Insulin plays a central role in the regulation of glucose homeostasis in part by stimulating glucose uptake and glycogen synthesis. The serine/threonine protein kinase Akt has been proposed to mediate insulin signaling in several processes. However, it is unclear whether Akt is involved in insulin-stimulated glucose uptake and which isoforms of Akt are responsible for each insulin action. We confirmed that expression of a constitutively active Akt, using an adenoviral expression vector, promoted translocation of glucose transporter 4 (GLUT4) to plasma membrane, 2-deoxyglucose (2-DG) uptake, and glycogen synthesis in both Chinese hamster ovary cells and 3T3-L1 adipocytes. Inhibition of Akt either by adenoviral expression of a dominant negative Akt or by the introduction of synthetic 21-mer short interference RNA against Akt markedly reduced insulin-stimulated GLUT4 translocation, 2-DG uptake, and glycogen synthesis. Experiments with isoform-specific short interference RNA revealed that Akt2, and Akt1 to a lesser extent, has an essential role in insulin-stimulated GLUT4 translocation and 2-DG uptake in both cell lines, whereas Akt1 and Akt2 contribute equally to insulin-stimulated glyogen synthesis. These data suggest a prerequisite role of Akt in insulin-stimulated glucose uptake and distinct functions among Akt isoforms.

The Akt (also referred as protein kinase B (PKB)1) was initially found to be an acute transforming component of the AKT8 virus isolated from a murine T cell lymphoma (1, 2). Its putative cellular homologue, Akt (c-Akt), encodes a serine/threonine protein kinase (3) whose catalytic domain, located in the carboxyl terminus of the protein, is closely related to that of protein kinase C (PKC) and protein kinase A (3, 4). The kinase activity of Akt is stimulated by a variety of growth factors including insulin (5). Recent extensive investigation revealed that Akt plays crucial roles in various cellular functions including cell survival, cell growth, cell differentiation, cell cycle progression, transcription, translation, and cellular metabolism through phosphorylation of target molecules (6).

Following insulin stimulation, insulin-receptor substrate proteins are phosphorylated, after which phosphoinositide 3-kinase (PI3-kinase) is activated. The pleckstrin homology (PH) domain of Akt binds to the lipid products of PI3-kinase and thereby mediates recruitment to the membrane in response to PI3-kinase activation (5, 7). The translocation of Akt allows for phosphorylation at Thr308 by another Ser/Thr protein kinase, 3-phosphoinositide-dependent protein kinase 1, in glucose uptake in 3T3-L1 adipocytes has been proposed (16), the opposite result has also been reported in the case of the same cell type (17). Thus, the role of atypical PKC also seems to be controversial.

An adenoviral gene transfer technique now in common use is thought to be a powerful strategy to introduce high levels of transgene expression into mammalian cells (18). The technique facilitates expression of inactive Akt in cells to overcome endogenous Akt activity, dominant negatively (14, 15). In addition, the participation of atypical protein kinase C (i.e. PKCδ and PKCζ), which is also thought to be activated by phosphoinositides and 3-phosphoinositide-dependent protein kinase 1, in glucose uptake in 3T3-L1 adipocytes has been proposed (16), the opposite result has also been reported in the case of the same cell type (17). Thus, the role of atypical PKC also seems to be controversial.

With regard to insulin action, Akt was shown to phosphorylate and inhibit glycogen synthase kinase 3 (GSK3) and subsequently promote glycogen synthesis in response to insulin (13). However, regarding another important insulin action, which is the stimulation of glucose uptake by the GLUT4 translocation, the role of Akt remained controversial (14, 15). In addition, although the participation of atypical protein kinase C (i.e. PKCδ and PKCζ), which is also thought to be activated by phosphoinositides and 3-phosphoinositide-dependent protein kinase 1, in glucose uptake in 3T3-L1 adipocytes has been proposed (16), the opposite result has also been reported in the case of the same cell type (17). Thus, the role of atypical PKC also seems to be controversial.

An adenoviral gene transfer technique now in common use is thought to be a powerful strategy to introduce high levels of transgene expression into mammalian cells (18). The technique facilitates expression of inactive Akt in cells to overcome endogenous Akt activity, dominant negatively (14, 15). According to other authors who have examined the role of Akt in glucose metabolism (14, 15), Akt-2A (Akt-T308A/S473A) and Akt-AAA (Akt-K179A/T308A/S473A) have been used. We used Akt-AAA (Akt-K179M/T308A/S473A) in the present study, since we con-
considered that Met (for Lys179; so-called catalytic Lys) has a relatively large side chain and is likely to impair kinase activity by occupying the ATP-binding pocket more efficiently compared with Ala as well as losing a positive charge for the stabilization of ATP binding and a hydrogen bond with the Glu in helix C.

