Role of Insulin Receptor Substrate-1 and pp60 in the Regulation of Insulin-induced Glucose Transport and GLUT4 Translocation in Primary Adipocytes*

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In muscle and fat, glucose transport occurs through the translocation of GLUT4 from an intracellular pool to the cell surface. Phosphatidylinositol (PI) 3-kinase has been shown to be required in this process. Insulin is thought to activate this enzyme by stimulating its association with tyrosine-phosphorylated proteins such as insulin receptor substrate (IRS)-1, IRS-2, Grb2-associated binder-1, and pp60. To study the role of these endogenous substrates in glucose transport, we analyzed adipocytes from IRS-1 null mice that we previously generated (Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S., Sekihara, H., Yoshioka, S., Horikoshi, H., Furuta, Y., Ikawa, Y., Kasuga, M., Yazaki Y., and Aizawa S. (1994) Nature 372, 182–186). In adipocytes from these mice, we showed that: 1) insulin-induced PI 3-kinase activity in the antiphosphotyrosine immunoprecipitates was 54% of wild-type; 2) pp60 was the major tyrosine-phosphorylated protein that associated with PI 3-kinase, whereas tyrosine phosphorylation of IRS-2 as well as its association with this enzyme was almost undetectable; and 3) glucose transport and GLUT4 translocation at maximal insulin stimulation were decreased to 52 and 68% of those from wild-type. These data suggest that both IRS-1 and pp60 play a major role in insulin-induced glucose transport in adipocytes, and that pp60 is predominantly involved in regulating this process in the absence of IRS-1.

In fat and muscle tissues, the major targets of insulin-induced glucose uptake, it is known that glucose transporter isoform GLUT4 translocates from a large intracellular pool to the cell surface in response to insulin (1–6), although the signaling mechanism regulating this process is not fully understood. Insulin enhances tyrosine phosphorylation of several endogenous substrates such as insulin receptor substrate (IRS)-1, IRS-2, Grb2-associated binder-1 (Gab1) and Shc (7–12). In addition, pp60, which has been reported to be present in primary adipocytes, is also phosphorylated on tyrosine residues in response to insulin (13). Except for Shc, these proteins have been shown to interact via their phosphotyrosine residues with Src homology (SH) 2 domain in the regulatory 85-kDa subunit (p85) of phosphatidylinositol (PI) 3-kinase (7, 8, 12–14). Inactivation of this enzyme by various methods has shown the enzyme to be important for insulin-stimulated glucose transport and GLUT4 translocation (15–18). Moreover, several reports have shown that insulin stimulation causes the association of IRS-1 with PI 3-kinase thereby targeting this enzyme to GLUT4-containing vesicles, which may explain why insulin, but not other growth factors such as platelet-derived growth factor, stimulates glucose transport (19–23). To investigate the relationship between these endogenous substrates and glucose transport, Quon et al. (24) previously analyzed rat adipocytes transiently transfected with an antisense ribozyme directed against IRS-1. They showed that the expression of this ribozyme reduced the sensitivity of GLUT4 translocation to insulin stimulus although the maximal response was unaffected. Recently, Morris et al. (25) have shown that microinjection of IRS-1-competitive reagents such as fusion protein containing phosphotyrosine binding domain of IRS-1, an anti-IRS-1 antibody, and an NPXY phosphopeptide did not affect the translocation of GLUT4 in response to insulin in 3T3-L1 adipocytes. Although these studies suggest that IRS-1 may not play an essential role in insulin-induced GLUT4 translocation, the analysis of primary adipocytes from genetically engineered mice can more critically evaluate the role of IRS-1 or other tyrosine-phosphorylated proteins in GLUT4 translocation under a physiological condition. In addition, since pp60 is not expressed in cultured cells such as 3T3-L1 adipocytes (13), the role of this protein in insulin signaling cannot be otherwise evaluated.

