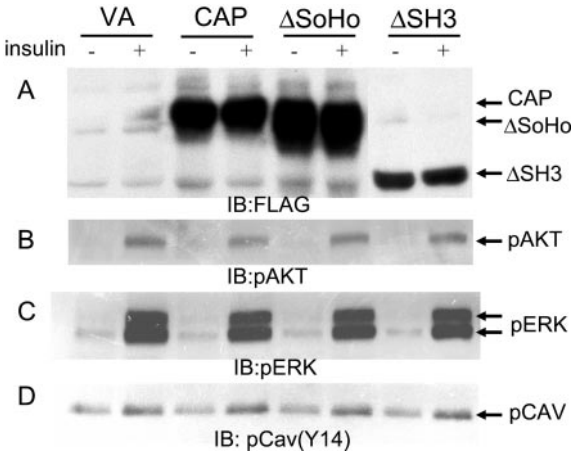

FIG. 2. **Insulin-stimulated tyrosine phosphorylation of caveolin-1 is independent of the Cbl·CAP pathway.** Fully differentiated 3T3L1 adipocytes were electroporated with 200 μg of vector control (*VA*), FLAG-tagged CAP (*CAP*), CAPΔSoHo (Δ*SoHo*), or CAPΔSH3 (Δ*SH3*) cDNA. Cells were placed in serum-free medium and starved for 3 h before treatment with 100 nM insulin for 5 min. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with FLAG (*A*), phospho-AKT (*B*), phospho-ERK (*C*), phospho-caveolin(Tyr^{14}) (*D*) and caveolin-1 antibodies (*E*). Shown is a representative experiment that was repeated three times.

(23). The insulin-stimulated translocation of phospho-Cbl to these microdomains is blocked by overexpression of deletion mutants of CAP lacking the Cbl-interacting SH3 domains (CAPΔSH3) or flotillin-binding SoHo domain (CAPΔSoHo) (23, 25). To determine whether Cbl·CAP signaling is involved in caveolin-1 phosphorylation, FLAG-tagged CAP, CAPΔSoHo, and CAPΔSH3 were expressed in 3T3-L1 adipocytes. The phosphorylation of caveolin-1 on Tyr^{14} in response to insulin was evaluated using a phosphospecific antibody (Fig. 2). Insulin stimulated the tyrosine phosphorylation of caveolin-1, using the phosphospecific antibodies to the sequences surrounding Tyr^{14} (Fig. 2*D*, *lanes 1* and *2*). Identical results were generated using anti-phosphotyrosine antibodies (data not shown). As previously reported (25), expression of CAP, CAPΔSoHo, or CAPΔSH3 did not effect the stimulation of Akt or MAPK phosphorylation in response to insulin. Similarly, expression of CAP, CAPΔSoHo, or CAPΔSH3 had no effect on insulin-stimulated caveolin phosphorylation (Fig. 2*D*, *lanes 3–8*), indicating that the Cbl·CAP pathway is not required for phosphorylation of caveolin-1. As controls, the relative expression levels of CAP, CAPΔSoHo, CAPΔSH3, and endogenous caveolin are shown.

Because the tyrosine phosphorylation of caveolin appears to be independent of the CAP/Cbl pathway, we sought to examine the potential role of the PI3K and MAPK pathways in this event (Fig. 3). 3T3L1 adipocytes were pretreated with PD98059 (Fig. 3, *lanes 3* and *4*), a pharmacological inhibitor of MEK that has been shown to block the MAPK pathway (34) as well as wortmannin (Fig. 3, *lanes 5* and *6*), an inhibitor of PI3K (35), followed by exposure to insulin. As expected, neither inhibitor blocked IRS-1 or insulin receptor tyrosine phosphorylation (Fig. 3*A*), although wortmannin blocked the phosphorylation of Akt (Fig. 3*B*) and PD98059 blocked the phosphorylation of MAPK (Fig. 3*C*). Moreover, the tyrosine phosphorylation of caveolin was unaffected by both inhibitors (Fig. 3*D*), indicating that this phosphorylation event is independent of the MAPK and PI3K pathways.

