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Cdc42 Is a Rho GTPase Family Member That Can Mediate Insulin Signaling to Glucose Transport in 3T3-L1 Adipocytes*

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We investigated the role of cdc42, a Rho GTPase family member, in insulin-induced glucose transport in 3T3-L1 adipocytes. Microinjection of anti-cdc42 antibody or cdc42 siRNA led to decreased insulin-induced and constitutively active G_q (CA-G_q; Q209L)-induced GLUT4 translocation. Adenovirus-mediated expression of constitutively active cdc42 (CA-cdc42; V12) stimulated 2-deoxyglucose uptake to 56% of the maximal insulin response, and this was blocked by treatment with the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor, wortmannin, or LY294002. Both insulin and CA-G_q expression caused an increase in cdc42 activity, showing that cdc42 is activated by insulin and is downstream of $G\alpha_{q/11}$ in this activation pathway. Immunoprecipitation experiments showed that insulin enhanced a direct association of cdc42 and p85, and both insulin treatment and CA-cdc42 expression stimulated PI3-kinase activity in immunoprecipitates with anti-cdc42 antibody. Furthermore, the effects of insulin, CA-G_q, and CA-cdc42 on GLUT4 translocation or 2-deoxyglucose uptake were inhibited by microinjection of anti-protein kinase Cλ (PKCλ) antibody or overexpression of a kinase-deficient PKCλ construct. In summary, activated cdc42 can mediate 1) insulin-stimulated GLUT4 translocation and 2) glucose transport in a PI3-kinase-dependent manner. 3) Insulin treatment and constitutively active G_q expression can enhance the cdc42 activity state as well as the association of cdc42 with activated PI3-kinase. 4) PKCλ inhibition blocks CA-cdc42, CA-G_q, and insulin-stimulated GLUT4 translocation. Taken together, these data indicate that cdc42 can mediate insulin signaling to GLUT4 translocation and lies downstream of $G\alpha_{q/11}$ and upstream of PI3-kinase and PKCλ in this stimulatory pathway.

Insulin stimulates glucose transport in skeletal muscle and adipose tissue by inducing the translocation of the GLUT4 glucose transporter from an intracellular pool to the plasma membrane (1, 2). Although the signaling pathways and the dynamics of GLUT4 movement have been intensively studied, the precise mechanisms of GLUT4 translocation remain incompletely understood. Insulin initiates its signal transduction cascade by activating the insulin receptor tyrosine kinase, leading to phosphorylation of phosphoprotein substrates, activation of PI3-kinase,[1] and stimulation of glucose transport (3–6). Phosphorylation of IRS-1, which binds to and activates PI3-kinase, is one mechanism by which insulin stimulates PI3-kinase to mediate glucose transport. However, several reports (7–12) using various approaches indicate that IRS-1 is not necessarily essential for transport stimulation and that other pathways exist. Thus, we have shown (6) that the activated insulin receptor can also phosphorylate the heterotrimeric protein component $G\alpha_{q/11}$, leading to activation of PI3-kinase and glucose transport stimulation. Others (13–15) have also found that $G\alpha_{q/11}$ can stimulate glucose transport but have failed to observe PI3-kinase dependence of this effect.

More recently, it has been recognized that a separate PI3-kinase-independent pathway is initiated by insulin stimulation that must complement the PI3-kinase-dependent pathway to achieve full GLUT4 translocation (16, 17). This PI3-kinase-independent pathway involves insulin-mediated localization of CAP-Cbl complexes to membrane rafts with subsequent recruitment of CrkII-C3G to these structures, leading to activation of the small Rho family GTPase, TC10. Insulin-stimulated cortical actin remodeling and polymerization are necessary for the final steps of GLUT4 movement to the plasma membrane, and it has been proposed that TC10 interacts with neural Wiskott-Aldrich syndrome protein in an insulin-dependent manner to mediate cortical actin polymerization (18).

Cdc42, another member of the Rho GTPase family, has 69% homology and 86% similarity to TC10 (16). Insulin can stimulate cdc42 activity, and cdc42 can facilitate actin rearrangement (19). Furthermore, bradykinin, a GPCR agonist that couples into $G\alpha_{q/11}$, can stimulate glucose transport and actin remodeling in 3T3-L1 adipocytes (20). Since it has been shown that cdc42 can mediate the effects of bradykinin-stimulated $G\alpha_{q/11}$ on actin remodeling (21), we hypothesized that cdc42 might also mediate signals to GLUT4 translocation. In the current study, we show a novel role for cdc42 as a downstream activator of $G\alpha_{q/11}$, which can mediate GLUT4 translocation in a PI3-kinase-dependent manner in 3T3-L1 adipocytes.

EXPERIMENTAL PROCEDURES

Materials—Mouse monoclonal anti-cdc42 and anti-p85 antibodies (N-SH2), cdc42 assay kit, and protein A-agarose were purchased from

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[1] The abbreviations used are: PI3-kinase, phosphatidylinositol 3-kinase; 2-DOG, 2-deoxyglucose; CA-G_q, constitutively active G_q; PKC, protein kinase C; m.o.i., multiplicity of infection; GFP, green fluorescent protein; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; DN-cdc42, dominant negative cdc42.

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Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse monoclonal anti-phosphotyrosine (PY20) antibody was from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-GLUT4 antibody was purchased from Chemicon International Inc. (Temecula, CA). Rabbit polyclonal anti-$G\alpha_{q/11}$, anti-p110α, and anti-cdc42 (P1) antibodies, goat polyclonal anti-cdc42 (C20) antibody, mouse monoclonal anti-cdc42 antibody (B8), and horseradish peroxidase-linked anti-rabbit and anti-mouse antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Sheep IgG and fluorescein isothiocyanate-conjugated and TRITC-conjugated anti-rabbit and anti-mouse IgG antibodies were from Jackson ImmunoResearch (West Grove, PA). Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Invitrogen. All radioisotopes were from ICN (Costa Mesa, CA). All other reagents were purchased from Sigma. The GTPase-deficient (constitutively active) Q209L mutant $G\alpha_q$ expression vector and recombinant adenoviruses were described elsewhere (6). Adenoviruses encoding constitutively active and kinase-deficient PKCλ were kindly provided by Dr. Wataru Ogawa (Kobe University, Japan), and adenoviruses encoding constitutively active cdc42 and dominant negative cdc42 were kindly provided by Dr. James R. Bamburg (Colorado State University).

Cell Culture and Cell Treatment—3T3-L1 cells were cultured and differentiated as described previously (6). Differentiated 3T3-L1 adipocytes were incubated with 100 μM LY294002, 100 or 300 nM wortmannin, 50 μM PD98059, or 0.1% dimethyl sulfoxide vehicle for 1 or 4 h before each assay. For adenovirus infection, 3T3-L1 adipocytes were transduced for 16 h in Dulbecco's modified Eagle's high glucose medium with 5% heat-inactivated serum with the following multiplicity of infection (m.o.i.) and with either the recombinant adenovirus of wild type-$G\alpha_q$ (40 m.o.i.), constitutively active mutant-$G\alpha_q$ (Q209L) (40 m.o.i.), constitutively active cdc42 (V12) (40 or 80 m.o.i.), dominant negative cdc42 (N17) (80 m.o.i.), constitutively active PI3-kinase (P110-CAAX) (40 m.o.i.), constitutively active PKCλ (80 m.o.i.) or kinase-deficient PKCλ (10–100 m.o.i.), or a control recombinant adenovirus of GFP. The total amount of adenovirus was adjusted to the same m.o.i. with control adenovirus in each experiment. Transduced cells were incubated for 48 or 60 h at 37 °C in 10% CO_2 and Dulbecco's modified Eagle's high glucose medium with 10% heat-inactivated serum, followed by incubation in the starvation media required for the assays. The efficiency of adenovirus-mediated gene transfer was above 90% as measured by histocytochemical staining of LacZ-infected cells with β-galactosidase, as we reported previously (6).