* This work was supported in part by Grant No. 192125 from the Juvenile Diabetes Foundation International (to T. K.), a grant for diabetes research from the Taisho Pharmaceutical Co., Ltd. (to T. K.), and Grant No. 07671148 from the Ministry of Education, Science, Sports and Culture, Japan (to S. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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1 The abbreviations used are: IRS, insulin receptor substrate; Gab1, Grb2-associated binder-1, SH, Src homology; p85, the regulatory 85-kDa subunit of phosphatidylinositol 3-kinase; PI, phosphatidylinositol; αGLUT4, antibodies specific for GLUT4; αPY, an antibody specific for phosphotyrosine; αPS5, antibodies specific for the 85-kDa subunit of phosphatidylinositol 3-kinase; αIRS-1 and -2, antibodies specific for insulin receptor substrate-1 and -2, respectively; PM, plasma membrane; DTT, dithiothreitol.
As reported in previous studies, we and others have generated mice with a targeted disruption of the IRS-1 gene that exhibited growth retardation and insulin resistance (9, 26). Our recent study (27) analyzing these mice showed that insulin signaling and biological actions such as PI 3-kinase activation, mitogen-activated protein kinase activation, and glucose transport are impaired in muscles from IRS-1 null mice, which was in contrast to the grossly normal signaling and actions in livers from these mice. Tyrosine phosphorylation of IRS-2 in livers from these mice was enhanced to almost the same degree as that of IRS-1 in livers from wild-type mice, whereas in muscles, tyrosine phosphorylation of IRS-2 from these mice was much less (20–30%) than that of IRS-1 in muscles from wild-type. Thus, the difference in the degree of insulin resistance in these two major insulin targets from IRS-1 null mice appeared to depend on the amount of tyrosine phosphorylation of IRS-2 (27). Different from that in muscles and livers, in adipocytes there exists another major tyrosine-phosphorylated protein, pp60, (13), and in these cells the insulin-induced translocation of GLUT4 can be readily examined. We therefore analyzed adipocytes from IRS-1 null mice and showed that insulin-stimulated PI 3-kinase activity, which was approximately 50% of that from wild-type, appeared to be principally mediated by pp60 but not by IRS-2, and that more than 50% of insulin-induced glucose transport as well as GLUT4 translocation was preserved in these cells. These data imply that not only IRS-1 but also pp60 plays a major role in mediating insulin actions in adipocytes.

EXPERIMENTAL PROCEDURES

Materials—Fraction V bovine serum albumin was from Intergen Co. Collagenase type I was from Worthington Biochemical Co. PI (bovine liver) was from Avanti Polar Lipids. Wortmannin was from Wako Pure Chemicals. Rabbit polyclonal antibodies against a 20-amino acids peptide corresponding to the COOH-terminal sequence of GLUT4 (αGLUT4) were kindly supplied by Hoffmann-La Roche. Mouse monoclonal 4G10 (an antibody specific for phosphotyrosine (aPY),) rabbit polyclonal antibodies against glutathione S-transferase fusion proteins containing full-length and NH2-terminal SH2 domain of the 85-kDa subunit of PI 3-kinase (pp85), rabbit polyclonal antibodies against a 14-amino acids peptide corresponding to the COOH-terminal sequence of rat IRS-1 (αIRS-1), and rabbit polyclonal antibodies specific for a glutathione S-transferase fusion protein containing a polypeptide corresponding to amino acids 976–1094 of mouse IRS-2 (αIRS-2) were purchased from Upstate Biotechnology. 125I-labeled protein A (Amersham International), 3-O-methyl-D-1-14C-glucose (NEN Life Science Products, Amersham International), silicon gel 60 plates (Merck), charcoal (Sigma), and α-32P-ATP (Amersham International) were also used in this study.

Preparation of Adipocytes—Isolated adipocytes were prepared from paraperidymal or parametrial fat pads from freshly fed, wild-type (C57 black), or IRS-1 null mice (26) (2 or 3 months old) as described (28). An equal ratio of male and female mice were used for each experiment. The diameter of adipocytes from these mice was different: wild-type was 29.65 ± 0.40 μm and IRS-1 null mice was 25.49 ± 0.47 μm (means ± S.E.), respectively. To analyze an equal amount of cells for each experiment, adipocytes were normalized by total protein content or cell volume suitable for each assay protocol as mentioned below.