Caveolin-1 was first identified as a tyrosine-phosphorylated protein in v-Src-transformed cells (36) and is known to be constitutively tyrosine-phosphorylated in Src- and Abl-transformed cells (33). Moreover, previous studies (30) revealed that the Src family kinase Fyn is abundant in Triton-insoluble low density complexes derived from 3T3L1 adipocytes. To examine if insulin-stimulated caveolin phosphorylation is a consequence of Fyn activity, 3T3L1 adipocytes were pretreated with the Src family kinase inhibitor PP2 prior to insulin treatment, and caveolin phosphorylation was evaluated (Fig. 4). This compound is known to specifically inhibit Src family tyrosine kinases in cells, including the kinase Fyn (37, 38). The compound

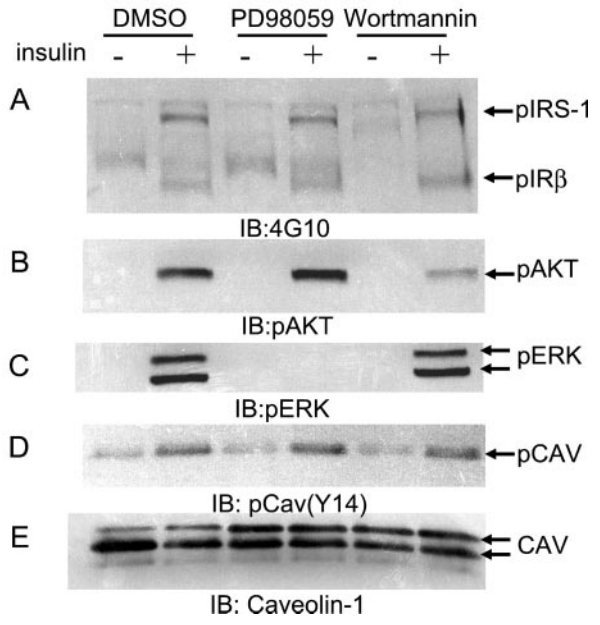

FIG. 3. **Insulin-stimulated tyrosine phosphorylation of caveolin-1 is PI3K- and MAPK-independent.** 3T3L1 adipocytes were placed in serum-free medium for 3 h and incubated for 30 min with 100 nM wortmannin or 10 μM PD98059 prior to 100 nM insulin treatment for 5 min. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with 4G10 (*A*), phospho-AKT (*B*), phospho-ERK (*C*), and phospho-caveolin(Tyr^{14}) antibodies (*D*). Shown is a representative experiment that was repeated three times.

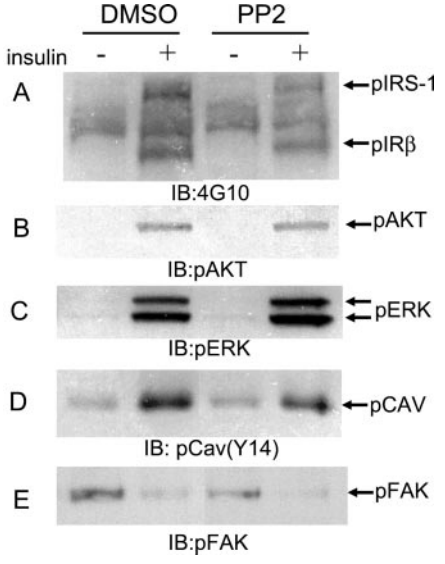

FIG. 4. **Insulin-stimulated tyrosine phosphorylation of caveolin-1 is PP2-insensitive.** Cells were placed in serum-free medium for 3 h and incubated for 20 min with 1 μM vehicle (Me_2SO (*DMSO*)) or PP2 prior to insulin stimulation at 100 nM for 5 min. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with 4G10 (*A*), phospho-AKT (*B*), phospho-ERK (*C*), phospho-caveolin(Tyr^{14}) (*D*), and phospho-FAK antibodies (*E*). Shown is a representative experiment that was repeated three times.

