During differentiation, expression of protein phosphatase-2Cα (PP2Cα) is increased in 3T3-L1 adipocytes. To elucidate the role of PP2Cα in insulin signaling, we overexpressed wild-type (WT) PP2Cα by adeno-virus-mediated gene transfer in 3T3-L1 adipocytes. Overexpression of PP2Cα-WT enhanced the insulin sensitivity of glucose uptake without any changes in the early steps of insulin signaling. Infection with adenovirus 5 expressing PP2Cα-WT increased phosphatidylinositol 3-kinase (PI3K) activities in the immunoprecipitate using antibody against the p85 or p110 subunit under both basal and insulin-stimulated conditions, followed by activation of downstream steps in the PI3K pathway, such as phosphorylation of Akt, glycogen synthase kinase-3, and atypical protein kinase C. In contrast, overexpression of the phosphatase-defective mutant PP2Cα(R174G) did not produce such effects. Furthermore, overexpression of PP2Cα-WT (but not PP2Cα(R174G)) decreased the 32P-labeled phosphorylation state as well as the gel mobility shift of the p85 subunit, suggesting that dephosphorylation of the p85 subunit by PP2Cα activation might stimulate PI3K catalytic activity. Moreover, knockdown of PP2Cα by transfection of small interfering RNA led to a significant decrease in Akt phosphorylation. In addition, microinjection of anti-PP2Cα antibody or PP2Cα small interfering RNA led to decreased insulin-stimulated GLUT4 translocation. In conclusion, PP2Cα is a new positive regulator of insulin sensitivity that acts through a direct activation of PI3K in 3T3-L1 adipocytes.

Phosphorylation state is regulated by both protein kinase and protein phosphatase and is critical for the regulation of cellular functions such as cell growth, differentiation, and metabolism (1, 2). The protein phosphatases of eukaryotic cells are structurally and functionally diverse enzymes that can be divided into two distinct families (serine/threonine phosphatases and protein-tyrosine phosphatases) based on their specificities for phosphoamino acids (3, 4). Serine/threonine phosphatases are further classified into four major groups (protein phosphatase (PP)-1, PP2A, PP2B, and PP2Cα) depending on their substrate specificities, bivalent cation dependences, and sensitivities to various inhibitor molecules such as protein inhibitor-1 and -2 and the tumor promoter okadaic acid (3, 5).

The role of protein-tyrosine phosphatases in insulin signaling has been well characterized in several recent studies (6–11). As does tyrosine phosphorylation, serine/threonine phosphorylation plays important roles in insulin signal transduction. A major role in the negative regulation of insulin action is attributed to agents that enhance serine/threonine phosphorylation of the receptor itself or its downstream effectors such as insulin receptor substrate-1 (IRS-1). Serine/threonine phosphorylation of such molecules induces insulin resistance. Furthermore, Akt and atypical protein kinase Cα (PKCa) are serine/threonine kinases, and their kinase activities are regulated through the serine/threonine phosphorylation states. These lines of evidence suggest that serine/threonine phosphatases have important roles in insulin signal transduction. However, their roles in insulin signaling are not completely understood (12, 13).

As differentiation proceeds, adipocytes become sensitive to insulin stimulation in terms of glucose uptake. As a proposed molecular mechanism for acquired insulin sensitivity during adipocyte differentiation, the expression levels of important molecules in insulin signaling such as the insulin receptor and IRS-1 have been reported to be up-regulated (14). Furthermore, the level of glucose transporter-4 (GLUT4) is also increased during adipocyte differentiation (15). In this study, we found that the amount of PP2A was decreased during differentiation in 3T3-L1 adipocytes. PP2A is reported to be a negative regulator of insulin signaling in terms of mitogenic signals (12). Thus, the decreased expression of PP2A may be responsible for acquired insulin sensitivity in 3T3-L1 adipocytes. In contrast, we observed that, during differentiation, PP2Cα protein expression was increased. Thus, this raises the interesting question of whether up-regulation of PP2Cα expression may be responsible for acquired insulin sensitivity by modulating ins-

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sulin signaling in 3T3-L1 adipocytes. Thus, we clarified the role of PP2Ca in the acquisition of additional insulin sensitivity in differentiated adipocytes by three distinct approaches: overexpression of PP2Ca by adenovirus-mediated gene transfer, knockdown of PP2Ca by transfection or microinjection of small interfering RNA (siRNA), and inhibition of PP2Ca function by microinjection of anti-PP2Ca antibody.

In this study, we found that overexpression of PP2Ca enhanced the insulin sensitivity of glucose uptake. Furthermore, microinjection of PP2Ca siRNA or anti-PP2Ca antibody led to decreased insulin-stimulated GLUT4 translocation. Decreased insulin sensitivity and decreased insulin responsiveness are two of the major characteristics of insulin resistance states. However, the molecular mechanism for moderation of insulin sensitivity is still unknown. Thus, we examined which step of insulin signaling is modulated by PP2Ca overexpression, and we found that PP2Ca overexpression directly activated phosophatidylinositol 3-kinase (PI3K), leading to sequential activation of Akt, glycogen synthase kinase-3 (GSK-3), and atypical protein kinase C (PKC). The resulting recombinant virus—adenovirus containing 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% FCS—was replication-defective (at least in cells lacking the E1 region of the adenovirus), but fully infectious. Ad5-lacZ and Ad5-p110-CAAX (where “A” is alphabetic amino acid) were described previously (21).