RNA interference (RNAi) is a process whereby double-stranded RNA (dsRNA) induces the degradation of cognate mRNA for prevention of the expression of alien genes. Originally, the RNAi phenomenon was discovered in Caenorhabditis elegans; however, recent studies revealed that such phenomena are evolutionarily conserved from plants to mammals (19–23). According to recent data, duplexes of 21-nucleotide short interference RNA (siRNA) with 2-nucleotide 3' overhangs are the most efficient triggers of sequence-specific mRNA degradation (24, 25). One fascinating aspect of RNAi is its extreme efficiency, since only a few trigger dsRNA molecules introduced into the cells suffice to inactivate a continuously transcribed cognate target mRNA for long periods of time (22). Although the introduction of dsRNA into mammalian cells has been known to cause RNA-dependent protein kinase-mediated apoptosis, the siRNA (e.g. 21-nucleotide dsRNA) has been shown not to cause such an effect (24). This important observation revealed new possibilities whereby one could prove gene function in mammalian cells.

In attempts to elucidate the roles of Akt in insulin actions, we used both adenoviral overexpression and RNAi in both CHO cells and 3T3-L1 adipocytes. Since it is unclear which isoforms of Akt are responsible for each insulin action, we investigated the role of each isoform in insulin actions using a specific RNAi against each Akt isoform.

**EXPERIMENTAL PROCEDURES**

**Materials**—γ-[32P]ATP and d-6-[3H]glucose were purchased from Amersham Biosciences. 2-Deoxy-d-ribofuranosyl-5'-guanosine 3'5'-cyclic monophosphate (an inhibitor of cyclic nucleotide phosphodiesterase) was from Moravek (Brea, CA). Phosphospecific antibodies against pAkt (Ser^473^), pErk-1/2 (Thr^202^/Tyr^204^), and pGSK3β (Ser^9^) were from Cell Signaling (Beverly, MA). Anti-insulin receptor, anti-14-3-3, anti-PKCζ, anti-phosphotyrosine (PY-99), and anti-Akt1-specific antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Erk-1/2, anti-GSK3β, anti-Akt2-specific, and anti-Akt3-specific antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). An anti-PKCζ antibody and anti-Akt antibody were obtained using rabbits immunized against synthetic peptides (CTMHPDHTQTVIPYNPSS and CVDSER- RPHFPQFSYSASGTA, respectively) (26). The anti-Akt antibody used was also from the AdEasy system (as described, 18). The recombinant adenosviruses were amplified in 293 cells. The 50% tissue culture infectious dose (TCID_{50}) was determined as pfu/ml, and either CHO cells or 3T3-L1 adipocytes were infected at the multiplicities of infection (MOI) of 10 pfu/cell (as indicated), respectively.

**Preparation of Recombinant Adenovirus**—The recombinant adenovirus encoding either dominant-negative (DN) Akt (Akt-MMA: K179M/T308A/S473A) or constitutively active (CA) Akt (myr-Akt1) was generated using the AdEasy system (as described, 18). The recombinant adenosviruses were amplified in 293 cells. The 50% tissue culture infectious dose (TCID_{50}) was determined as pfu/ml, and either CHO cells or 3T3-L1 adipocytes were infected at the multiplicities of infection (MOI) of 10 pfu /cell (as indicated), respectively.

**Adenoviral Gene Transduction**—CHO-GLUT4myc cells or 3T3-L1 adipocytes (the 8th day after initiation of differentiation) were infected with adenovirus for 2 h with tilting every 30 min; we then added the growth medium and incubated the preparation for 32 h followed by serum starvation with serum-free medium (i.e. F-12 or Dulbecco's modified Eagle's medium) for 16 h. Experiments were performed 48 h after the infection.