2-Deoxyglucose Uptake—The procedure for glucose uptake was described previously (22) with some modifications. After 60 h of adenovirus infection, 3T3-L1 adipocytes were serum-starved for 3 h, and the cells were stimulated with 0.5 or 17 nM insulin in KRP-Hepes buffer (10 mM Hepes, pH 7.4, 131.2 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 2.5 mM NaH_2PO_4) for 30 min at 37 °C. The procedure for stimulation by osmotic shock was the same as described previously (23). Glucose uptake was determined in triplicate at each point after the addition of 2-[^{3}H]deoxyglucose (0.1 μCi, final concentration 0.1 mM) in KRP-Hepes buffer for 5 min at 37 °C.

Microinjection of Antibodies and siRNAs—Microinjection was carried out using a semiautomatic Eppendorf microinjection system. Antibodies for microinjection were concentrated and dissolved at 5 mg/ml in microinjection buffer containing 5 mM sodium phosphate, pH 7.2, 100 mM KCl and were injected into the cytoplasm. Five mg/ml sheep IgG was injected into the control cells. siRNA for cdc42 (guuauccacagacagaugutt), silencing mediator for retinoid and thyroid hormone receptor (cgagaguucucgcuggacutt), and insulin receptor (tataccatgaattccagcaactt) were purchased from Dharmacon. siRNAs were dissolved at 5 μM in microinjection buffer.

Immunostaining and Immunofluorescence Microscopy—Immunostaining of GLUT4 was performed essentially as described (6). 3T3-L1 adipocytes were stimulated with insulin for 20 min at 37 °C and were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. Following washing, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked with 2% fetal calf serum in PBS for 10 min. The cells were then incubated with anti-GLUT4 antibody in PBS with 2% fetal calf serum overnight at 4 °C. After washing, GLUT4 and injected IgG were detected by incubation with TRITC-conjugated donkey anti-rabbit IgG antibody and fluorescein isothiocyanate-conjugated donkey anti-mouse or anti-sheep antibody, respectively, followed by observation under an immunofluorescence microscope. In all counting experiments, the observer was blinded to the experimental condition of each coverslip.

Western Blotting—Serum-starved 3T3-L1 cells were stimulated with 17 nM insulin at 37 °C for various times as indicated in each experiment. The cells were lysed in solubilizing buffer containing 20 mM Tris, 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 mM NaF, pH 7.5, for 15 min at 4 °C. The cell lysates were centrifuged to remove insoluble materials. For Western blot analysis, whole cell lysates (20–50 μg of protein) were denatured by boiling in Laemmli sample buffer containing 100 mM dithiothreitol and were resolved by SDS-PAGE. Gels were transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA) using Transblot apparatus (Bio-Rad). For immunoblotting, membranes were blocked and probed with specific antibodies. Blots were then incubated with horseradish peroxidase-linked secondary antibodies followed by chemiluminescence detection, according to the manufacturer's instructions (Pierce).

PI3-kinase Assay—After 48 h of adenovirus infection, 3T3-L1 adipocytes were starved for 16 h and stimulated with insulin (17 nM) for 10 min, washed once with ice-cold PBS, lysed, and subjected to immunoprecipitation (300–500 μg of total protein) with anti-cdc42 or anti-p110α antibody for 4 h at 4 °C. Immunocomplexes were precipitated with protein A-plus agarose (Upstate Biotechnology Inc.). The immunoprecipitates were washed with the following buffers: (i) PBS, containing 1% Nonidet P-40, 100 μM sodium orthovanadate, pH 7.4; (ii) 100 mM Tris, 0.5 M LiCl, 100 μM sodium orthovanadate, pH 7.4; and (iii) 10 mM Tris, 100 mM NaCl, 100 μM sodium orthovanadate, pH 7.4. Immunoprecipitates were washed mildly (once with each washing buffer) only in the experiment using LY294002, whereas they were washed more strictly (twice with each buffer) in the other experiments, because LY294002 was a reversible inhibitor. The washed immunocomplexes were incubated with phosphatidylinositol for 5 min and then with [γ-^{32}P]ATP (3000 Ci/mmol) for 5 min at room temperature. Reactions were stopped with 20 μl of 8 N HCl, mixed with 160 μl of $CHCl_3$:methanol (1:1). Samples were centrifuged, and the lower organic phase was applied to a silica gel TLC plate that had been coated with 1% potassium oxalate. TLC plates were developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.3:2), dried, and exposed to an x-ray film. PI3-kinase activity was quantitated by scanning the film using NIH Image.

Cdc42 Assay—Cdc42 activity was measured according to the manufacturer's instructions (Upstate Biotechnology, Inc.). After 48 h of adenovirus infections, 3T3-L1 adipocytes were starved for 16 h and stimulated with 17 nM insulin for the indicated times, washed once with ice-cold PBS, and lysed with lysis buffer containing 25 mM Hepes, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 25 mM NaF for 15 min at 4 °C. Insoluble materials were removed by centrifugation. For a negative control, cell lysate was incubated with 1 mM GDP for 15 min at 30 °C. Five μg of PAK1-agarose beads, which specifically bound to active cdc42 (24), were added to the cell lysates and incubated for 1 h at 4 °C. Agarose beads were washed with lysis buffer three times and boiled in 2× Laemmli sample buffer. Samples were resolved by SDS-PAGE and immunoblotted with anti-cdc42 antibody.

RESULTS

Cdc42 Plays a Role in Insulin-induced GLUT4 Translocation and 2-DOG Uptake—To evaluate the role of cdc42 in insulin-stimulated GLUT4 translocation, we conducted single cell microinjection studies using mouse monoclonal (B8) or goat polyclonal (C20) anti-cdc42 antibody, followed by immunofluorescence staining with GLUT4 antibody in 3T3-L1 adipocytes (Fig. 1*A*). In the basal state, most of the cells display GLUT4 staining in the perinuclear region, and after insulin stimulation there is a marked increase in the proportion of cells demonstrating GLUT4 localization at the plasma membrane as a circumferential ring, as described previously (6). Microinjection of either mouse monoclonal or goat polyclonal anti-cdc42 antibody decreased 1.7 nM insulin-stimulated GLUT4 translocation by 60–75%. To demonstrate further the importance of cdc42 for insulin-stimulated GLUT4 translocation, we utilized siRNA to knock down cdc42 expression followed by measurement of GLUT4 translocation (Fig. 1*B*). siRNA directed against cdc42 was microinjected into the cytoplasm of 3T3-L1 adipocytes, and 72 h later, GLUT4 translocation was measured. As seen in Fig. 1*B*, cdc42 siRNA led to a 65% decrease in insulin-induced GLUT4 translocation. As a positive control, siRNA against the insulin receptor was injected, which completely abolished insulin stimulation. As a negative control, siRNA against silencing mediator for retinoid and thyroid hormone receptor, a tran-