Generation of His-tagged Wild-type and Mutant p85 Subunits of PI3K—Full-length bovine p85 was provided by Dr. Masato Kasuga (Kobe University, Kobe, Japan) (22). A QuickChange kit was used for the construction of the chimeric construct. The chimeric construct was cloned into a plasmid vector and transformed into DH10B (Invitrogen). The resulting recombinant plasmids were ligated into pGEX-2T containing 3-5′ phosphorylation of PP2Cα in 3T3-L1 Adipocytes

EXPERIMENTAL PROCEDURES

Materials—Human insulin was provided by Lilly. Anti-phospho-Akt Thr308 antibody, anti-phospho-Akt Ser473 antibody, anti-phospho-PKA-Thr163 antibody, and anti-phospho-PKCα-Thr63 antibody were from New England Biolabs Inc. (Beverly, MA). Anti-p110α antibody, anti-Akt antibody, horseradish peroxidase-linked anti-rabbit and anti-mouse antibodies, protein A-Sepharose, and protein G-Sepharose were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PP2A antibody, anti-PP2B antibody, anti-PP2Ca antibody, anti-PP2C antibody, anti-IRS-1 antibody, anti-phosphoserine antibody 4G10, anti-IRS-3 antibody, and anti-PP2C p85 N-terminal SH2 domain antibody were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-hemagglutinin (HA) antibody was from Roche Applied Science. Anti-phosphotyrosine antibody RC-20 and anti-PKCα antibody were from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-linked anti-sheep antibody and polyvinylidene difluoride membrane were obtained from Millipore Corp. (Bedford, MA). Anti-His antibody, Dulbecco’s modified Eagle’s medium (DMEM), and fetal calf serum (FCS) were obtained from Invitrogen. XAR-5 film was obtained from Eastman Kodak Co. All other reagents and chemicals were from standard suppliers.

Cell Culture—3T3-L1 fibroblasts (American Type Culture Collection, Manassas, VA) were grown and maintained in high glucose DMEM containing 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% FCS in a 10% CO2 environment. The cells were allowed to grow until 2 days post-confluence and then were differentiated as described previously (18). The adipocytes were trypsinized and seeded in the appropriate culture dishes. The transfected adipocytes were stained with an adipocyte phenotype for at least 10 days after replating as evaluated by noting the extent of lipid droplets by oil red O staining (data not shown). Cells of the adenovirus E1A-transformed human embryonic kidney line 293 cells were cultured in high glucose DMEM containing 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% FCS in a 10% CO2 environment. The cells were allowed to grow until 2 days post-confluence and then were differentiated as described previously (16). The adipocytes were trypsinized and seeded in the appropriate culture dishes. The transgenic adipocytes retained an adipocyte phenotype for at least 10 days after replating as evaluated by noting the extent of lipid droplets by oil red O staining (data not shown).

Generation of Mutant PP2Ca and Preparation of Recombinant Adenovirus—The recombinant vector containing HA-tagged mouse PP2Ca cDNA (18) as described previously was used for directed mutagenesis of the E1A gene. An oligonucleotide in which an Ar (T)17 amino acid mouse PP2Ca was replaced with Gly was used as the primer in the in vitro mutagenesis reaction. Wild-type (WT) and phosphatase-defective (R174G) were subcloned into pACCMVpLpASR(+)/pλ plasmid (11). This plasmid contains 13 map units of the adenovirus 5 (Ad5) left end, cytomegalovirus early promoter, pUC19 polylinker site, and SV40 poly(A) sequences, followed by map units 9–18 of the Ad5 genome. The resulting recombinant plasmids were then cotransfected with the pJM17 plasmid (19) into packaging 293 cells, which carry Ad5 genomic DNA, and propagated as described previously (20). Mature recombinant Ad5 adenoviruses encoding PP2Ca-WT and PP2Ca(R174G) were thus generated after in vivo homologous recombination between these two plasmids. Because 293 cells were originally derived from adenovirus transformation, the missing E1 gene function of pJM17 was provided in trans. The resulting recombinant viruses containing PP2Ca-WT were designated Ad5-PP2Ca-WT and Ad5-PP2Ca(R174G), and replication-defective (in at least in cells lacking the E1 region of the adenovirus) were designated Ad5-PP2Ca for microinjection into 3T3-L1 adipocytes. The cells were transfected with 2 μg of each expression plasmid by the LipofectAMINE method. Cells cultured for 48 h were used in the experiments.

Microinjection of Antibodies and siRNAs—Microinjection was carried out using a semiautomated 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) and 100 mM KCl and were injected into the cytoplasm. Sheep IgG (5 mg/ml) was injected into the control cells. siRNA was dissolved at 5 μM in microinjection buffer (23) and injected into the cytoplasm of 3T3-L1 adipocytes.

Northern Blotting—The cells were starved for 16 h in regular glucose DMEM containing 0.2% bovine serum albumin; stimulated with 0–100 nM insulin for 5–10 min at 37 °C; and then lysed in solubilization buffer containing 20 μM Tris (pH 7.5), 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40, 50 units/ml aprotinin, 1 mM NaN3, 1 mM phenylmethylsulfonyl fluoride, and 50 mM NaF for 30 min at 4 °C. The cell lysates were centrifuged to remove insoluble materials. Whole cell lysates (20 μg of protein/lane) were denatured by boiling in Laemmli sample buffer containing 100 mM dithiothreitol and resolved by SDS-PAGE. Gels were transferred to nitrocellulose by electroblotting in Towbin buffer containing 20% methanol. For immunoblotting, membranes were blocked and probed with specific antibodies. The blots were then incubated with horseradish peroxidase-linked second antibody; followed by chemiluminescence detection using the ECL system (Amersham Biosciences). The Western blot data were quantitated by scanning the film using NIH Image.

Northern Blot Analysis—Total RNA was isolated with TRIzol reagent (Invitrogen), and 20 μg of RNA samples were run on a 1% agarose gel containing formaldehyde and transferred to a nylon membrane (NEN). Northern blot analysis was performed as described previously (18). A QuikClone lambda concatemer was generated by PP2Ca expression vector by enzyme digestion. A DNA probe labeled with 32P-dCTP (PerkinElmer Life Sciences) using a labeling kit (Takara, Shiga, Japan) was hybridized to UV-cross-linked DNA of total RNA, and then washed and exposed to UV films. The bands were replicated onto Hyperfilm MR film at ~80 °C. The signal was quantitated with a densitometer, and loading differences were normalized to the signal generated with a probe for 18 S ribosomal RNA.