**Akt in Vitro Kinase Assay**—Akt kinase activity was measured using AktDte-2T (26) as a substrate, as described (33). Cells seeded on either six-well or 24-well plates were incubated in serum-free medium for 16 h and then stimulated with 100 mM insulin for 10 min. Cells were washed once with PBS and lysed in solubilizing buffer containing 20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.5 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, and 1% (v/v) Nonidet P-40. Cell lysates were then sonicated, clarified by centrifugation, and the supernatant was subjected to immunoprecipitation using either the anti-Akt antibody employed for Akt kinase activity measurement or the control antibody (26). The precipitated Akt fraction was then separated by SDS-PAGE and analyzed by autoradiography. In the case of measuring Akt kinase activity, the Akt protein bands were excised from the gels and subjected to in-gel protein kinase assay of Akt, AKTide-2T (ARKRERTYSFGHAA), was as described (33). Cells seeded on either six-well or 24-well plates were incubated with OPTI-MEM (Invitrogen) containing the indicated MOI of recombinant adenovirus for 2 h with tilting every 30 min; we then added the growth medium and incubated the preparation for 32 h followed by serum starvation with serum-free medium (i.e. F-12 or Dulbecco's modified Eagle's medium) for 16 h. Experiments were performed 48 h after the infection.

**GLUT4 Translocation**—Insulin-stimulated GLUT4 translocation to plasma membrane was examined by determination of the exposure of Myc epitope (GLUT4myc) to the outer surface of plasma membrane in response to insulin, as described (34). In brief, cells on 24-well plates were

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The above text is a detailed and comprehensive description of the experimental procedures used to study the roles of Akt in insulin actions, focusing on the use of RNA interference and adenoviral transduction techniques.
FIG. 1. Effects of CA- and DN-Akt on insulin actions in CHO-GLUT4myc cells. Cells were infected with the indicated MOI (pfu/cell) of adenovirus, and insulin-stimulated (100 nM, 10 min) Akt kinase activity (A and E; the antibody used for immunoprecipitation can recognize all Akt isoforms), GLUT4 translocation (B and F), 2-DG uptake (C and G), and glycogen synthesis (D and H) were measured. Data are expressed by mean ± S.E. from 4–6 experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control (GFP). For 2-DG uptake, 28 pmol/min/2 × 10^5 cells was regarded as 1 arbitrary unit.
FIG. 2. Effects of CA- and DN-AKT on insulin actions in 3T3-L1-GLUT4myc adipocytes. Cells were infected with the indicated MOI (pfu/cell) of adenovirus, and insulin-stimulated Akt kinase activity (A and E; the antibody used for immunoprecipitation can recognize all Akt isoforms), GLUT4 translocation (B and F), 2-DG uptake (C and G), and glycogen synthesis (D and H) were measured. Data are expressed by mean ± S.E. from 4–6 experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control (GFP). For 2-DG uptake, 8.6 pmol/min/2 × 10⁵ cells was regarded as 1 arbitrary unit.
Differential Roles of AKT/PKB Isoforms in Insulin Actions

**RESULTS**

Constitutively Active Akt Mimicked and Dominant Negative Akt Inhibited Insulin Actions in Both CHO Cells and 3T3-L1 Adipocytes—We generated recombinant adenovirus encoding either constitutively active Akt (CA-Akt; myr-AKT) or dominant negative Akt (DN-Akt; Akt-K179M/T308A/T473A) and examined effects of these Akt mutants on insulin actions, including GLUT4 translocation to plasma membrane, 2-deoxyglucose (2-DG) uptake, and glycogen synthesis. As shown in Figs. 1A and 2A, infection of CA-Akt adenovirus markedly enhanced Akt kinase activity in both CHO cells and 3T3-L1 adipocytes. In parallel, the infection mimicked effects of insulin on GLUT4 translocation, 2-DG uptake, and glycogen synthesis as shown in Figs. 1, B–D, and 2, B–D. On the other hand, the control adenovirus that encoded green fluorescent protein (GFP) had little effect on these actions as well as on Akt kinase activity. Interestingly, although CA-Akt enhanced glycogen synthesis in quiescent cells, the maximum rate of glycogen syntheses in insulin-stimulated cells was blunted in both CHO cells and 3T3-L1 adipocytes (Figs. 1D and 2D; see “Discussion”).