Assay of PI 3-kinase activity in immunoprecipitates with aPY—After treatment with insulin for 2 min, the cells were washed three times with ice-cold Krebs-Ringer bicarbonate-Hepes buffer (120 mM NaCl, 4 mM KH2PO4, 1 mM MgSO4, 1 mM CaCl2, 10 mM HCO3−, and 30 mM Hepes, pH 7.4) and lysed in buffer A (25 mM Tris-HCl, pH 7.4, 100 mM NaF, 10 mM Na2VO4, 10 mM Na3PO4, 2 mM phenylmethlysulfonyl fluoride, 10 mM EDTA, and 1% Triton X-100) containing 1% solid 125I-protein A, and the bands were visualized by autoradiography.

Glucose Transport Activity—Glucose transport activity was measured in intact adipocytes by the 3-O-methylglucose uptake technique as described previously (28). To examine the inhibition of insulin-induced glucose transport by unlabeled 3-O-methylglucose or wortmannin, adipocytes were preincubated with various concentrations of unlabeled 3-O-methylglucose or 100 nm wortmannin for 30 min before measurement of [3H]glucose uptake as described (30).

Quantification of Translocated GLUT4—The amount of GLUT4 in each membrane fraction was assessed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with αGLUT4 as described (31). Blots were evaluated by measuring the radioactivity in the corresponding bands.

RESULTS

Assay of PI 3-Kinase Activity in Immunoprecipitates with aPY—To examine the contribution of IRS-1 and other signaling proteins in insulin-induced PI 3-kinase activation, we measured PI 3-kinase activity in the aPY-immunoprecipitates from adipocytes (Fig. 1). The fold stimulation of PI 3-kinase activity in wild-type was 2.72 and 8.79 at 10−7 and 10−8 M insulin, respectively, whereas that in adipocytes from IRS-1 null mice was 2.16 and 4.77 at 10−7 and 10−8 M insulin, respectively; this indicated that more than 50% of insulin-induced PI 3-kinase activity was preserved even in the absence of IRS-1. Thus, there exist other tyrosine-phosphorylated proteins that also play a role in insulin-stimulated PI 3-kinase activation in adipocytes.

Immunoblotting of Phosphotyrosine-containing Proteins from Insulin-treated Adipocytes with aPY—The phosphotyrosine-containing proteins in adipocytes from wild-type and...
IRS-1 null mice were examined by immunoprecipitating the cell lysates with antibodies followed by immunoblotting with αPY (Fig. 2, A and B). In both cell types, insulin stimulated tyrosine phosphorylation of the 95-kDa protein corresponding to the insulin receptor β-subunit (Fig. 2A). In contrast, the major band of the 170-kDa tyrosine-phosphorylated protein corresponding to IRS-1 was observed only in adipocytes from wild-type mice (Fig. 2, A and B). The weak band of the 190-kDa tyrosine-phosphorylated protein corresponding to IRS-2 (8) was detectable in adipocytes from both mouse types (Fig. 2, A and B). However, results using the specific antibodies showed that tyrosine phosphorylation of IRS-2 was much lower than that of IRS-1 in adipocytes from wild-type mice and that IRS-2 was not a major endogenous substrate even in the absence of IRS-1 (Fig. 2B). In contrast, the major band of an approximately 60-kDa tyrosine-phosphorylated protein was observed in adipocytes from both wild-type and IRS-1 null mice in an almost equal amount (Fig. 2A). These results indicate that this 60-kDa protein, but not IRS-2, is the major insulin-induced phosphotyrosine protein in adipocytes from IRS-1 null mice.