3P Labeling of 3T3-L1 Adipocytes—3T3-L1 adipocytes infected with...
FIG. 1. Expression levels of protein phosphatases during 3T3-L1 differentiation. Differentiation of 3T3-L1 fibroblasts started on day 0 and was completed by day 7. Adipocytes were cultured in medium with 10% FCS for another 7 days. On each day indicated, cells were lysed and analyzed by SDS-PAGE, followed by Western blotting with corresponding antibodies. A, data from a representative experiment are shown. The graph shows the mean ± S.E. of three independent experiments, and data are presented as the percentage abundance of PP1, PP2A, PP2B, and PP2Ca on day 14. The content on day 0 was taken as 100%. IB, immunoblot. B, on each day, RNA samples were isolated, and 20 μg of RNA were run on a 1% agarose gel and transferred to a nylon membrane. A PP2Ca probe labeled with [α-32P]dCTP was hybridized to the blots. The signal was quantified with a densitometer. The graph shows the mean ± S.E. of three independent experiments, and data are presented as the -fold increase in mRNA levels compared with that on day 0, when loading differences were normalized to the signal generated with a probe for 18 S ribosomal RNA.

Ad5-ctrl, Ad5-PP2Ca-WT, or Ad5-PP2Ca(R174G) were starved for 16 h in regular glucose DMEM containing 0.2% bovine serum albumin, followed by phosphate starvation for 2 h in phosphate-free DMEM containing 0.2% bovine serum albumin, [32P]Orthophosphate (0.25 mCi/ml; Amersham Biosciences) was then added, and the cells were cultured for an additional 2 h. The cells were lysed and subjected to immunodepletion with protein A-agarose for 2 h at 4 °C. The resulting supernatants were incubated with anti-p85 antibody and protein A-agarose for 16 h at 4 °C. The immunoprecipitates were denatured by boiling and resolved by SDS-PAGE. The gels were dried and exposed to Kodak Biomax MR film. There was no loading difference as determined by Coomassie Brilliant Blue staining (data not shown).

PP2Ca Phosphatase Activity—Phosphatase activity was measured using p-nitrophenyl phosphate as a substrate (24) with a phosphatase assay kit (Upstate Biotechnology, Inc.) as described previously (12). Starved cells were lysed in 50 mM HEPES (pH 8.0) containing 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1.5 mM magnesium chloride, 1% Triton X-100, 1 μg/ml leupeptin, 50 units/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were incubated with anti-PP2Ca or anti-HA antibody and protein A-agarose for 2 h at 4 °C. The immunoprecipitates were washed twice with lysis buffer and once with assay buffer (50 mM Tris (pH 8.0) and 0.1 mM calcium chloride), resuspended in assay buffer containing 2.5 mM nickel chloride and 900 μg/ml p-nitrophenyl phosphate in the absence or presence of 10 mM Mn2⁺ and 60 mM Mg2⁺, and incubated for 30 min at 30 °C because PP2Ca phosphatase activity is known to be dependent on Mn2⁺ and Mg2⁺. The amount of p-nitrophenol produced was determined by measuring the absorbance at 405 nm.

PP2Ca Phosphatase Treatment—The p85 subunit was immunoprecipitated using anti-p85 antibody from lysates of 3T3-L1 adipocytes. The immunoprecipitates were washed twice with lysis buffer and twice with assay buffer, followed by incubation with phosphatase buffer (1 mM EGTA, 0.1% 2-mercaptoethanol, 20 mM imidazole, 1 mg/ml bovine serum albumin, and 20 mM magnesium acetate) with or without 0.4 milliunits of recombinant PP2Ca (Upstate Biotechnology, Inc.) for 30 min at 30 °C. After several washes, samples were denatured by boiling and subjected to Western blotting.

Immunostaining and Immunofluorescence—Staining of LaCZ or immunostaining of GLUT4 was performed as described (21, 25). Immunofluorescence microscopy was performed as described previously (23).

2-Deoxyglucose Uptake—The procedure for evaluating glucose transport was a modification of a method described previously (26). Glucose uptake was determined in triplicate at various time points after the addition of 10 μM of substrate. 2-[3H]Deoxyglucose or L-[3H]glucose (0.1 μCi, 0.01 nm final concentration) was added to provide a concentration at which cell membrane transport is rate-limiting. The value for L-glucose was subtracted to correct each sample for the contributions of diffusion and trapping.

PI3K Assay—Serum-starved cells (16 h for 3T3-L1 adipocytes and 24 h for HIRc B cells) were incubated in the absence (basal conditions) or presence of 100 nM insulin for 5 min; washed once with ice-cold phosphate-buffered saline; lysed; and subjected to immunoprecipitation (500 μg of total protein) with anti-p110β (2 μg), anti-p85 (4 μg), anti-PP2Ca (4 μg), or anti-His (2 μg) antibody for 2 h at 4 °C. Immunocomplexes were precipitated from the supernatant with protein A or G and washed as described (27). The washed immunocomplexes were incubated with phosphatidylinositol (Avanti Polar Lipids) and [γ-32P]ATP (3 mCi/mm) for 10 min at room temperature. Reactions were stopped with 20 μl of 0.1 n HCl and 180 μl of CHCl3/methanol (1:1); the reaction mixtures were centrifuged, and the lower organic phases were removed and applied to a silica gel TLC plate (Merck) that had been coated with 1% potassium oxalate. TLC plates were developed in CHCl3/CH3OH/H2O/NH4OH (60:47:11.3:2), dried, visualized, and quantitated on a PhosphorImager (Amersham Biosciences).

Statistics—The values are expressed as means ± S.E., unless otherwise stated. Scheffe’s multiple comparison test was used to determine the significance of any differences among more than three groups. p < 0.05 was considered significant.