We next examined the effect of DN-Akt on insulin actions in these cells. As shown in Figs. 1E and 2E, DN-Akt adenovirus efficiently inhibited insulin-stimulated Akt kinase activity and insulin-stimulated GLUT4 translocation, 2-DG uptake, and glycogen synthesis in an MOI-dependent manner in both cell lines. In the case of 3T3-L1 adipocytes, although DN-Akt (at MOI 80) inhibited Akt activity almost completely, insulin-stimulated metabolic activities, including 2-DG uptake and glycogen synthesis, still remained, which suggests an additional pathway in 3T3-L1 adipocytes (see “Discussion”). Expression of DN-Akt had little effect on either phosphorylation of Erk-1/2 (by immunoblotting with phosphospecific antibody) or activation of PI3-kinase activity (by in vitro kinase assay) in insulin-stimulated cells (data not shown).

RNAi-mediated Gene Silencing of Akt—We then carried out RNAi-mediated gene silencing using a synthetic 21-mer oligonucleotide RNA duplex (siRNA) to determine the requirement of Akt for insulin actions. We designed a pair of oligonucleo-
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Fig. 4. Functional silencing of endogenous Akt by RNA interference. A, schematic explanation of the luciferase system. The specific (against Akt; siAKTc) and/or nonspecific (against GFP; siGFP) synthetic 21-mer dsRNAs (total of 0.5 μg/well) were co-transfected with FKHR1 (0.01 μg/well), BP1-luc (0.5 μg/well), and pRL-SV40 (0.1 μg/well) using LipofectAMINE 2000 into either COS-7 cells (B) or 3T3-L1 adipocytes (C) plated on a 24-well plate. After 48 h of transfection, luciferase activity was measured using dual luciferase reporter assay kits. A PI3-kinase inhibitor, LY294002 (100 μM), was also exposed to the cells 1 h before the assay. Normalized relative luciferase activity (Pp-luc/Rr-luc) was expressed. The data are expressed by mean ± S.E. from three experiments. **, p < 0.01; ***, p < 0.001 compared with control (siGFP; 0.5 μg).

Fig. 3. We then co-transfected siRNAs. As shown in Fig. 3, matches against the target sequence of siAKTc, as shown in Fig. 3, contain either single (siAKTc-1m) or triple (siAKTc-3m) mismatches within the corresponding sequences of rat Akt3, human Akt3, and mouse Akt2 and Akt3. COS-7 cells were then co-transfected with the siAKTc and Akt expression plasmid that also expressed GFP driven by an independent CMV promoter as well as CMV-driven HA-tagged rat Akt1. As shown in Fig. 3B, the introduction of synthetic oligo-RNA (siAKTc) suppressed the expression of HA-tagged rat Akt1, but not GFP, in a dose-dependent manner. In our preliminary examination using fluorescein isothiocyanate-labeled siAKTc, siRNA was observed in the nucleus within 1 h after transfection (data not shown). To test the specificity of the siRNA, we also designed another two pairs of oligo-RNA duplexes that contain either single (siAKTc-1m) or triple (siAKTc-3m) mismatches against the target sequence of siAKTc, as shown in Fig. 3C. As shown in Fig. 3D, we then co-transfected siRNAs with either HA-tagged Akt1 or Akt2 or an Akt3 expression construct. Although siAKTc-1m had only a single mismatch, the siAKTc-1m did not show any RNAi effect. The siAKTc-3m as well as siAKT-1m also showed no effect. Consistent with this, since siAKTc has a single mismatch against rat Akt3, it showed no RNAi effect (Fig. 3D). Thus, we confirmed the specific silencing against the cognate target gene by siRNA.

To determine whether siRNA would affect endogenous Akt activity, we performed the FOXO3a/FKHR1 reporter assay that has been shown to be a model system reflecting endogenous Akt activity (30, 31, 36). According to reported data, Akt phosphorylates and down-regulates the function of forkhead transcription factors, including FOXO3a/FKHR1, and subsequently inhibits IGF-1-BP (insulin-like growth factor 1-binding protein) reporter activity (37) shown as a scheme in Fig. 4A. As shown in Fig. 4, B and C, siAKTc as well as LY294002 (PI3-kinase inhibitor) enhanced luciferase activities of IGF-1-BP1 in COS-7 cells and 3T3-L1 adipocytes in a dose-dependent manner. Thus, we also confirmed that the siRNA silenced endogenous Akt as well as exogenously transfected Akt.