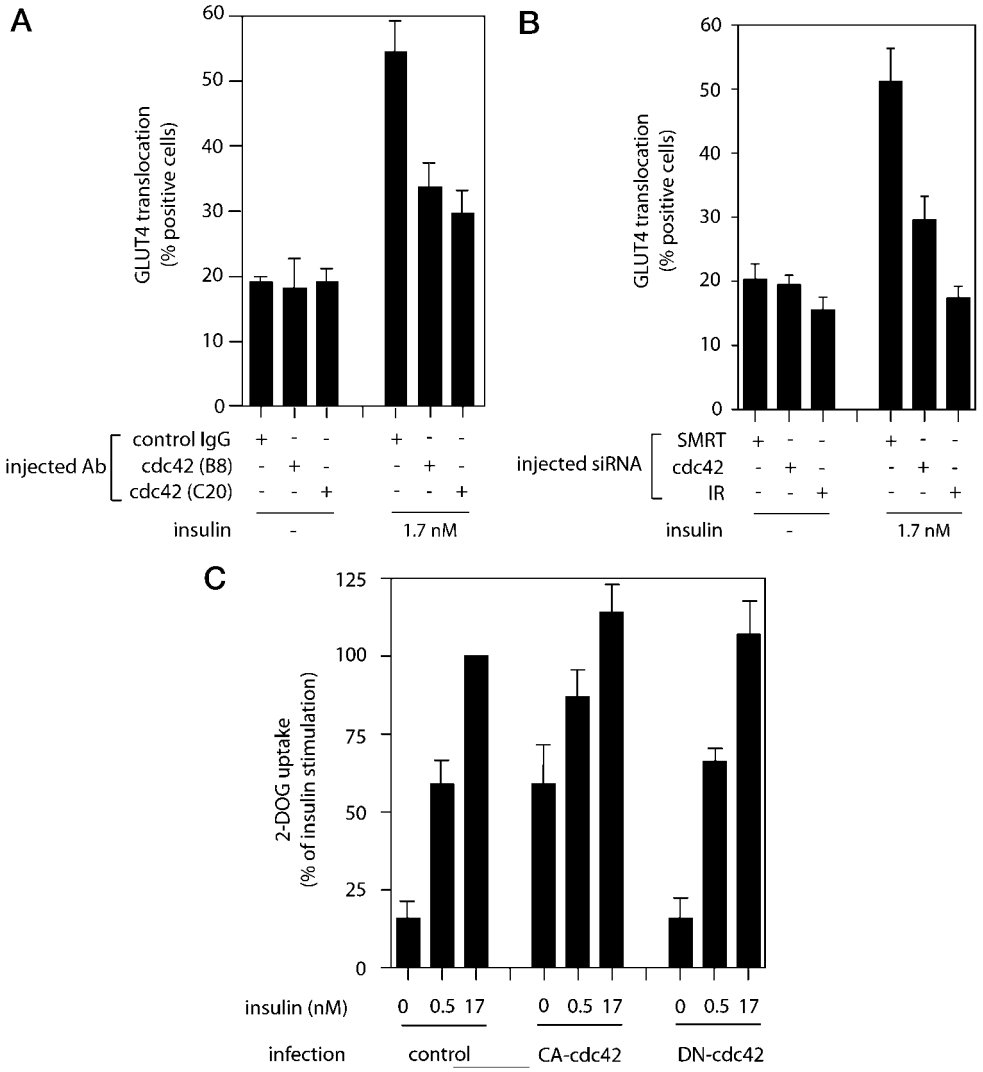

FIG. 1. **Involvement of cdc42 in insulin-induced glucose transport in 3T3-L1 adipocytes.** *A*, 3T3-L1 adipocytes on coverslips were serum-starved for 4 h, and mouse monoclonal (*B8*) or goat polyclonal (*C20*) anti-cdc42 antibody (*Ab*) or sheep IgG was microinjected. Cells were stimulated with or without 1.7 nM insulin for 20 min. GLUT4 was stained as described under "Experimental Procedures." The percentage of cells positive for GLUT4 translocation was calculated by counting at least 100 cells at each point. The data are the mean ± S.E. from three independent experiments. *B*, after 72 h of microinjection of cdc42, insulin receptor (*IR*), or silencing mediator for retinoid and thyroid hormone receptor siRNA, 3T3-L1 adipocytes on coverslips were serum-starved for 4 h and were stimulated with or without 1.7 nM insulin for 20 min. Cells were then stained for GLUT4 localization. Data represent the mean ± S.E. of three independent experiments. *C*, 3T3-L1 adipocytes were infected with adenoviruses expressing constitutively active (*CA-cdc42*) or dominant negative cdc42 (*DN-cdc42*) or control GFP (*control*). After 48 h of infection, these cells were serum-starved for 3 h and stimulated with 0.5 or 17 nM insulin for 30 min, and 2-[^{3}H]deoxyglucose uptake was measured as described under "Experimental Procedures." The data are the mean ± S.E. from four independent experiments.

scription co-regulating molecule, was also injected and had no effect.

To examine further the role of cdc42 in insulin-induced glucose transport, we next measured 2-DOG uptake in 3T3-L1 adipocytes infected with adenovirus vectors containing either constitutively active cdc42 (CA-cdc42; V12) or dominant negative cdc42 (DN-cdc42; N17). As shown in Fig. 1*C*, infection of CA-cdc42 at 80 m.o.i. increased 2-DOG uptake to 61% of the maximal insulin response in the basal state and further enhanced 2-DOG uptake stimulated by submaximal insulin (0.5 nM). Interestingly, adenovirus-mediated expression of DN-cdc42 was without effect on insulin stimulation of glucose transport. Taken together, these results suggest that cdc42 plays an important role in insulin signaling leading to GLUT4 translocation.

cdc42 Is Downstream of G$\alpha_{q/11}$ in the Insulin-GLUT4 Translocation Signaling Pathway—As we reported recently (6), an adenovirus encoding constitutively active G_q (CA-G_q; Q209L) stimulated GLUT4 translocation to 60% of the maximal insulin response, showing a role for G$\alpha_{q/11}$ in this insulin action. In order to examine whether cdc42 is downstream of G$\alpha_{q/11}$, we microinjected mouse monoclonal anti-cdc42 antibody (B8) into cells infected with CA-G_q or CA-cdc42, and similar to the results with insulin- or CA-cdc42 stimulation, anti-cdc42 antibody injection inhibited CA-G_q-induced GLUT4 translocation (Fig. 2). To assess further the relative loci of G$\alpha_{q/11}$ and cdc42 in this pathway, we microinjected anti-G$\alpha_{q/11}$ antibody into cells stimulated by CA-cdc42, CA-G_q, or insulin. Anti-G$\alpha_{q/11}$ antibody injection inhibited insulin- or CA-G_q-induced GLUT4 translocation but did not inhibit the effects of CA-cdc42 (Fig. 2). These data provide further evidence that cdc42 is downstream of G$\alpha_{q/11}$ in mediating insulin-stimulated GLUT4 translocation.

Constitutively Active Cdc42 Stimulates 2-DOG Uptake in a PI3-kinase-dependent Manner—We next examined the effects of the PI3-kinase inhibitors, wortmannin and LY294002, on

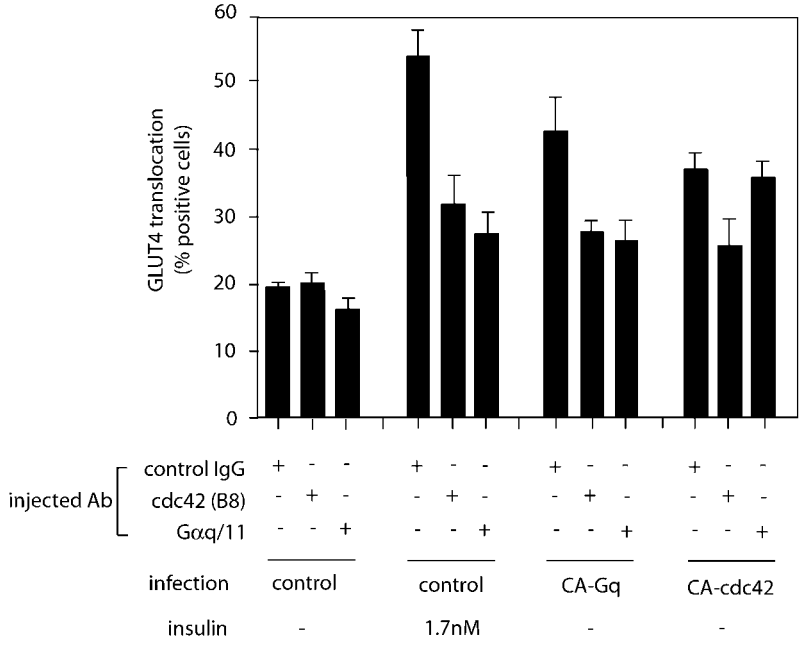

FIG. 2. **Effects of microinjection of anti-cdc42 or anti-G$\alpha_{q/11}$ antibody on GLUT4 translocation in 3T3-L1 adipocytes.** 3T3-L1 adipocytes were infected with adenoviruses expressing constitutively active cdc42 (*CA-cdc42*), constitutively active G_q (*CA-Gq*), or control GFP (*control*). After 48 h of infection, anti-G$\alpha_{q/11}$ antibody (*Ab*), anti-cdc42 antibody, or control sheep IgG was microinjected into the cells on coverslips. Cells were serum-starved for 4 h and stimulated with 1.7 nM insulin for 20 min. GLUT4 in the cells was stained as described under "Experimental Procedures." The percentage of cells positive for GLUT4 translocation was calculated by counting at least 100 cells at each point. The data are the mean ± S.E. from three independent experiments.