RESULTS

Expression of Protein Phosphatases During Differentiation in 3T3-L1 Adipocytes—Several proteins have been reported to be up-regulated upon 3T3-L1 adipocyte differentiation, including the insulin receptor and IRS-1 (14) and GLUT4 (15), which contribute to the regulation of insulin responsiveness and insulin sensitivity. To compare the relative abundance of protein phosphatases, the expression levels of PP1, PP2A, PP2B, and PP2Ca were measured during differentiation of 3T3-L1 fibroblasts into adipocytes. PP2Ca protein expression increased time dependently during differentiation (days 0–7) and...
reached a level comparable with that in well differentiated adipocytes (days 7–14) (Fig. 1A, upper panel). In contrast, the levels of PP1, PP2A, and PP2B decreased during differentiation. In well differentiated 3T3-L1 adipocytes, the expression levels of PP1, PP2A, and PP2B were only 11.7–32.0% of those in 3T3-L1 fibroblasts (Fig. 1A, lower panel). In contrast, the expression level of PP2C in adipocytes was increased to 230% compared with that in cells infected with Ad5-ctrl. Each Western blot is representative of three independent experiments. IB, immunoblot. C, cell lysates were prepared and assayed for PP2C activity in the absence (−) or presence (+) of Mn2+ and Mg2+ as described under “Experimental Procedures.” Data are presented as the -fold increase in phosphatase activity compared with that in cells infected with Ad5-ctrl obtained using anti-PP2C antibody and represent the mean ± S.E. of five independent experiments. IP, immunoprecipitate.

Expression of PP2Ca in 3T3-L1 Adipocytes—Gene transfer into 3T3-L1 adipocytes by conventional methods is inefficient. To achieve sufficient levels of expression, we used adenovirus-mediated gene transfer in terminally differentiated 3T3-L1 adipocytes. To assess infection efficiency, we performed LacZ staining to examine expression of recombinant adenovirus containing the bacterial β-galactosidase gene (Ad5-lacZ) following infection of 3T3-L1 adipocytes at m.o.i. = 50. The infection efficiency was assessed by determining the percentage of cells expressing Ad5-lacZ. >90% of the 3T3-L1 adipocytes showed positive staining on post-infection day 3 as judged by the number of blue cells/200 cells, whereas control adenovirus (Ad5-ctrl)-infected cells exhibited no blue color in the presence of the β-galactosidase chromogenic substrate (Fig. 2A). We next overexpressed PP2Ca by adenovirus-mediated gene transfer in 3T3-L1 adipocytes. 3T3-L1 adipocytes were infected with Ad5-ctrl or with recombinant adenovirus expressing HA-tagged wild-type PP2Ca (Ad5-PP2Ca-WT) or phosphatase-defective PP2Ca (Ad5-PP2Ca(R174G)). Following a 72-h incubation, the cells were lysed and analyzed by Western blotting with anti-PP2Ca or anti-HA antibody (Fig. 2B).
PP2Ca were observed in 3T3-L1 adipocytes infected with Ad5-ctrl alone. PP2Ca-WT and PP2Ca(R174G) were expressed in a dose-dependent manner (Fig. 2B). Furthermore, a 43-kDa band was observed only in Ad5-PP2Ca-WT- or Ad5-PP2Ca(R174G)-infected cells when immunoblotting was performed using anti-HA antibody (Fig. 2B). A 6-fold increase in PP2Ca protein expression was observed in 3T3-L1 adipocytes infected with Ad5-PP2Ca-WT or Ad5-PP2Ca(R174G) at m.o.i. = 50 compared with that in control cells. To evaluate the effect of PP2Ca overexpression on PP2Ca phosphatase activity, cell lysates from 3T3-L1 adipocytes infected with Ad5-ctrl, Ad5-PP2Ca-WT, or Ad5-PP2Ca(R174G) were immunoprecipitated with anti-PP2Ca antibody, and in vitro phosphatase activity was measured using p-nitrophenyl phosphate as a substrate with or without Mn²⁺ and Mg²⁺ (Fig. 2C). Overexpression of PP2Ca-WT led to increased phosphatase activity in a dose- and Mn²⁺-dependent manner to as much as 5-fold at m.o.i. = 50. These enzymatic activities paralleled the PP2Ca protein expression levels. Expression of PP2Ca(R174G) did not increase phosphatase activity compared with that of PP2Ca-WT (Fig. 2C). We measured PP2Ca phosphatase activity in the immunoprecipitate using anti-HA antibody to avoid contamination of endogenous PP2Ca activity. Overexpression of PP2Ca-WT also led to a dose-dependent increase in phosphatase activity. The cells infected with Ad5-ctrl or Ad5-PP2Ca(R174G) had no phosphatase activity (Fig. 2C). These results indicate that expression of PP2Ca-WT specifically increases PP2Ca enzymatic activity and that PP2Ca(R174G) is a phosphatase-defective mutant.

Effect of PP2Ca Overexpression on Insulin-stimulated Glucose Uptake in 3T3-L1 Adipocytes—To examine whether upregulated PP2Ca activity affects insulin-stimulated glucose uptake, we measured insulin-stimulated 2DOG uptake in 3T3-L1 adipocytes infected with Ad5-PP2Ca-WT or Ad5-PP2Ca(R174G) (Fig. 3A). Insulin stimulated 2DOG uptake in a dose-dependent manner with ED₅₀ = 0.49 ± 0.11 nM in Ad5-ctrl-infected cells. Overexpression of PP2Ca-WT at m.o.i. = 50 increased both the basal and insulin-induced 2DOG uptakes compared with those in control cells (Fig. 3A). Furthermore, overexpression of PP2Ca-WT enhanced the insulin sensitivity of 2DOG uptake in an m.o.i.-dependent manner (Fig. 3, B and C). On the other hand, the basal and insulin-induced 2DOG uptakes as well as the insulin sensitivity of 2DOG uptake were not affected by overexpression of PP2Ca(R174G) (Fig. 3, A–C). We did not observe any change in GLUT4 expression in any of these cells (Fig. 3D).