Effect of siRNA on Insulin Actions—To elucidate the roles of Akt on insulin actions, we introduced siRNA into cultures of CHO cells and 3T3-L1 adipocytes. Before the experiments, since the half-life of protein levels of Akt was thought to be a key factor to determine the optimal incubation period from the transfection to biological assays, we estimated that the half-life of endogenous Akt protein was around 4 h in cycloheximide-treated CHO cells (data not shown). As shown in Fig. 5A, siAKTc, but not control siRNA (siGFP), down-regulates endogenous protein kinase activity of Akt in both quiescent and insulin-stimulated cells. In parallel with this, siAKTc inhibited insulin-stimulated GLUT4 translocation to plasma membrane, 2-DG uptake, and glycogen synthesis in CHO cells. We also observed the effect of siAKTc on GLUT4 translocation in an immunocytochemical analysis (data not shown).

Since 3T3-L1 cells were a mouse-derived line, siAKTc could silence only Akt1 and not Akt2, as shown in Fig. 5E, because of a mismatch (see Fig. 3A). However, as shown in Fig. 5, the suppression of Akt1 in 3T3-L1 adipocytes by siAKTc could suppress all of these insulin actions only weakly compared with CHO cells yet significantly. Regarding the expression of Akt3 in these cells, we did not observe the expression of Akt3 by immunoblotting using an anti-Akt3-specific antibody, whereas the expression could be seen in the lysate from brain-derived U251MG cells, as shown in Fig. 8B.

Isoform-specific Gene Silencing of AKT by siRNA—Three Akt isoforms (Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ) are known, and we carried out isoform-specific silencing using isoform-specific siRNA against each Akt isoform, as summarized in Fig. 6A. We introduced each RNAi as well as nonspecific control siRNA (i.e. siGFP) with the indicated expression con-
FIG. 5. Effect of siRNA targeting Akt on insulin actions in CHO-GLUT4myc cells and 3T3-L1-GLUT4myc adipocytes. 4 h (CHO-GLUT4myc cells) or 6 h (3T3-L1-GLUT4myc adipocytes) before assay, cells were transfected with the specific (siAKTc) and/or nonspecific (siGFP) siRNAs using LipofectAMINE 2000 (2 μl/150 μl of serum-free medium, 24 wells, 5 μl/400 μl/6 wells) and insulin-stimulated (100 nM, 10 min) Akt kinase activity (A and E; cells on six-well plates were analyzed, adjusted amounts of siRNA to 24-well plates are indicated), GLUT4 translocation (E and F; 24-well plates were used), 2-DG uptake (C and G; cells on 24-well plates were analyzed), and glycogen synthesis (D and H; cells on 24-well plates were analyzed) were measured. Data are expressed by mean ± S.E. from four experiments. * p < 0.05 versus control (siGFP). For 2-DG uptake, 30 pmol/min/2 × 10⁵ cells (CHO-GLUT4myc cells) and 11 pmol/min/2 × 10⁷ cells (3T3-L1-GLUT4myc adipocytes) are regarded as 1 arbitrary unit.
structs, including serum- and glucocorticoid-inducible protein kinase, PKCα, and PKCγ, into COS-7 cells. As shown in Fig. 6B, each siRNA clearly silenced only each cognate gene. We also examined the effect of isoform-specific siRNA on endogenous isoforms of Akt in both CHO cells and 3T3-L1 adipocytes as shown in Fig. 8, A and E. Although the expression of Akt3 in both lines was not detectable (Fig. 8I), we did detect a significant expression of both Akt1 and Akt2 in these lines, and each isoform-specific siRNA did inhibit each cognate target gene (Figs. 7 and 8, A and E). The siAKTc silenced Akt2 in CHO cells but not in 3T3-L1 adipocytes. Thus, we demonstrated the sequence-specific gene silencing of both exogenous and endogenous Akt using isoform-specific siRNA.

Specific Silencing of Akt Signaling by siRNA—We attempted to confirm the effect of isoform-specific siRNA on insulin signaling in CHO-GLUT4myc cells. Although single transfection revealed the effect of RNAi, as shown in Fig. 4, to interpret the roles of each isoform more clearly, we used dual transfection (6 and 24 h before the assay) in these experiments, and the effect could be clearly observed on immunoblotting. As shown in Fig. 7, siAKTc, siAKT1, and siAKT2, but not siAKT3, down-regulated not only each endogenous cognate isoform of Akt but also total Akt expression (the anti-Akt antibody recognized all Akt isoforms) and insulin-stimulated phosphorylation (Ser(P)473) of Akt compared with control siRNA (siGFP). In parallel with this, phospho-Ser9/Ser21 of GSK3α/β (known as the phospho-

Fig. 6. Isoform-specific gene silencing of Akt by RNA interference. A, the sequences of synthetic siRNA duplexes targeting each Akt isoform are shown. B, the indicated siRNAs (0.5 μg/24 wells) were co-transfected with either HA-Akt1–3, HA-tagged serum- and glucocorticoid-inducible protein kinase, or PKCα or PKCγ expression vectors into COS-7 cells on a 24-well plate, and after 48 h of the transfection, these cells were harvested, and the obtained total cell lysates were analyzed by immunoblotting using either anti-HA antibody or specific antibodies. Data are representative of at least three independent experiments.