CA-cdc42-induced 2-DOG uptake (Fig. 3*A*). In the absence of insulin, expression of CA-cdc42 increased 2-DOG uptake in a dose-responsive manner with 40 and 80 m.o.i. stimulating to 39 and 56% of the maximal insulin response, respectively. Incubation of the cells with 100 nM wortmannin or 100 μM LY294002 for 1 h did not alter 2-DOG uptake stimulated by CA-cdc42 expression, whereas it completely inhibited insulin-stimulated glucose uptake. However, when the cells were incubated with these inhibitors for 4 h, not only insulin-induced but also CA-cdc42-induced 2-DOG uptake was inhibited to basal levels. As reported previously (6), overexpression of CA-G_q enhanced 2-DOG uptake, and this was also inhibited by incubation with 100 nM wortmannin or 100 μM LY294002 for 4 h but not for 1 h. As expected, incubation with a MEK1 inhibitor, PD98059, for 4 h did not affect 2-DOG uptake stimulated by insulin, CA-cdc42, or CA-G_q expression (Fig. 3*A*). These results suggest that activated cdc42 and G$\alpha_{q/11}$ can stimulate glucose uptake in a PI3-kinase-dependent manner in 3T3-L1 adipocytes.

Because the lower doses of wortmannin and LY294002 took 4 h to reach their maximal effects, we further examined the time course and dose dependence of PI3-kinase inhibition on 2-DOG uptake in cells expressing CA-cdc42 as well as constitutively active PI3-kinase (p110-CAAX). As seen in Fig. 3*B*, 48 h after expression of CA-cdc42, cells were treated with 100 or 300 nM wortmannin for 1–4 h, and 2-DOG uptake was measured. The CA-cdc42-induced increase in glucose transport was maximally inhibited at 300 nM at the first time point, and at the lower dose (100 nM) transport was inhibited in a gradual, time-dependent manner reaching maximal inhibition by 4 h. Constitutively active PI3-kinase (p110-CAAX) expression is known to stimulate 2-DOG uptake in 3T3-L1 cells, and the same inhibitory experiments were performed in p110-CAAX-expressing cells with essentially identical results (Fig. 3*C*). These data indicate that in cells manifesting chronic PI3-kinase activation, there is a time- and dose-dependent effect of these inhibitors to suppress glucose transport. This is in contrast to acute effects of insulin to stimulate transport, which can be inhibited rapidly at a low or high dose of PI3-kinase inhibitors.

To exclude the possibility that the decrease in CA-cdc42- or CA-G_q-induced 2-DOG uptake after 4 h of treatment with the PI3-kinase inhibitors might be because of the toxicity or the nonspecific effects on general trafficking systems, 2-DOG uptake stimulated by osmotic shock was measured after 4 h of treatment with the PI3-kinase inhibitors. Osmotic shock stimulates glucose transport predominantly through a PI3-kinase-independent mechanism, and as seen in Fig. 3*D*, pretreatment with 100 nM wortmannin or 100 μM LY294002 for 4 h did not inhibit osmotic shock-induced glucose transport.

Insulin and Constitutively Active Gα_q Stimulate Cdc42 Activity—Because our data indicate that glucose uptake induced by CA-cdc42 and CA-G_q involves common mechanisms, we further examined the relationship between cdc42 and G$\alpha_{q/11}$ before and after insulin stimulation. We measured the time course of insulin-induced cdc42 activation using GST-PAK1 as a substrate, which specifically binds to active cdc42 (24). As shown in Fig. 4*A*, insulin rapidly stimulated cdc42 activity with a maximal response at 1 min, returning to basal levels thereafter. We showed recently (6) that insulin treatment led to tyrosine phosphorylation of G$\alpha_{q/11}$, and this could activate G$\alpha_{q/11}$ as a positive signaling molecule. Interestingly, the time course of insulin-stimulated tyrosine phosphorylation of G$\alpha_{q/11}$ was comparable with the time course of cdc42 activation (Fig. 4*A*). Cdc42 activity was also measured in 3T3-L1 adipocytes after adenovirus expression of wild type or constitutively active G_q, before and after insulin stimulation for 1 min (Fig. 4*B*). Expression of CA-G_q stimulated cdc42 activity in the basal state and enhanced the effect of insulin compared with control cells. These data show that cdc42 is activated by insulin and G$\alpha_{q/11}$, further suggesting the concept that cdc42 is downstream of G$\alpha_{q/11}$ in the insulin signaling cascade. We also assessed the activity of DN-cdc42 by using this method. As can be seen in Fig. 4*C*, expression of CA-cdc42 resulted in increased cdc42 activity in the absence of insulin, whereas expression of DN-cdc42 at 80 m.o.i. inhibited the effect of insulin to activate cdc42.

Cdc42 Stimulates PI3-kinase Activity—Since it has been shown in other systems that cdc42 can bind to the p85 regulatory subunit of PI3-kinase and stimulate PI3-kinase activity (25, 26), we examined the relationship between cdc42 and PI3-kinase in 3T3-L1 adipocytes. Fig. 5*A* shows the co-precipitation of p85 in anti-cdc42 immunoprecipitates after insulin stimulation. Association of p85 with cdc42 was barely detected