PP2Ca Directly Activates p85-associated PI3K—To clarify the molecular mechanism for the increased insulin sensitivity of glucose uptake induced by PP2Ca overexpression, we next investigated the effect of PP2Ca overexpression on the early steps of insulin signaling in 3T3-L1 adipocytes. Compared with the control 3T3-L1 adipocytes, overexpression of PP2Ca-WT or PP2Ca(R174G) affected neither the degree of insulin-induced tyrosine phosphorylation of either the insulin receptor β-subunit or IRS-1 nor IRS-1 association with the p85 subunit of PI3K (data not shown). These results indicate that PP2Ca may not affect the early steps of insulin signaling. To further investigate the insulin signaling, we assessed PI3K activity. Consistent with no change in the association of IRS-1 with the p85 subunit of PI3K, PI3K activity in the immunoprecipitate using anti-phosphotyrosine antibody was not affected by infection with either Ad5-PP2Ca-WT or Ad5-PP2Ca(R174G) under the basal and insulin-stimulated conditions (data not shown). We next measured PI3K activity in the immunoprecipitate using antibody against the p85 subunit of PI3K. In cells overexpressing PP2Ca-WT, the PI3K activity in the immunoprecipitate using anti-p85 subunit antibody was unexpectedly increased by 3-fold under the basal conditions (Fig. 4A). Insulin further increased PI3K activity by 4.5-fold (Fig. 4A). The PI3K activity in the immunoprecipitate using an antibody against the p110 subunit of PI3K was also increased by 2- and 3-fold under the basal and insulin-stimulated conditions, respectively (Fig. 4B). However, the increment of PI3K stimulated by insulin was comparable with that in control cells. Preincubation with 1 μM wortmannin (PI3K inhibitor) blocked the PI3K activity associated with the p110 subunit in cells overexpressing PP2Ca-WT (data not shown). On the other hand, PP2Ca(R174G) did not affect PI3K activity (Fig. 4, A and B). Moreover, we observed significant PI3K activity in the immunoprecipitate using anti-PP2Ca antibody (Fig. 4C). These results confirm that PP2Ca directly enhances PI3K activity itself.
Effect of PP2Ca Overexpression on Akt, GSK-3, and PKC\(\alpha\) Phosphorylation in 3T3-L1 Adipocytes—To assess the effect of PP2Ca overexpression on further downstream signaling of the PI3K pathway, 3T3-L1 adipocytes overexpressing either PP2Ca-WT or PP2Ca(R174G) were stimulated with insulin for 10 min, lysed, and analyzed by Western blotting using anti-phospho-Akt, anti-GSK-3, or anti-PKC\(\alpha\) antibody (Fig. 5, A). Overexpression of PP2Ca-WT stimulated Akt phosphorylation at Thr\(^{308}\) and Ser\(^{473}\) in both the absence and presence of insulin. Overexpression of PP2Ca-WT also stimulated GSK-3 and PKC\(\alpha\) phosphorylation (Fig. 5, B and C). On the other hand, PP2Ca(R174G) expression did not affect Akt, GSK-3, or PKC\(\alpha\) phosphorylation (Fig. 5, A–C). Furthermore, we observed the enhanced insulin sensitivity of Akt phosphorylation as well as of insulin-stimulated glucose uptake (Fig. 5D). Taken together, these results suggest that PP2Ca may activate both the PI3K/Akt/GSK-3 and PI3K/PKC\(\alpha\) pathways in 3T3-L1 adipocytes. Furthermore, PI3K activity stimulated by PP2Ca directly enhances its downstream signal, without any interaction with the IRS molecule.

Effect of Knockdown of PP2Ca on Insulin Signaling in 3T3-L1 Adipocytes—We used distinct approaches to clarify the role of endogenous PP2Ca functions in insulin signaling. We transfected siRNA into 3T3-L1 adipocytes to knockdown the PP2Ca mRNA. Forty-eight hours after transfection, the amount of PP2Ca protein was decreased by 37.7 ± 5.4% in PP2Ca siRNA-transfected cells compared with that in control siRNA-transfected cells (p < 0.01), whereas expression of PP2A and PP2B was unaffected (Fig. 6A). Akt phosphorylation in adipocytes transfected with PP2Ca siRNA was significantly inhibited compared with that in cells transfected with control siRNA (Fig. 6B). To demonstrate the role of endogenous PP2Ca in glucose uptake, we next performed a microinjection study with PP2Ca siRNA or anti-PP2Ca antibody, followed by measurement of GLUT4 translocation, which is rate-limiting for glucose uptake. PP2Ca siRNA or anti-PP2Ca antibody was microinjected into the cytoplasm of 3T3-L1 adipocytes; and 48 and 72 h later, GLUT4 translocation was measured. Microinjection of PP2Ca siRNA or anti-PP2Ca antibody decreased insulin-stimulated GLUT4 translocation compared with that in control cells (Fig. 6, C and D). Taken together, these results suggest that PP2Ca plays an important role in insulin signaling in glucose metabolism.

**PP2Ca Affects Glucose Uptake via PI3K in 3T3-L1 Adipocytes**—To confirm that PP2Ca affects glucose uptake via PI3K activation, we used two approaches. First, we assessed whether constitutively active PI3K could compensate for knockdown of PP2Ca. Targeting of the PI3K catalytic subunit to the membrane by the addition of the CAAX signal (p110-CAAX) is known to result in the constitutively active form of PI3K (21). The 3T3-L1 adipocytes were infected with Ad5-ctrl or Ad5-p110-CAAX for 16 h, followed by transfection with control or PP2Ca siRNA. Forty-eight hours after transfection, we measured Akt phosphorylation and 2DOG uptake. In the cells infected with Ad5-p110-CAAX, phosphorylation of Akt was enhanced. However, knockdown of PP2Ca did not affect Akt phosphorylation induced by constitutively active PI3K (Fig. 7A). Concomitantly, 2DOG uptake induced by p110-CAAX was not affected by knockdown of PP2Ca (Fig. 7B). Second, we evaluated whether the PI3K inhibitor wortmannin could block PP2Ca-induced phosphorylation of Akt and 2DOG uptake. As shown in Fig. 7 (C and D), wortmannin inhibited PP2Ca-enhanced phosphorylation of Akt and 2DOG uptake. Taken together, these findings confirm that the ef-
Effects of PP2C/H9251 on glucose uptake are mediated via PI3K activation.