Fig. 7. Effect of siRNA against each Akt isoform on endogenous Akt expression and insulin signaling. At both 24 and 6 h before the assay, CHO-GLUT4myc cells on a 24-well plate were transfected with 0.5 μg of the indicated siRNAs. Cells were stimulated with 100 nM insulin for 10 min and harvested, and then the obtained total cell lysates were analyzed by immunoblotting using the indicated specific antibodies. Anti-14-3-3 blotting was also done as a loading control. Data are representative of at least three independent experiments.
rylation sites by Akt) was also inhibited, but the expression of GSK3β itself was not measurably affected. On the other hand, either the upstream (i.e. Tyr phosphorylation of insulin receptor) or the distinct signaling (i.e. phosphorylation of Erk1/2) was not measurably affected. Thus, the siRNA against Akt was likely to specifically silence only Akt signalings.

**Effect of Isoform-specific siRNA on Insulin Actions in CHO-GLUT4myc Cells or 3T3-L1-GLUT4myc Adipocytes**—Finally, we examined the effect of isoform-specific siRNA against Akt on insulin actions. As shown in Fig. 8, A and E, we observed a clear silencing effect of each specific siRNA on each cognate isoform of Akt, and then we did biological assays under the same conditions. The silencing of Akt2 affected both insulin-stimulated GLUT4 translocation (Fig. 8, B and F) and 2-DG uptake (C and G), and glycogen synthesis (D and H) most prominently in both lines. On the other hand, silencing of Akt1 and Akt2 equally affected insulin-stimulated glycogen synthesis (Fig. 8, D and H). We did not observe any significant effect in control siRNA-transfected cells compared with either mock-transfected cells or untransfected cells (data not shown).
In the case of CHO-GLUT4myc cells, siAKTc silenced both Akt1 and Akt2 simultaneously and showed additive inhibitory effects on all of these insulin actions compared with either siAKT1 or siAKT2 alone as shown in Fig. 8. By contrast, in the case of 3T3-L1-GLUT4myc adipocytes, siAKTc silenced only Akt1 because of single mismatch of siAKTc to mouse Akt2 (Fig. 3A). We also tested the effect of siAKT1 and siAKT2 simultaneously in 3T3-L1-GLUT4myc adipocytes and observed additive effects on insulin-stimulated GLUT4 translocation and 2-DG uptake but not significantly on glycogen synthesis (data not shown).

**DISCUSSION**

To elucidate the roles of Akt and each of its isoforms in insulin actions, we used adenoviral gene transfer and RNA interference techniques. Based on the data using adenovirus, CA-Akt mimicked and DN-Akt inhibited insulin actions. Although these data strongly suggested that Akt is an essential mediator of insulin actions, there may be unexpected effects, because we overexpressed either CA-Akt or DN-Akt in the adenovirus gene transfer. Thus, we next investigated the role of Akt using the RNAi technique by synthetic siRNA. These siRNAs specifically and potently down-regulated not only endogenous expression and the protein kinase activity of Akt, but also insulin-stimulated insulin actions including GLUT4 translocation to plasma membrane, 2-DG uptake and glycogen synthesis. On the other hand, distinct signals from the Akt signal (i.e. Erk1/2) or the upstream signal of Akt (insulin receptor) were not affected. These data strongly suggested that activation of Akt is essential for these insulin actions. Based on experiments with isoform-specific siRNA, Akt2 and, to a lesser extent, Akt1, have an essential role in insulin-stimulated GLUT4 translocation and 2-DG uptake, whereas Akt1 and Akt2 are likely to contribute equally to insulin-stimulated glycogen synthesis.