Association of PI 3-Kinase with Phosphotyrosine-containing Proteins from Insulin-treated Adipocytes—To analyze phosphotyrosine-containing proteins associated with PI 3-kinase, the cell lysates from insulin-treated adipocytes were immunoprecipitated with αp85, and the immunoprecipitates were immunoblotted with αPY (Fig. 3). Immunoblotting the sheet with αp85 revealed that the amount of p85 expressed in adipocytes from IRS-1 null mice was nearly identical to that in adipocytes from wild-type mice (data not shown). The major band of the 170-kDa tyrosine-phosphorylated protein corresponding to IRS-1 was observed only in adipocytes from wild-type mice. The 190-kDa tyrosine-phosphorylated protein corresponding to IRS-2 was undetectable in adipocytes from either wild-type or IRS-1 null mice under our assay conditions. In contrast, the 60-kDa protein, which was comparable with tyrosine-phosphorylated IRS-1 in adipocytes from wild-type mice, was observed as a major p85-binding protein in adipocytes from both mouse types in an almost equal amount. When the supernatant from the total lysates immunoprecipitated with αp85 were re-immunoprecipitated with αPY, immunoblotting with αPY revealed that most of the 60-kDa protein was immunoprecipitated with αp85 (data not shown). As this protein was abundantly present in primary adipocytes and bound to p85, we presumed that the majority of this 60-kDa substrate was pp60 (13). There were no other tyrosine-phosphorylated proteins detectable, including a polypeptide of 115-kDa corresponding to Gab1 in adipocytes from either wild-type or IRS-1 null mice, suggesting that this protein or other tyrosine-phosphorylated proteins did not appear to have a major function in insulin-induced activation of PI 3-kinase in adipocytes. These results indicate that pp60 plays a principal role in insulin-induced PI 3-kinase activation in adipocytes from IRS-1 null mice.

Glucose Transport Activity—We studied dose response curves of insulin-stimulated glucose transport to analyze the contribution of IRS-1 and other tyrosine-phosphorylated proteins in this process (Fig. 4). Basal glucose transport activity in adipocytes from IRS-1 null mice was significantly elevated as compared with that in cells from wild-type although the reason was unknown. In contrast, the maximally-stimulated glucose transport in adipocytes from these mice was 52.0% of that in cells from wild-type. The sensitivity to insulin was also impaired because the half-maximal response occurred at 9.5 × 10⁻¹⁰ M of insulin in adipocytes from IRS-1 null mice as compared with 3.62 × 10⁻¹⁰ M in those from wild-type. Similar curves were shown in adipocytes from both mice for the inhibition of glucose uptake by unlabeled 3-O-methylglucose, indicating that an apparent affinity for hexose was unchanged (data not shown). Thus, these results show that approximately 50% of insulin-induced glucose transport activity was preserved by a pathway mediated by other signaling proteins. As pp60 is the predominant protein that is tyrosine-phosphorylated and binds to PI 3-kinase in adipocytes from IRS-1 null mice (Figs. 2 and 3), these data also suggest that this protein may play a major role in mediating insulin-stimulated glucose transport in the absence of IRS-1.

Inhibition of Glucose Transport by Wortmannin—Recent studies using inhibitors or expression of mutant proteins have verified that PI 3-kinase is important for insulin-sensitive glu-
We measured glucose uptake in the presence of the PI 3-kinase inhibitor wortmannin to determine whether the preserved glucose transport activity in adipocytes from IRS-1 null mice was also dependent on PI 3-kinase activation (Fig. 5). As described previously (15, 17), exposure of cells to 100 nM wortmannin, a concentration which has been shown to abolish PI 3-kinase activity, completely inhibited insulin-stimulated glucose transport in adipocytes from wild-type mice. The inhibitor also suppressed insulin stimulation of glucose transport in adipocytes from IRS-1 null mice. These data indicate that PI 3-kinase activation is also essential for glucose transport regulated by an IRS-1-independent pathway, and substantiate our hypothesis that pp60 is a key factor in this process.