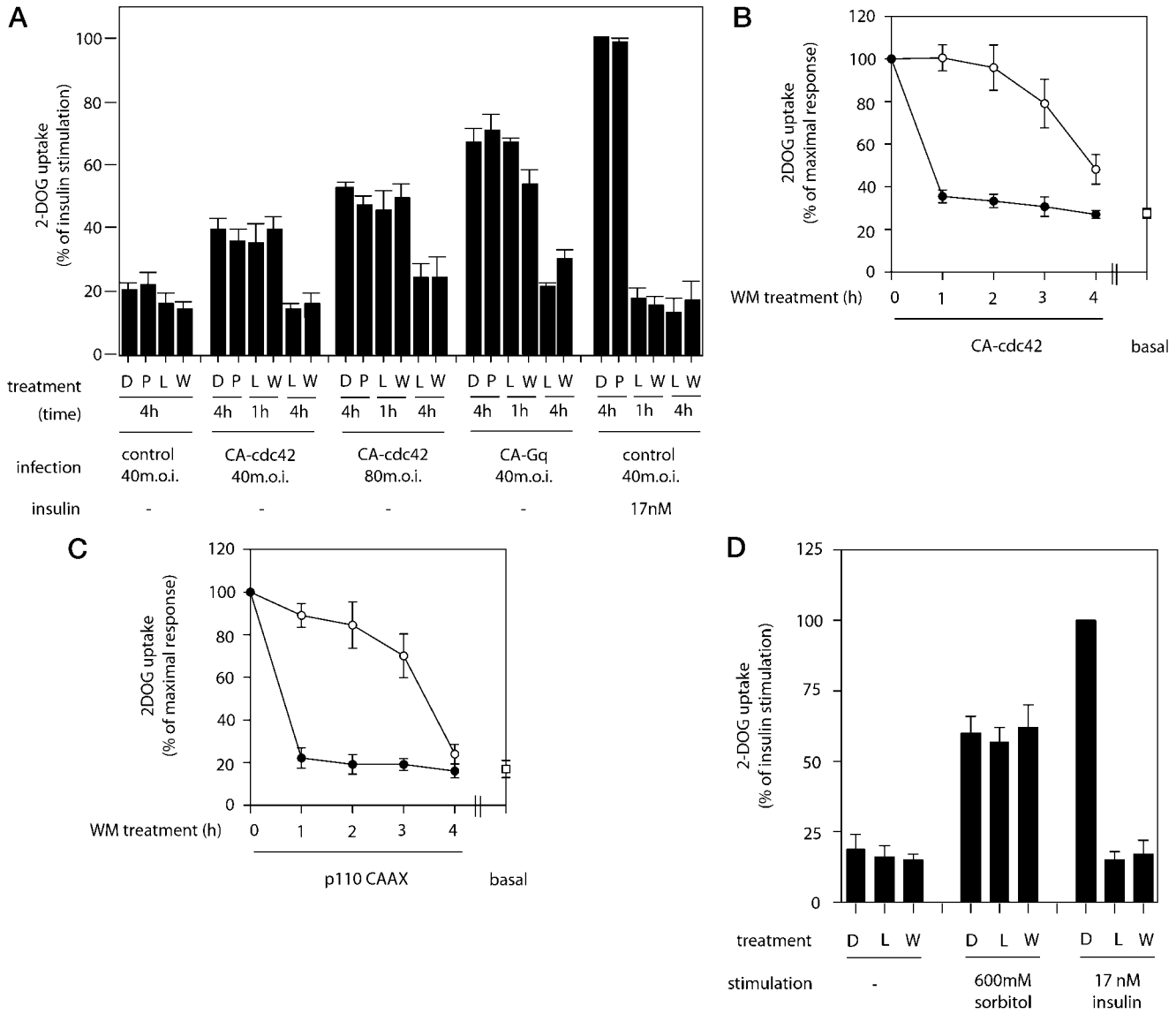

FIG. 3. **Effects of PI3-kinase inhibitors on constitutively active cdc42-, constitutively active G_q-, insulin-, or osmotic shock-induced 2-DOG uptake into 3T3-L1 adipocytes.** *A*, 3T3-L1 adipocytes were infected with adenoviruses expressing constitutively active cdc42 (*CA-cdc42*) or constitutively active G_q (*CA-Gq*) or control GFP (*control*) with indicated m.o.i. After 48 h of infection, cells were serum-starved and incubated with 100 μM LY294002 (*L*), 100 nM wortmannin (*W*), 50 μM PD98059 (*P*), or 0.1% DMSO (*D*) for 1 or 4 h. Some cells were stimulated by 17 nM insulin for 30 min (insulin). 2-[^{3}H]Deoxyglucose uptake was measured as described under "Experimental Procedures." *B* and *C*, 3T3-L1 adipocytes were infected with adenoviruses expressing CA-cdc42 (*B*) or constitutively active PI3-kinase (*p110-CAAX*) (*C*). After 48 h of infection, cells were serum-starved and incubated with 100 (*open circle*) or 300 nM wortmannin (*WM*) (*closed circle*) for the indicated times. 2-[^{3}H]Deoxyglucose uptake was measured as described under "Experimental Procedures." *Open square* indicates 2-[^{3}H]deoxyglucose uptake in basal without virus infection. *D*, 3T3-L1 adipocytes were serum-starved and incubated with 100 μM LY294002 (*L*), 100 nM wortmannin (*W*), or 0.1% DMSO (*D*) for 4 h. They were incubated with 600 mM sorbitol or 17 nM insulin for 30 min, and 2-[^{3}H]deoxyglucose uptake was measured as described under "Experimental Procedures." The data are the mean ± S.E. from four (*A*) or three (*B–D*) independent experiments.

in the basal state and was increased by insulin stimulation, with a maximal association at 10 min. Next, we directly measured PI3-kinase activity associated with cdc42 (Fig. 5, *B* and *C*). Insulin increased the PI3-kinase activity in anti-cdc42 immunoprecipitates by 2.8-fold with a maximal response by 10 min. Expression of CA-cdc42 and CA-G_q (40 m.o.i.) stimulated PI3-kinase activity in the absence of insulin by 4.4- and 4.2-fold, respectively. Incubation of the cells with 100 nM wortmannin for 4 h inhibited PI3-kinase activity stimulated by either CA-G_q, CA-cdc42, or insulin (Fig. 5*B*), while 1 h of treatment with wortmannin inhibited only insulin stimulation (data not shown). Comparable results were obtained when 100 μM LY294002 was used (Fig. 5*C*).

As with the glucose transport data presented in Fig. 3, inhibition of cdc42-associated PI3-kinase activity was not complete until 4 h after treatment with 100 nM wortmannin or 100 μM LY294002. To study the dose response and time course of this inhibition, CA-cdc42 and p110-CAAX-expressing cells were treated with wortmannin at 100 or 300 nM for up to 4 h, followed by measurement of cdc42-associated PI3-kinase activity. As seen in Fig. 5, *D* and *E*, 300 nM wortmannin led to maximal inhibition at the first time point studied, while the inhibition was more gradual, reaching the maximal effect at 4 h at the low dose (100 nM) of wortmannin. These results are completely consistent with the dose response and time course of 2-DOG inhibition shown in Fig. 3.

Constitutively Active Cdc42 Stimulates GLUT4 Translocation and 2-DOG Uptake in a PKCλ-dependent Manner—It has been reported (27, 28) that PKCλ is a component of the insulin signaling cascade and plays an important role in insulin-in-