PP2C/H9251 Dephosphorylates the p85 Subunit of PI3K and Activates PI3K Activity—Several studies have shown that phosphorylation of the p85 subunit regulates PI3K activity (22, 28, 29). Dhand et al. (22) have shown that the p110 subunit of PI3K has serine/threonine kinase activities as well as lipid kinase activity and that the p110 subunit phosphorylates Ser608 of the p85 subunit, resulting in decreased PI3K activity in an in vitro experiment. Thus, we hypothesized that PP2C/H9251 might modulate phosphorylation of the p85 subunit and enhance PI3K activity. To test this hypothesis, we assessed whether PP2C could modulate phosphorylation of the p85 subunit by performing an in vivo [32P]orthophosphate labeling study. 3T3-L1 adipocytes were infected with Ad5-ctrl (ctrl), Ad5-PP2Cα-WT (WT), or Ad5-PP2Cα(R174G) (R/G) at 50 m.o.i. for 16 h and grown in medium containing 2% heat-inactivated serum for 56 h. The cells were serum-starved for 16 h and incubated in the absence (−) or presence (+) of 0.1, 1, and 10 nM (D) or 100 nM (A–C) insulin for 10 min. Then, the cells were lysed, and Western blotting was performed with anti-phospho-Akt Ser473 (A and D), anti-phospho-Akt Thr308 (A), anti-phospho-GSK-3 (B), of anti-phospho-PKCα (C) antibody. The membranes were stripped and rebotted with anti-Akt, anti-GSK-3, or anti-PKCα antibody. Data are presented as the percentage of phosphorylation compared with insulin-stimulated Ad5-ctrl-infected cells and represent the mean ± S.E. of three independent experiments. The graph in D shows the mean ± S.E. of the percentage of the maximal effect. *, p < 0.05 compared with the control. IB, immunoblot.

Role of PP2Cα in 3T3-L1 Adipocytes

FIG. 5. Effect of PP2Cα overexpression on Akt, GSK-3, and PKCα phosphorylation in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were infected with Ad5-ctrl (ctrl), Ad5-PP2Cα-WT (WT), or Ad5-PP2Cα(R174G) (R/G) at 50 m.o.i. for 16 h and grown in medium containing 2% heat-inactivated serum for 56 h. The cells were serum-starved for 16 h and incubated in the absence (−) or presence (+) of 0.1, 1, and 10 nM (D) or 100 nM (A–C) insulin for 10 min. Then, the cells were lysed, and Western blotting was performed with anti-phospho-Akt Ser473 (A and D), anti-phospho-Akt Thr308 (A), anti-phospho-GSK-3 (B), of anti-phospho-PKCα (C) antibody. The membranes were stripped and rebotted with anti-Akt, anti-GSK-3, or anti-PKCα antibody. Data are presented as the percentage of phosphorylation compared with insulin-stimulated Ad5-ctrl-infected cells and represent the mean ± S.E. of three independent experiments. The graph in D shows the mean ± S.E. of the percentage of the maximal effect. *, p < 0.05 compared with the control. IB, immunoblot.
FIG. 6. **Effect of PP2Cα knockdown on insulin signaling in 3T3-L1 adipocytes.** 3T3-L1 adipocytes were transfected with control or PP2Cα siRNA using LipofectAMINE 2000, grown in serum-free medium for 48 h, and incubated in the absence (−) or presence (+) of 100 nM insulin for 10 min. Then, the cells were lysed, and Western blotting was performed with anti-PP2A, anti-PP2B, or anti-PP2Cα antibody (A) or anti-phospho-Akt Ser473 antibody (B). The membranes were stripped and reblotted with anti-Akt antibody (B'). Data are presented as percentages compared with insulin-stimulated control cells and represent the mean ± S.E. of three independent experiments (A and B). IB, immunoblot. 3T3-L1 adipocytes on coverslips were serum-starved for 4 h, and control or PP2Cα siRNA (C) or anti-PP2Cα antibody (Ab) or control sheep IgG (D) was microinjected. Cells were stimulated with (+) or without (−) 5 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. Data are the mean ± S.E. of three independent experiments. *, p < 0.01 compared with the control.

FIG. 7. **PP2Cα affects glucose uptake via PI3K in 3T3-L1 adipocytes.** 3T3-L1 adipocytes were infected with Ad5-ctrl (−) or Ad5-p110-CAAX (+) for 16 h, followed by transfection with control or PP2Cα siRNA. A, 48 h after transfection, the cells were lysed, and Western blotting was performed with anti-phospho-Akt antibody. The membranes were stripped and reblotted with anti-Akt antibody. IB, immunoblot. B, 2DOG uptake was measured as described under "Experimental Procedures." The graph shows the mean ± S.E. of four independent experiments, and the values are expressed as fold increases in glucose uptake compared with those observed in Ad5-ctrl-infected and control siRNA-transfected cells. *, p < 0.05 compared with the control value. C, differentiated 3T3-L1 adipocytes were infected with Ad5-ctrl (ctrl) or Ad5-PP2Cα-WT (PP2C). The cells were serum-starved for 16 h and pretreated with (+) or without (−) 300 nM wortmannin. The cells were lysed, and Western blotting was performed with anti-phospho-Akt antibody. The membranes were stripped and reblotted with anti-Akt antibody. D, 2DOG uptake was measured. The graph shows the mean ± S.E. of four independent experiments, and the values are expressed as fold increases in glucose uptake compared with those observed in Ad5-ctrl-infected cells. *, p < 0.05 compared with the control value.
unit is thought to be caused by increased phosphorylation of the p85 subunit. Overexpression of PP2Cα-WT led to subtle but significant reduction of the mobility shift of the p85 subunit (Fig. 8B, lanes 1 and 2), suggesting decreased phosphorylation of the p85 subunit in cells overexpressing PP2Cα-WT. In contrast, PP2Cα(R174G) overexpression did not affect the mobility.
shift of the p85 subunit (Fig. 8B, lanes 1 and 3). Moreover, the immunoprecipitate using anti-p85 subunit antibody pretreated with 0.4 milliliters of recombinant PP2Ca showed the same reduction in mobility shift as in the PP2Ca-WT-overexpressing cells (Fig. 8B, lanes 2 and 5). Tyrosine phosphorylation of the p85 subunit in the immunoprecipitate using anti-p85 antibody was not affected by infection with either virus (data not shown). These data show that the decreased gel mobility indicates dephosphorylation of the serine/threonine residue of the p85 subunit. To further elucidate whether PP2Ca might dephosphorylate the putative serine phosphorylation site (Ser<sup>608</sup>) of the p85 subunit and enhance PI3K activity, we generated His-tagged wild-type p85 and mutant p85 in which Ser<sup>608</sup> was replaced with Ala (His-p85(S608A)) and transfected them into HIReB cells. His-p85-WT and His-p85(S608A) were expressed at identical levels (Fig. 8C). HIReB cells cotransfected with His-p85-WT or His-p85(S608A) with control, PP2Ca-WT, or PP2Ca(R174G) vectors were analyzed by SDS-PAGE, followed by Western blotting with anti-His antibody (Fig. 8D). The gel mobility shift assay showed that His-p85(S608A) led to a significant decrease in mobility shift as well as coexpression of p85-WT with PP2Ca-WT (Fig. 8D, lanes 2 and 4). We were able to measure the PI3K activity associated with the His-tagged p85 subunit in the immunoprecipitate using anti-His antibody so as to avoid contaminating the activity of the endogenous p85 subunit. As shown in Fig. 8E (lanes 1–3), expression of PP2Ca-WT augmented the PI3K activity associated with p85-WT, whereas PP2Ca(R174G) had no effect. The protein levels of His-p85 and HA-PP2Ca in each sample were identical (Fig. 8E). Interestingly, the p85(S608A)-associated PI3K activity was 3-fold greater than the p85-WT-associated PI3K activity under basal conditions (Fig. 8E, lanes 1 and 4). These results support the idea that phosphorylation of the p85 subunit at Ser<sup>608</sup> may inhibit PI3K activity and that replacement of this serine residue with Ala may prevent phosphorylation of PI3K by serine phosphorylation. Moreover, expression of PP2Ca-WT did not further activate p85(S608A)-associated PI3K activity (Fig. 8E, lanes 4 and 5). In addition, PP2Ca-WT did not further decrease the mobility shift of p85(S608A) (Fig. 8D). We have demonstrated here that PP2Ca may modulate the phosphorylation states of the p85 subunit and activate PI3K activity.