had no effect on insulin receptor or IRS-1 tyrosine phosphorylation (Fig. 4*A*) or on the activation of Akt (Fig. 4*B*) or MAPK (Fig. 4*C*) in response to insulin. Furthermore, PP2 pretreatment failed to inhibit basal and insulin-stimulated phosphorylation of caveolin-1 (Fig. 4*D*). In contrast, PP2 pretreatment completely blocked the constitutive phosphorylation of FAK, a known substrate of Src family kinases such as Fyn (Fig. 4*E*), although the dephosphorylation of the protein produced by insulin (39) was not affected. Additionally, addition of PP2

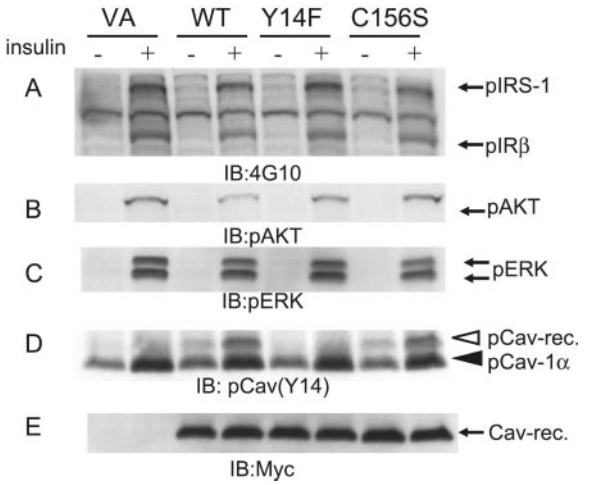

FIG. 5. **Palmitoylation of caveolin-1 at Cys^{156} is not required for insulin-stimulated phosphorylation.** Fully differentiated 3T3L1 adipocytes were electroporated with 200 μg of vector control, Myc-tagged caveolin-1 wild type (*WT*), caveolin-1 Y14F mutant (*Y14F*), or caveolin-1 C156S mutant (*C156S*). Thirty-six hours after electroporation, cells were placed in serum-free medium for 3 h before treatment with 100 nM insulin for 5 min. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with 4G10 (*A*), phospho-AKT (*B*), phospho-ERK (*C*), phospho-caveolin (Tyr^{14}) (*D*), and myc antibodies (*E*). Anti-phospho-caveolin(Tyr^{14}) antibodies recognized both expressed caveolin-1 and endogenous-caveolin-1 α forms upon phosphorylation. Anti-myc antibodies recognized only expressed caveolin-1. Shown is a representative experiment that was repeated three times.

blocked the constitutive tyrosine phosphorylation of paxillin and Pyk2 in these cells (data not shown).

Previous reports have demonstrated that palmitoylation-deficient caveolin-1 cannot be phosphorylated on Tyr^{14} by c-Src (33). Moreover, Cys^{156} appears to be required for the interaction of caveolin-1 with Src family kinases, because substitution of Cys^{156} with Ser inhibited the c-Src-catalyzed phosphorylation of the protein (33). To further explore the role of Fyn in insulin-induced phosphorylation of caveolin-1, we expressed mutant forms of caveolin-1 in 3T3L1 adipocytes and analyzed tyrosine phosphorylation (Fig. 5). Cells were transfected by electroporation (23, 25) with vector alone (*VA*, *lanes 1* and *2*), wild type caveolin-1 (*WT*, *lanes 3* and *4*), a caveolin mutant in which Tyr^{14} was mutated to phenylalanine (*Y14F*, *lanes 5* and *6*), and a caveolin-1 mutant in which Cys^{156} was mutated to serine (*C156S*, *lanes 7* and *8*). All of these constructs encoded proteins that were myc-tagged and were expressed at levels roughly equal to those of the endogenous caveolin, as detected by blotting with anti-caveolin antibodies (Fig. 5*E*). Expression of wild type caveolin, Cav(Y14F) or Cav(C156S) had no effect on the stimulation of IR, IRS-1, Akt, and ERK phosphorylation in response to insulin (Fig. 5, *A–C*). Furthermore, the ectopic expression of these proteins did not inhibit the tyrosine phosphorylation of endogenous caveolin-1 (Fig. 5*D*). Myc-tagged wild type caveolin was phosphorylated in response to insulin as was Cav(C156S). In contrast, Cav(Y14F) did not undergo phosphorylation in response to insulin. Probing this blot with anti-phosphotyrosine antibodies also revealed no phosphorylation (data not shown), indicating that insulin stimulates the phosphorylation of caveolin primarily on Tyr^{14}. Interestingly, expression of the Y14F mutant of caveolin-1 did not produce a dominant-interfering phenotype regarding phosphorylation of the endogenous protein, suggesting that significant overexpression of the protein will be required to observe this effect.