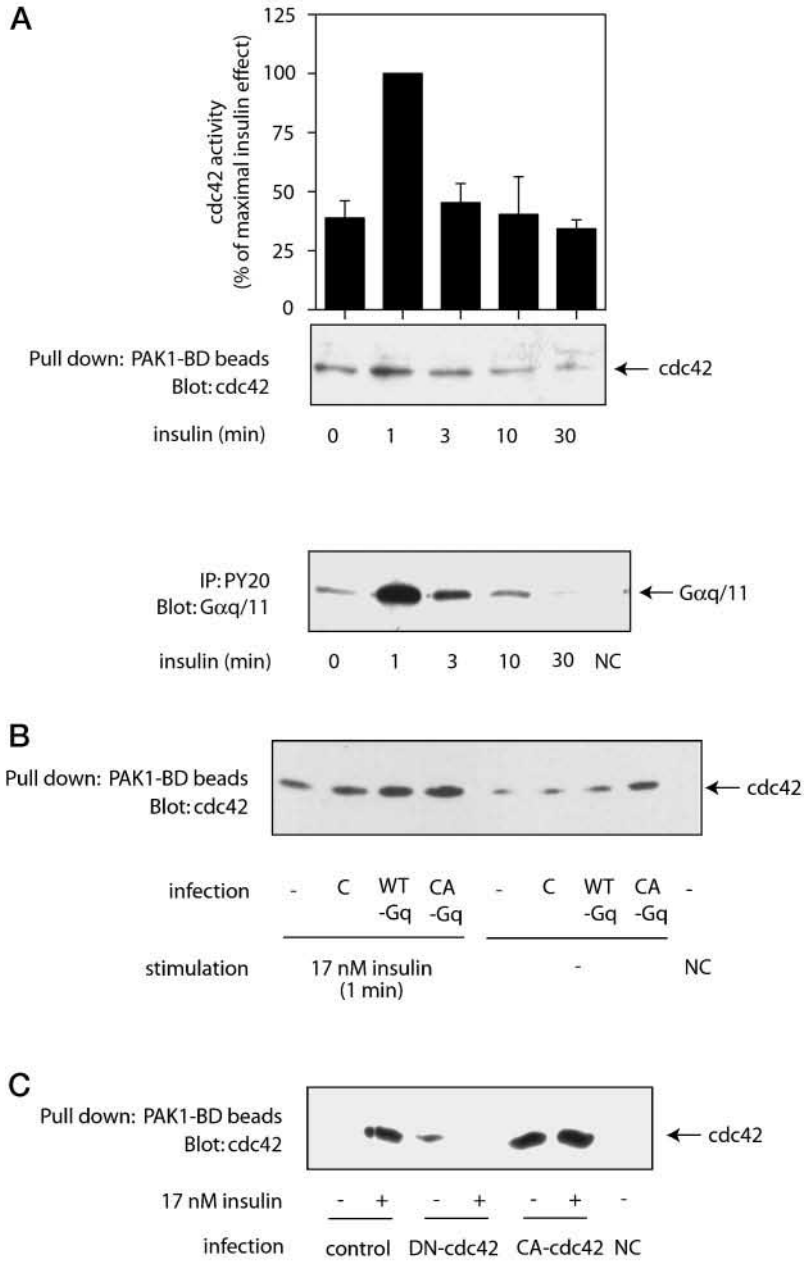

FIG. 4. **Insulin- or constitutively active G_q-induced cdc42 activity.** *A*, 3T3-L1 adipocytes were serum-starved for 16 h and stimulated with 17 nM insulin for the indicated times. Cdc42 activities were measured by mixing the cell lysates with GST-PAK1 beads that specifically recognize active cdc42. Samples were analyzed by Western blotting using anti-cdc42 antibody. The activity was quantitated by scanning the film using NIH Image. Tyrosine-phosphorylated $G\alpha_{q/11}$ was detected by immunoprecipitating (*IP*) with or without (negative control (*NC*)) anti-phosphotyrosine antibody (PY20) and Western blotting using anti-$G\alpha_{q/11}$ antibody as described under "Experimental Procedures." Representative blots are shown from five (cdc42) or three ($G\alpha_{q/11}$) independent experiments. *B*, 3T3-L1 adipocytes were infected with adenoviruses expressing wild type G_q (*WT-Gq*), constitutively active G_q (*CA-Gq*), or control GFP (*C*). After 48 h of infection, 3T3-L1 adipocytes were serum-starved for 16 h, stimulated with or without insulin for 1 min, and lysed. Cdc42 activity was analyzed as described above. These experiments were repeated twice. *C*, 3T3-L1 adipocytes were infected with adenoviruses expressing dominant negative cdc42 (*DN-cdc42*), constitutively active cdc42 (*CA-cdc42*), or control GFP (*control*). After 48 h of infection, 3T3-L1 adipocytes were serum-starved for 16 h, stimulated with or without insulin for 1 min, and lysed. Cdc42 activity was analyzed as described above. A sample for a negative control (*NC*) was prepared as described under "Experimental Procedures." Representative blot is shown from three independent experiments.

duced GLUT4 translocation downstream of PI3-kinase. Since we recently showed that PKCλ was also required for CA-G_q-induced GLUT4 translocation (6), we determined whether PKCλ was a participant in the cdc42 pathway leading to GLUT4 translocation and glucose uptake. First, we measured the effect of anti-PKCλ antibody microinjection on GLUT4 translocation stimulated by insulin, CA-cdc42, or CA-G_q. Anti-PKCλ antibody injection completely blocked CA-cdc42-stimulated GLUT4 translocation, similar to the results with insulin stimulation or CA-G_q expression. Adenovirus-mediated expression of constitutively active PKCλ stimulated GLUT4 translocation to the same extent as insulin, further arguing that PKCλ is a participant in the insulin signaling pathway leading to GLUT4 translocation (Fig. 6*A*). Next, we examined the effect of a kinase-deficient mutant of PKCλ (K273E) on insulin- or CA-cdc42-induced 2-DOG uptake (Fig. 6*B*). Adenoviral gene transfer of this mutant PKCλ resulted in a dose-dependent inhibition of 2-DOG uptake stimulated by either 17 nM insulin or CA-cdc42 expression. Taken together, these results further argue that both $G\alpha_{q/11}$ and cdc42 mediate insulin-stimulated GLUT4 translocation and glucose transport by a common signaling pathway in which $G\alpha_{q/11}$ lies upstream of cdc42 and that both are upstream of PKCλ.

DISCUSSION

One of the major actions of insulin is to stimulate GLUT4 translocation in order to increase glucose uptake into target cells. This is accomplished by a complicated, multistep signaling pathway, which remains incompletely understood. In the current study, we have shown an important role for cdc42, a Rho GTPase family member, in this process. We show that insulin stimulates cdc42 activity and that interfering with cdc42 function by microinjection of anti-cdc42 antibody or siRNA into 3T3-L1 adipocytes inhibits insulin-stimulated GLUT4 translocation. Furthermore, we show that cdc42 is downstream of another insulin-stimulated activator of glucose transport, $G\alpha_{q/11}$, and that the effects of cdc42 to facilitate GLUT4 translocation are mediated through PI3-kinase. Taken together, these results describe a novel role for cdc42 as a