**DISCUSSION**

In the four major families of serine/threonine phosphatases, it has been reported that PP1 and PP2A may be involved in insulin signaling (12, 13). On the other hand, PP2B and PP2Ca have been little studied. In this study, we examined the protein expression of serine/threonine phosphatases during 3T3-L1 differentiation and the effects of PP2Ca on the insulin signaling pathway. Our study demonstrated that expression of PP2Ca was increased during the differentiation of 3T3-L1 adipocytes. Furthermore, adenovirus-mediated overexpression of PP2Ca in 3T3-L1 adipocytes significantly enhanced PI3K activity in the immunoprecipitate as evaluated using antibodies against the p85 and p110 subunits of PI3K. We also found increased phosphorylation of Akt, GSK-3, and PKC<sub>a</sub>, resulting in enhanced insulin sensitivity (reducing ED<sub>50</sub> for stimulation) of glucose uptake. However, we found that stimulation of mitogen-activated protein kinase was unaffected by PP2Ca overexpression (data not shown), indicating that PP2Ca selectively enhances the PI3K pathway (Fig. 9). On the other hand, expression of the phosphatase-defective mutant PP2Ca(R174G) did not have such effects. Finally, we demonstrated that microinjection of PP2Ca siRNA or anti-PP2Ca antibody led to decreased insulin-stimulated GLUT4 translocation.

During differentiation of 3T3-L1 fibroblasts into adipocytes, the cells acquire insulin responsiveness and insulin sensitivity in glucose uptake. In this study, we found that the protein abundance of PP1, PP2A, and PP2B was decreased, whereas that of PP2Ca was unexpectedly up-regulated (Fig. 1). Although sequential expression of various genes in adipocyte differentiation has been extensively investigated (30, 31), there is no report on the differentiation-dependent expression pattern of these serine/threonine phosphatases. An earlier study reported that PP2Ca is expressed in various human tissues (32), but PP2Ca has not been examined in adipose tissue. Thus, we first demonstrated the possible importance of PP2Ca in the acquisition by adipocytes of insulin sensitivity during the differentiation process. Molecular cloning has identified PP2Ca as a molecule distinct from PP1, PP2A, and PP2B because it constitutes a separate gene family and is a monomeric enzyme (3). Recently, investigations of PP2Ca function have revealed a wide range of potential physiological functions (33–36). However, the lack of a specific inhibitor for PP2Ca hinders the study of its functions. Thus, we performed a PP2Ca overexpression study by the adenovirus-mediated gene transfer technique (Fig. 2). Das et al. (37) examined the molecular structure of PP2Ca by crystallographic analysis and reported that PP2Ca consists of two domains, an N-terminal catalytic domain with six α-helices and 11 β-strands and a 90-residue C-terminal domain with three α-helices. Arg<sup>174</sup> is present in the 60-residue segment in the catalytic channel of PP2Ca. Marley et al. (38) reported that deletion of the C-terminal residues (including Arg<sup>174</sup>) results in a completely inactive protein. In addition, replacement of Arg<sup>178</sup> (corresponding to Arg<sup>174</sup> of PP2Ca) with Gly in PP2Cβ results in a nearly complete loss of enzymatic activity (18). Therefore, Arg<sup>174</sup> may be necessary for the phosphatase activity of the protein. Thus, we constructed a PP2Ca mutant in which Arg<sup>174</sup> was replaced with Gly (PP2Ca(R174G)). Although the protein expression level of the mutant was similar to that of PP2Ca-WT, the phosphatase activity of PP2Ca(R174G) obtained with the anti-PP2Ca immunoprecipitate was similar to that obtained in untransfected control cells, and no phosphatase activity was obtained with anti-HA antibody. Thus, we conclude that this mutation disrupted phosphatase activity. Furthermore, this mutant did...
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not have a dominant-negative property. Thus, we used PP2Ca(R174G) as a phosphatase-dead enzyme to examine the significance of PP2Ca expression as an insulin sensitizer.