The data described above suggest that caveolin phosphorylation is likely to be catalyzed directly by the insulin receptor. To explore this possibility in more detail, we sought to evaluate

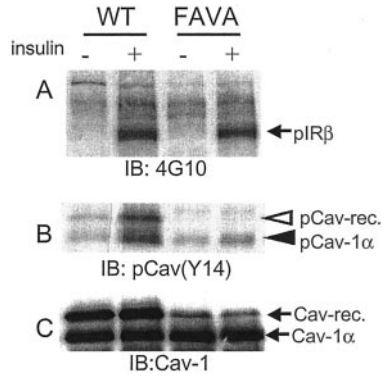

FIG. 6. **Caveolin scaffolding domain is required for insulin-stimulated phosphorylation.** Fully differentiated 3T3-L1 adipocytes were electroporated with 200 μg of Myc-tagged caveolin-1 wild type (*WT*) or caveolin-1 F92A/V94A mutant (*FAVA*). Cells were incubated with 100 nM insulin for 5 min, 36 h after electroporation. The Triton-insoluble pellet fraction (see "Experimental Procedures") were separated by SDS-PAGE, transferred to nitrocellulose, and blotted for 4G10 (*A*), phospho-caveolin(Tyr^{14}) (*B*) or anti-caveolin-1 antibodies (*C*). Shown is a representative experiment that was repeated three times.

a domain on caveolin that is thought to interact with proteins in caveolae. Caveolin contains a short stretch of amino acids in its amino terminus around residues 80–100 that may mediate these interactions (40), including those with the insulin receptor (41). To determine whether direct interactions between caveolin-1 and the insulin receptor are required for insulin-stimulated phosphorylation of caveolin, a mutant form of the protein was constructed in which Phe^{92} and Val^{94} were mutated to alanine (Cav(FAVA)). This construct was transfected into 3T3L1 adipocytes, along with wild type caveolin-1 (Fig. 6). Although the expression levels of Cav(FAVA) were significantly lower than that observed for the wild type form of the protein, it was possible to detect the protein by blotting with anti-myc or anti-caveolin antibodies (Fig. 6*C*). Cav(FAVA) was detected at the plasma membrane and localized in Triton-insoluble fractions, typical of wild type caveolin. Moreover, overexpression of this mutant had no effect on the distribution of the insulin receptor in these low density fractions (data not shown). Expression of wild type caveolin or Cav(FAVA) had no effect on insulin receptor autophosphorylation (Fig. 6*A*) or on levels of endogenous caveolin (Fig. 6*C*). However, the tyrosine phosphorylation of endogenous caveolin-1 was significantly inhibited by expression of Cav(FAVA) (Fig. 6*B*). Furthermore, expressed Cav(FAVA) was not phosphorylated in response to insulin, whereas wild type caveolin did undergo tyrosine phosphorylation.

DISCUSSION

Recent evidence suggests that signaling molecules and pathways are restricted to discrete compartments in cells by specific protein interactions. Lipid raft subdomains have been implicated as sites for signal initiation in numerous cell types (42). In adipocytes and muscle cells, much attention has focused on the caveolae, a subset of lipid raft microdomains. These small invaginations of the plasma membrane are often enriched in signaling molecules, glycolipids, and cholesterol and have been proposed to act as signaling organelles (43). Based on the identification of a signaling complex in caveolae involving APS, Cbl, CAP, and TC10, we have hypothesized that the segregation to these domains might provide a potential explanation for the specificity of signal transduction in insulin action (2). Disruption of caveolae with cholesterol-extracting drugs, such as β-cyclodextrin, or expression of a dominant negative form of caveolin-3 (28) blocked specifically the initiation of this pathway and prevented the stimulation of glucose transport and Glut4 translocation in response to insulin (44).