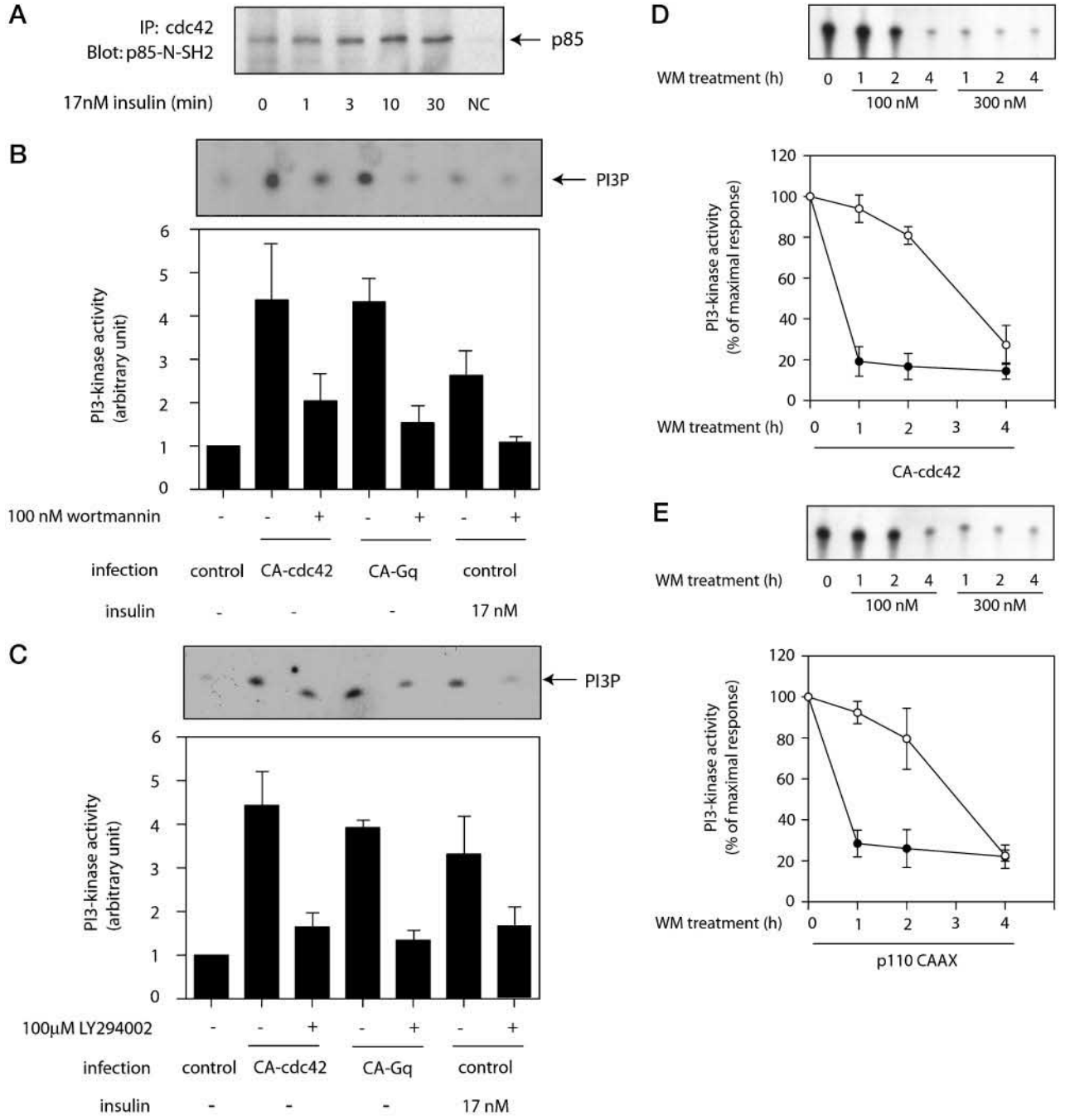

FIG. 5. **Association of p85 and PI3-kinase activity in immunoprecipitates with cdc42 antibody.** *A*, 3T3-L1 adipocytes were serum-starved for 16 h, stimulated with 17 nM insulin for the indicated times, and immunoprecipitated (*IP*) with or without (negative control (*NC*)) anti-cdc42 antibody. Immunoprecipitates were analyzed by Western blotting using anti-p85 antibody as described under "Experimental Procedures." A representative blot is shown from three independent experiments. *B* and *C*, 3T3-L1 adipocytes were infected with adenoviruses expressing constitutively active cdc42 (*CA-cdc42*), constitutively active G_q (*CA-Gq*), or control GFP (*control*). After 48 h of infection, 3T3-L1 adipocytes were serum-starved for 16 h and incubated with wortmannin (*B*) or LY294002 (*C*) for 4 h. PI3-kinase activity in immunoprecipitates with cdc42 antibody was measured as described under "Experimental Procedures." A representative film is shown, and the *bar graph* shows the mean ± S.E. from four independent experiments. *PI3P*, phosphatidylinositol 3-phosphate. *D* and *E*, 3T3-L1 adipocytes were infected with adenoviruses expressing CA-cdc42 (*D*) or constitutively active PI3-kinase (p110-CAAX) (*E*). After 48 h of infection, cells were serum-starved for 16 h and incubated with 100 (*open circle*) or 300 nM wortmannin (*closed circle*) for 1, 2, or 4 h. PI3-kinase activity in immunoprecipitates using cdc42 antibody (*D*) or p110α antibody (*E*) was measured as described under "Experimental Procedures." A representative film is shown, and the *graph* represents the mean ± S.E. of four independent experiments.

signaling molecule within the insulin action cascade ultimately mediating GLUT4 translocation.

TC10 is a small Rho family GTPase that participates in insulin stimulation of glucose transport and GLUT4 translocation (17). It has been suggested that TC10 helps mediate cortical actin polymerization, possibly through interaction with neural Wiskott-Aldrich syndrome protein (18). Cdc42 is also a member of the Rho GTPase family, with a high degree of homology (69%) to TC10 (17), and a role for cdc42 in mediating actin rearrangement has been suggested (19). With respect to bradykinin-stimulated actin remodeling, data exist placing cdc42 downstream of $G\alpha_{q/11}$ in this process (21). Because bradykinin can also stimulate glucose transport under certain conditions (20), and because we have previously demonstrated

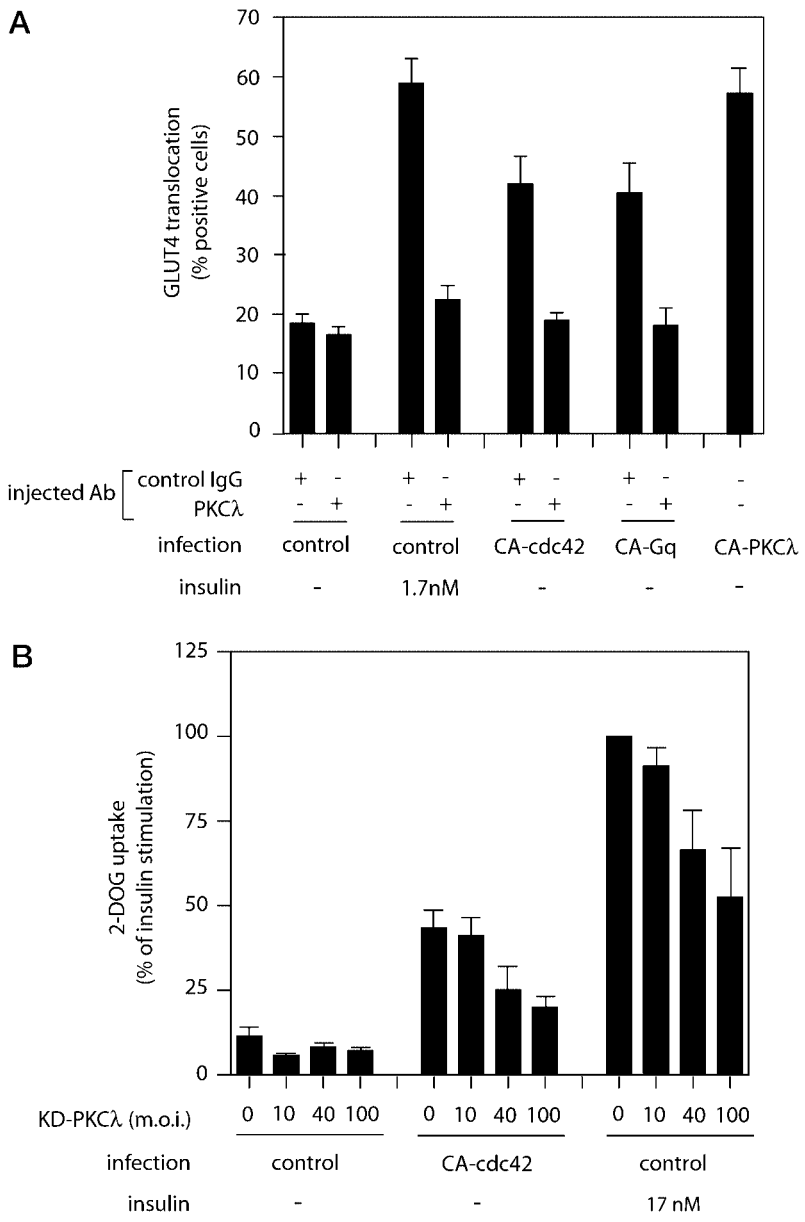

FIG. 6. **Effects of microinjection of anti-PKCλ antibody on CA-cdc42-induced GLUT4 translocation and effects of overexpression of kinase-deficient PKCλ on CA-cdc42-induced glucose uptake in 3T3-L1 adipocytes.** *A*, 3T3-L1 adipocytes were infected with adenoviruses expressing constitutively active cdc42 (*CA-cdc42*), constitutively active G_q (*CA-Gq*), constitutively active PKCλ (*CA-PKC*λ), or control GFP (*control*). After 48 h of infection, anti-cdc42 antibody (*Ab*) (+) or sheep IgG (−) was microinjected into the cells on coverslips. Cells were serum-starved for 4 h and incubated with 1.7 nM insulin for 20 min. GLUT4 in the cells was stained as described under "Experimental Procedures." The percentage of cells positive for GLUT4 translocation was calculated by counting at least 100 cells at each point. The data are the mean ± S.E. from three independent experiments. *B*, 3T3-L1 adipocytes were infected with adenovirus expressing kinase-deficient PKCλ (*KD-PKC*λ) with the indicated m.o.i. Adenoviruses expressing constitutively active cdc42 (*CA-cdc42*) with 40 m.o.i. or control adenovirus (*control*) were also co-infected to adjust the total amount of adenovirus. After 48 h of infection, these cells were serum-starved for 3 h and stimulated by 17 nM insulin for 30 min, and 2-[^{3}H]deoxyglucose uptake was measured as described under "Experimental Procedures." The data are the mean ± S.E. from three independent experiments.