Regarding the role of Akt in the translocation of GLUT4 to the plasma membrane, there are arguments. In 1993, we reported that insulin-stimulated glucose uptake was mediated by the PI3-kinase-dependent pathway (38), and this observation was given agreement. In 1996, Kohn et al. (39) reported that constitutively active Akt mimics insulin-stimulated glucose uptake, thereby suggesting the participation of Akt in the action. However, over the next 7 years, the issue has been controversial. We speculate that the endogenous Akt kinase activity seems to difficult to eradicate, especially in 3T3-L1 adipocytes. In addition, 3T3-L1 adipocytes express low levels of the adenovirus receptor (i.e. Coxsackie virus and adenovirus receptor). Kitamura et al. (14) reported that dominant negative Akt (Akt-2A; Akt-T308A/S473A) inhibited the kinase activity of Akt by ~80% but did not impair insulin-stimulated glucose uptake in 3T3-L1 adipocytes. However, there was residual Akt activity, which may be sufficient to support insulin-stimulated glucose uptake (15). On the other hand, Wang et al. (15) reported that dominant-negative Akt (Akt-AAA; Akt-K179A/T308A/S473A) did impair both endogenous Akt kinase activity and insulin-stimulated glucose uptake in L6 myoblasts. In our present study, we used Akt-K179M/T308A/S473A (Akt-MAA), which impaired endogenous kinase activity dominant negatively as did the other dominant negative form of Akt-AAA. This DN-Akt adenovirus was recognized to inhibit signalings via all Akt isoforms, because the Akt kinase assay (Figs. 1E and 2E) reflected total Akt activity. The antibody we used in this assay recognized all isoforms of Akt (data not shown). In addition, we also confirmed that insulin-stimulated Ser\(^{473}\) phosphorylation of Akt2 in 3T3-L1-GLUT4myc adipocytes was attenuated by the DN-Akt (data not shown). In our study, although DN-Akt (at MOI 80) inhibited Akt activity almost completely, residual metabolic activities could still be slightly observed (Fig. 2, E–H). Therefore, there may be an additional pathway in 3T3-L1 adipocytes (e.g. atypical PKC). Although we observed little effect of adenoviral overexpression on either upstream (PI3-kinase) or distinct signaling (Erk), we further used the RNAi technique that was considered to be more specific.

With RNAi targeting each Akt isoform (i.e. Akt1–3), isoform-specific silencing was observed in both CHO-GLUT4myc cells and 3T3-L1-GLUT4myc adipocytes (Fig. 8, A and E). The inhibitory effects of siRNA on insulin actions were modest com-
lipofection is limited, whereas adenovirus can infect all cells late this is because the transfection efficiency of siRNA by compared with that of adenovirus but were significant. We specu-
i.e.

Akt1-deficient mice display growth reduction but no metabolic defect, including glucose metabolism (43, 44). In an intact body, Akt1 and Akt2 are likely to be key molecules for growth and metabolism, respectively. In accordance with their data, our data also suggested that Akt2 is a dominant isoform among the Akt family in insulin-stimulated glucose uptake as well as GLUT4 translocation but also suggested a modest role for Akt1 at least at the cell level. The suppression of Akt1 also affected insulin-stimulated GLUT4 translocation and glucose uptake.

The roles of Akt3 in these cells are unclear. Although Akt1 and Akt2 have been shown to be expressed ubiquitously even in insulin-responsive tissues (i.e. liver, skeletal muscle, and adipose tissue) (45), the distribution of Akt3 seems to be relatively limited (i.e. testis and brain). We also observed high levels of Akt3 expression in mouse tissues, including brain and testis, but extremely lower levels in adipose tissues, only trace Akt3 expression in mouse tissues, including brain and testis, and Akt2 have been shown to be expressed ubiquitously even in insulin-responsible tissues (i.e.

Akt1 at least at the cell level. The suppression of Akt1 also be similar (28). Kim et al. reported that Akt1, but not Akt2, co-localized with JIP1, a scaffold protein of the c-Jun N-terminal kinase pathway, through the PH domain of Akt1 (50). We reported that TCL1 is a co-activator of Akt by promotion of oligomerization (51). The PH domain of Akt (52) mediates the binding of Akt to TCL1, and Akt1 and Akt2 bind to all of the known TCL1 family, including TCL1, MTPC, and TCL1b, yet Akt3 binds to only TCL1 (53). Thus, the cellular function of each Akt isoform may be mediated by its binding partner, not by substrate specificity. Yet to be identified proteins (40, 54) on the GLUT4 vesicle that bind dominantly to Akt2 may be candidates to direct GLUT4 translocation in response to insulin.
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