Quantification of Translocated GLUT4—The role of IRS-1 and other tyrosine-phosphorylated proteins in GLUT4 translocation was investigated by membrane fractionation and immunoblotting with αGLUT4 (Fig. 6). The amount of GLUT4 expressed in adipocytes from IRS-1 null mice was nearly identical to that in adipocytes from wild-type mice (data not shown). Similar to basal glucose transport activity (Fig. 4), the amount of GLUT4 in plasma membrane (PM) of unstimulated adipocytes from IRS-1 null mice was higher than that from wild-type. The amount of GLUT4 translocated to PM in response to $10^{-7}$ M and $10^{-9}$ M insulin in adipocytes from IRS-1 null mice was reduced to 67.9 and 42.1% of that from wild-type, respectively (Fig. 6, and data not shown). Thus, the absence of IRS-1 partially impaired insulin-induced GLUT4 translocation, as
munoprecipitated with phosphorylated proteins have been identified (13, 32–38). Among them, mice (Fig. 2). Recently, a variety of 60-kDa tyrosine-phosphorylated protein in adipocytes from IRS-1 null insulin actions in adipocytes from IRS-1 null mice. In fact, our there should be other substrates that mediate the residual actions in adipocytes from wild-type mice. On the other hand, pp60 played a role in mediating at least 30–50% of these insulin decrease in glucose transport was due to the defect in GLUT4 translocation. Moreover, we demonstrated that PI 3-kinase activated by insulin was decreased because 1) insulin stimulated more PI 3-kinase activity at $10^{-7}$ m in IRS-1 null mice than it did at $10^{-9}$ m in wild-type mice, but glucose transport activity at $10^{-7}$ m insulin in IRS-1 null mice was only about 50% of that at $10^{-9}$ m insulin in wild-type; and 2) even though $10^{-9}$ m insulin in IRS-1 null mice stimulated less than 50% of the glucose transport activity it did in wild-type mice, at this concentration of insulin there was no significant difference in PI 3-kinase activity between these mouse types (Figs. 1 and 4). Therefore, these data also indicate that glucose transport does not simply parallel PI 3-kinase activity although PI 3-kinase is essential for this process. These discrepancies may be due to the existence of signaling deficits other than PI 3-kinase activation in adipocytes from IRS-1 null mice, which may potentially reflect the lower efficiency of pp60 to target PI 3-kinase to GLUT4-containing vesicles or the presence of other additional signaling molecules interacting with IRS-1 but not pp60.

Comparing the extent of GLUT4 translocation to plasma membrane with that of glucose transport activity, the impairment of GLUT4 translocation at $10^{-7}$ m insulin in these cells appeared to be unexpectedly small as compared with that of glucose transport activity (Figs. 4 and 6). In contrast, at $10^{-9}$ m insulin, which caused half-maximal activation of glucose transport in these cells, the degree of impairment of GLUT4 translocation to PM was in parallel with the extent of decrease in glucose transport activity. As the discrepancy between glucose transport activity and GLUT4 translocation is likely to be due to the presence of glucose transporters that are catalytically inactive or partially occluded in plasma membrane (5, 41), the present data may indicate that the IRS-1-mediated pathway is also involved in the activation of these glucose transporters. However, it is also possible that the plasma membrane is contaminated by other membrane fractions that may cause this discrepancy.

After submitting this paper, Lavan et al. (42) reported the isolation of the cDNA of pp60 which was designated as IRS-3. The predicted amino acid sequence of IRS-3 contains an NH$_2$-terminal pleckstrin homology domain followed by a tyrosine-phosphorylation domain and a group of potential tyrosine phos-

![Figure 6. Subcellular distribution of GLUT4 protein in insulin-stimulated adipocytes from wild-type (WT) and IRS-1 null mice. PM and low density microsome (LDM) fractions from adipocytes treated or untreated with $10^{-7}$ m insulin were prepared, and GLUT4 was immunoblotted as described under "Experimental Procedures." Autoradiography and quantitation of the amount of GLUT4 in intracellular fractions in adipocytes are shown. The amount of GLUT4 translocated to plasma membrane in response to insulin ($\Delta$PM) is also shown in this figure. Values represent the results of the means and S.E. of three independent experiments. *, $p < 0.05$ versus wild-type; **, $p < 0.01$ versus wild-type.]

well as glucose transport, in adipocytes. The results imply that not only IRS-1 but also pp60 takes part in stimulating translocation of GLUT4 in these cells.