One of the major metabolic actions of insulin is stimulation of glucose transport that is mediated in a PI3K-dependent manner. In this study, overexpression of PP2Ca-WT enhanced the insulin sensitivity of glucose uptake and also increased both the basal and insulin-stimulated glucose uptakes. In several studies of insulin stimulation of glucose uptake, an approach in which constitutively active signal molecules were expressed in 3T3-L1 adipocytes was adopted (21, 39–44). All of these constitutively active molecules, including PI3K, Akt, and PKC, stimulate 2DOG uptake in the basal state. However, the results are controversial in terms of insulin-stimulated glucose uptake (21, 40–44). Thus, in our opinion, one of the most interesting findings of this study is the evidence that insulin sensitivity is regulated at a site downstream of the insulin receptor and IRS-1 because tyrosine phosphorylation of both proteins was not affected by overexpression of PP2Ca. Impaired insulin sensitivity is one of the major characteristics of insulin resistance. Down-regulation of the insulin receptor due to hyperinsulinemia causes decreased insulin sensitivity (45). In contrast, increased expression of the insulin receptor leads to increased insulin sensitivity (17). Furthermore, expression of a dominant-negative kinase-defective insulin receptor impairs tyrosine phosphorylation of IRS-1 (17, 46), resulting in decreased insulin sensitivity. Moreover, increased expression of protein-tyrosine phosphatase-1B decreases tyrosine phosphorylation of both the insulin receptor and IRS-1 and decreases the insulin sensitivity of insulin-stimulated amino acid uptake in the high glucose-induced insulin-resistant state (10). However, the molecular mechanisms that modulate insulin sensitivity are still unclear. This study demonstrates that PP2Ca is a positive regulator of insulin sensitivity at the post-IRS-1 level in 3T3-L1 adipocytes, and we propose that modulation of PP2Ca activity may be a new therapeutic approach to ameliorate insulin resistance.

Overexpression of PP2Ca-WT led to a much higher level of PI3K activity than seen with insulin stimulation alone, but it had only a partial effect on stimulating downstream signaling. Consistent with this, Frevert and Kahn (39) showed that coexpression of both the p110 subunit and the inter-SH2 region of p85 leads to the same results. In addition, when PI3K was activated by thiophosphorylated peptides, only a minor effect on GLUT4 translocation was observed (40). Taken together with our previous result that overexpression of the membrane-localized version of p110-CAAX stimulates glucose uptake in the basal state (21), these studies support the conclusion that active PI3K is sufficient to stimulate insulin signaling only if it is targeted to the proper subcellular membrane compartment. Thus, localization of the p85-p110 complex in the appropriate subcellular compartment is critical for mediating insulin signaling as well as activation of PI3K activity.

In this study, the basal and insulin-stimulated PI3K activities in the immunoprecipitate obtained using anti-p85 and anti-p110 antibodies were increased without any effect on phosphorylation of the insulin receptor and IRS-1 in Ad5-PP2Ca-WT-infected cells. These results clearly show the importance of PP2Ca in this process, and this raises the question of how PP2Ca expression could enhance PI3K activity. The 32P labeling study indicated that PP2Ca-WT led to a significant decrease in the phosphorylation state of the p85 subunit. Furthermore, gel mobility shift assay showed that overexpression of PP2Ca-WT and pretreatment of recombinant PP2Ca led to a significant decrease in p85 subunit phosphorylation. Taken together with the unchanged tyrosine phosphorylation of the p85 subunit, the decreased gel mobility indicated dephosphorylation of the serine/threonine residue of the p85 subunit. These data confirm that PP2Ca modulates the phosphorylation state of the p85 subunit.

We further assessed the effect of PP2Ca expression on the PI3K activity associated with the p85 subunit. We expressed His-tagged wild-type and putative serine phosphorylation site-mutated p85 subunits, and we successfully measured the PI3K activity associated with the His-tagged p85 subunit to distinguish between the activities of the transfected and endogenous p85 subunits. Cotransfection with His-tagged p85-WT and PP2Ca-WT increased PI3K activity. However, cotransfection with His-tagged p85(S608A) and PP2Ca-WT had no effect (Fig. 8), suggesting that PP2Ca might dephosphorylate the p85 subunit and activate PI3K activity. Furthermore, we demonstrated that PP2Ca-WT activated PI3K, but that the phosphatase-dead form did not, suggesting that phosphatase activity is important for enhancement of PI3K activity and that Ser608 of the p85 protein may be the site of dephosphorylation by PP2Ca.

We attempted several times to perform mass spectroscopy analysis in vivo to determine the exact phosphorylation site(s). However, the protein amount of the p85 subunit was not sufficient for analysis. Furthermore, we were not able to detect any phosphorylation site(s) of the p85 subunit because of weak signals. It is also possible that the phosphorylated peptide may be degraded by the laser beam during mass spectroscopy analysis and that highly negatively charged peptides from the p85 protein may not be appropriate for mass spectroscopy study. Thus, it is methodologically difficult to obtain direct evidence of the phosphorylation site of serine/threonine by mass spectroscopy analysis of whole cells. A phospho-specific antibody to the putative phosphorylation site is also required to determine the exact sites of dephosphorylation by PP2Ca. Thus, determination of the phosphorylation sites of the p85 subunit will require further investigation. Taken together with the results of the 32P labeling study, the gel mobility shift assay, and the mutagenesis study of the p85 subunit, we have demonstrated here that PP2Ca activity may directly regulate PI3K activity by dephosphorylating the p85 subunit, although the exact sites of dephosphorylation of p85 require further investigation.

In summary, this study clearly demonstrates that PP2Ca increases the insulin sensitivity of glucose uptake and enhances the PI3K pathway. In addition, our results are consistent with the view that PP2Ca may be a positive regulator of insulin signaling (Fig. 9). Finally, they demonstrate a novel regulation mechanism for insulin sensitivity through direct activation of PI3K by the serine/threonine phosphatase PP2Ca.

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Protein Phosphatase-2Cα as a Positive Regulator of Insulin Sensitivity through Direct Activation of Phosphatidylinositol 3-Kinase in 3T3-L1 Adipocytes
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