The caveolin family of proteins appears to play a particularly important role in the formation of caveolae and possibly in signaling activities that occur in these microdomains. Disruption of the gene encoding caveolin-1 results in a failure of lung epithelial cells and adipocytes to form caveolae (45, 46), although the impact of this manipulation on signal transduction has not been studied. Numerous studies have demonstrated that caveolin interacts with a number of signaling molecules that are thought to be enriched in caveolae, including certain small and heterotrimeric G proteins (40, 47), endothelial nitric-oxide synthase (48, 49), and a subset of tyrosine kinase receptors (50), including the insulin receptor (41, 51). Caveolin is also known to undergo tyrosine phosphorylation in some cases (30–33). We first demonstrated that insulin could specifically stimulate the phosphorylation of caveolin in 3T3L1 adipocytes (31). This effect of the hormone was rapid, dose-dependent, and specific for insulin. Interestingly, the effect was not observed in 3T3L1 pre-adipocytes, which express both caveolin and the insulin receptor, suggesting that caveolin phosphorylation correlates with metabolic responsiveness to insulin (30).

Although the mechanism involved in the insulin-stimulated phosphorylation of caveolin has not been established, previous studies suggested that the Src family kinase Fyn might catalyze this event (30). Fyn is highly expressed n 3T3L1 adipocytes and co-purifies with caveolin in Triton-insoluble low density complexes. Moreover, caveolin undergoes hyperphosphorylation upon overexpression of Fyn in these cells (30). However, the data presented in this study strongly support a direct insulin receptor-mediated tyrosine phosphorylation of caveolin on Tyr^{14} of the protein. This phosphorylation does not require the activation of PI3K or MAPK and is independent of the phosphorylation and translocation of Cbl to the lipid raft microdomain. Furthermore, this phosphorylation event does not rely on the activity of the Fyn tyrosine kinase, because inhibitors of this enzyme have no inhibitory effect on caveolin phosphorylation. Finally, expression in cells of a mutant form of caveolin that cannot be phosphorylated by Src family kinases still undergoes insulin-stimulated phosphorylation, whereas expression of a mutant form of the protein that cannot interact with the insulin receptor is not phosphorylated in these cells. Moreover, the insulin receptor is localized to plasma membrane domains that are highly enriched in caveolin. Taken together, these observations strongly suggest that caveolin is a direct substrate of the insulin receptor.

What is the biological significance of increased caveolin tyrosine phosphorylation that occurs after insulin stimulation in adipocytes? Unfortunately, it was not possible to block this event by overexpression of a phosphorylation-deficient mutant form of caveolin (Y14F), because we were unable to attain the expression levels required to block phosphorylation of the endogenous protein. Thus, expression of a dominant negative form of caveolin in cells metabolically responsive to insulin will likely require a knock-out/knock-in strategy. Although there are no data on the functional alterations in caveolin as a consequence of phosphorylation on Tyr^{14}, it is possible to speculate about phosphotyrosine-dependent interactions that might recruit proteins to these domains in an insulin-dependent manner, perhaps via SH2 interactions. In this regard, Grb7 and -14 have been proposed to interact with caveolin in a tyrosine phosphorylation-dependent manner (33), although we have been unable to detect insulin-dependent interactions with these adapter proteins in adipocytes (data not shown). It is also possible that caveolin might itself regulate the activity of the insulin receptor, increasing its activity toward other substrates

(41). Finally, the possibility remains that the tyrosine phosphorylation of caveolin by the insulin receptor is merely a result of the interaction of these two molecules and, further, that the binding of caveolin to the receptor helps to recruit the latter protein to caveolae, where it initiates the CAP/Cbl signaling pathway. These possibilities will be the subject of future studies.

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