an important role for $G\alpha_{q/11}$ as a mediator of insulin-stimulated GLUT4 translocation, we postulated a role for cdc42 in insulin-stimulated glucose transport. The current studies fully support this idea and also argue strongly that cdc42 lies downstream of $G\alpha_{q/11}$ in this pathway. Thus, insulin stimulates $G\alpha_{q/11}$ as well as cdc42, and both of these events are necessary for full stimulation of GLUT4 translocation. Adenovirus-mediated expression of CA-cdc42 increased GLUT4 translocation and glucose transport independent of insulin, and these effects of CA-cdc42 were attenuated by inhibitors of PI3-kinase. Moreover, microinjection of cdc42 antibody inhibits insulin- and CA-G_q-stimulated GLUT4 translocation, and CA-G_q expression stimulates cdc42 activity. Furthermore, microinjection of $G\alpha_{q/11}$ antibody inhibits insulin- but not CA-cdc42-stimulated GLUT4 translocation. These results place cdc42 downstream of $G\alpha_{q/11}$ in this insulin stimulatory cascade. Finally, insulin stimulates PKCλ activity in a PI3-kinase-dependent manner (6, 27, 28), and microinjection of anti-PKCλ antibody blocks insulin, CA-G_q-, and CA-cdc42-stimulated GLUT4 translocation, indicating that all of these molecules participate in a common signaling pathway. These results do not argue in any way against a role for TC10 as a mediator of GLUT4 translocation, and it is quite possible that both of these small Rho family GTPase proteins participate and may comprise parallel or redundant steps to mediate this important biologic effect of insulin.

It should be noted that some recent studies have shown that transfection of dominant negative cdc42 (DN-cdc42) does not affect insulin-induced glucose transport into 3T3-L1 adipocytes (17), and, at first approximation, this is not consistent with our above described results. However, in our own study, we also find that expression of DN-cdc42 did not inhibit insulin-stimulated glucose transport, and this result may have to do with the specific nature of this dominant negative construct. The DN-cdc42 used in the current studies, as well as in the previous reports (17), contains a point mutation impairing its ability to phosphorylate PAK1, one of the target molecules of cdc42. Indeed, we demonstrate that DN-cdc42 inhibits insulin-stimulated cdc42 activity toward PAK1. However, cdc42 may have multiple target molecules (19), and it is unknown whether this mutant has a dominant negative effect on actions of cdc42 other than PAK1 phosphorylation. Thus, DN-cdc42 inhibits the ability of endogenous cdc42 to bind to GST-PAK1 beads but does not inhibit insulin-stimulated glucose transport or insulin-stimulated cdc42-associated PI3-kinase activity (data not

shown). These results raise the possibility that cdc42 may stimulate PI3-kinase and glucose uptake through a mechanism independent from its ability to interact with PAK1, and this could provide an explanation for why DN-cdc42 did not inhibit insulin-induced glucose transport in the studies by Chiang *et al.* (17) or in our own experiments.

Based on numerous studies (29–32), it is quite clear that activation of PI3-kinase is a necessary step for stimulation of GLUT4 translocation and glucose transport. However, the connection between the activated insulin receptor and PI3-kinase stimulation is somewhat more involved. IRS-1 is a major substrate of the insulin receptor, and tyrosine-phosphorylated IRS-1 can bind to the Src homology 2 domain of the p85 regulatory subunit of PI3-kinase, resulting in PI3-kinase enzymatic activation (3). However, a number of studies, using different approaches, have indicated that IRS-1 is not strictly essential for glucose transport stimulation and that other signaling pathways may be involved (7–12). For example, inhibition of IRS-1 activity in 3T3-L1 adipocytes does not impair insulin-stimulated glucose transport (9), and IRS-1 knockout animals are only mildly insulin-resistant, and adipocytes from these animals show only a partial defect in insulin-stimulated glucose transport (10, 11). Additionally, when IRS-1 is activated independently of insulin, glucose transport is not stimulated (12). Furthermore, we have shown previously (6) that insulin can cause tyrosine phosphorylation of $G\alpha_{q/11}$ and that microinjection of $G\alpha_{q/11}$ inhibitory reagents into 3T3-L1 adipocytes blocks insulin-stimulated glucose transport. We also found that constitutively active $G\alpha_q$ stimulates glucose transport by itself in a PI3-kinase-dependent manner (6). Additionally, other ligands such as bradykinin and ET-1, which receptors couple into $G\alpha_{q/11}$, can also stimulate GLUT4 translocation (33). Some of these studies have shown that the effects of $G\alpha_{q/11}$ are PI3-kinase-dependent (6, 33), whereas others (13–15) have been unable to demonstrate this linkage. In the current study, we find that adenovirus-mediated expression of CA-G_q leads to activation of cdc42 and that CA-G_q as well as CA-cdc42 can stimulate glucose transport. These stimulatory effects are inhibited by 1 h of treatment with 300 nM wortmannin, whereas 100 nM wortmannin took 4 h to reach full inhibitory effect. Because these constitutively active proteins were expressed in cells through adenovirus-mediated gene transfer, and the assays were performed 48 h after infection, CA-G_q and CA-cdc42 had considerable time to exert their effects. Because both wortmannin and LY294002 inhibit ATP binding to PI3-kinase (34), in the case of constitutively active preactivation, the ATP-binding site of PI3-kinase is already occupied by endogenous ATP before the treatment with the inhibitors. It is possible that higher concentrations of inhibitors or longer term treatments are necessary to replace ATP under these conditions. The dose response and time course studies for inhibition of 2-DOG and PI3-kinase activity are fully consistent with this idea (Fig. 3, *B* and *C*, and Fig. 5, *D* and *E*). This may explain why some of the earlier studies were unable to show PI3-kinase dependence of $G\alpha_{q/11}$ effects. Another possibility is that insulin and cdc42 could utilize somewhat different PI3-kinase isoforms to mediate their effects and that different isoforms of PI3-kinase could have different sensitivity to inhibition by wortmannin. In any event, under the current experimental conditions, the effects of CA-cdc42 as well as CA-G_q are clearly inhibited by wortmannin and LY294002.

Supporting this line of reasoning, and consistent with our current results, it has already been reported that some of the biologic actions of cdc42 are mediated through activation of PI3-kinase. For example, cdc42, as well as Rac1, modifies actin organization leading to increased motility and invasiveness in epithelial cells (35). The activation of PAK1 and c-Jun NH_2-terminal kinase, both of which are known target molecules of cdc42, is not required for these functions, but PI3-kinase activation is necessary (35). It has also been suggested that the Bcr homology domain of p85 directly interacts with cdc42 and Rac1 (26, 36), and it is quite possible that the biological actions of cdc42, which require PI3-kinase activation, are mediated through the interaction of cdc42 with the Bcr homology domain of p85.

In summary, we have demonstrated an important role for cdc42 as a novel signaling molecule in the insulin action pathway leading to GLUT4 translocation and stimulation of glucose transport. We find that cdc42 is downstream of $G\alpha_{q/11}$ in this signaling system and lies upstream of PI3-kinase and PKCλ. Our experiments show that the various components of this pathway are necessary for full and efficient insulin stimulation of glucose transport in 3T3-L1 adipocytes. Because stimulation of glucose transport is a major action of insulin, and because insulin resistance to glucose transport stimulation is a central component of a number of disease states such as Type II diabetes, obesity, etc., it is possible that functional abnormalities in this pathway may participate in the mechanisms of insulin resistance in the pathophysiologic conditions in man.

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