**DISCUSSION**

In this study, we analyzed adipocytes from IRS-1 null mice to clarify the role of IRS-1 and other endogenous substrates in insulin-stimulated translocation of GLUT4. As shown in Figs. 4 and 6, we clarified that the decrease in glucose transport was due to the defect in GLUT4 translocation. Moreover, we demonstrated that PI 3-kinase activated by insulin was decreased by 50% (Fig. 1). From these data, we estimated that IRS-1 played a role in mediating at least 30–50% of these insulin actions in adipocytes from wild-type mice. On the other hand, there should be other substrates that mediate the residual insulin actions in adipocytes from IRS-1 null mice. In fact, our data demonstrated that the 60-kDa protein was the major tyrosine-phosphorylated protein in adipocytes from IRS-1 null mice (Fig. 2). Recently, a variety of 60-kDa tyrosine-phosphorylated proteins have been identified (13, 32–38). Among them, we considered this 60-kDa protein as pp60 (13) since 1) a major part of the tyrosine-phosphorylated 60-kDa protein was immunoprecipitated with pp65 (Fig. 3, and data not shown), and 2) this protein was detected in adipocytes but not in livers or muscles from wild-type and IRS-1 null mice (Figs. 2 and 3) (27). Of note, unlike muscles and livers in which IRS-2 was the alternate substrate in the absence of IRS-1 (27, 39), the amount of tyrosine-phosphorylated IRS-2 in adipocytes was very low (Figs. 2 and 3) (40). Thus, we conclude that pp60 but not IRS-2 is the major substrate that principally associates with PI 3-kinase in adipocytes from IRS-1 null mice. We can presume from these data that the pp60/PI 3-kinase complex regulates the insulin-induced translocation of GLUT4 in adipocytes from IRS-1 null mice. Interestingly, the amount of pp60 tyrosine phosphorylation does not appear to increase in adipocytes from IRS-1 null mice (Fig. 2), which also suggests that IRS-1 and pp60 are additively involved in mediating insulin actions in adipocytes. This is in contrast with the livers and muscles in which tyrosine phosphorylation of IRS-2 is enhanced by the lack of IRS-1 (27, 39), indicating that IRS-1 but not IRS-2 is preferentially phosphorylated on tyrosine residues in wild-type mice.

As shown in Fig. 5, treatment of the cells with the PI 3-kinase inhibitor wortmannin abolished insulin-sensitive glucose transport in both cell types, which suggested that PI 3-kinase activity is also essential for this IRS-1-alternative pathway that appears to be regulated by pp60 (Figs. 3 and 5). However, in this study we found that when PI 3-kinase activity was compared with glucose transport, glucose transport was not strictly correlated with the extent of PI 3-kinase activation because 1) insulin stimulated more PI 3-kinase activity at $10^{-7}$ m in IRS-1 null mice than it did at $10^{-9}$ m in wild-type mice, but glucose transport activity at $10^{-7}$ m insulin in IRS-1 null mice was only about 50% of that at $10^{-9}$ m insulin in wild-type; and 2) even though $10^{-9}$ m insulin in IRS-1 null mice stimulated less than 50% of the glucose transport activity it did in wild-type mice, at this concentration of insulin there was no significant difference in PI 3-kinase activity between these mouse types (Figs. 1 and 4). Therefore, these data also indicate that glucose transport does not simply parallel PI 3-kinase activity although PI 3-kinase is essential for this process. These discrepancies may be due to the existence of signaling deficits other than PI 3-kinase activation in adipocytes from IRS-1 null mice, which may potentially reflect the lower efficiency of pp60 to target PI 3-kinase to GLUT4-containing vesicles or the presence of other additional signaling molecules interacting with IRS-1 but not pp60.
phosphorylation sites, four of which are in the YYXM motif that binds to the SH2 domains of PI 3-kinase. Although the tissue distribution of IRS-3 or the functions of this protein in insulin-induced signal transduction have not been demonstrated, its structure indicates that IRS-3 may interact with the activated insulin receptor and associate via its phosphorylated tyrosine residues with SH2-containing proteins such as PI 3-kinase (42). These findings are consistent with our hypothesis that pp60 plays a role in insulin-induced glucose transport in adipocytes.

Taken together, the analysis of adipocytes from IRS-1 null mice showed that 1) at least 30–50% of insulin-induced PI 3-kinase activity, glucose transport, and GLUT4 translocation in adipocytes from wild-type mice appears to be regulated by IRS-1; 2) insulin-induced PI 3-kinase, glucose transport, and GLUT4 translocation in adipocytes from IRS-1 null mice, which are approximately 50% of that from wild-type, may be principally mediated by pp60 but not by IRS-2 or other endogenous substrates; and 3) not only IRS-1 but also pp60 may play a major role in mediating insulin-stimulated glucose transport in adipocytes.

Acknowledgments—We thank Hoffmann-La Roche for providing us aGLUT4 antibodies. We also thank Dr. Osamu Hazeki (Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo) for helping with the immunoblotting of phosphotyrosine-containing proteins from adipocytes.

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