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A novel IRS-1-associated protein, DGK ζ regulates GLUT4 translocation in 3T3-L1 adipocytes

TingYu Liu, BuChin Yu, Mamoru Kakino, Hitoshi Fujimoto, Yasutoshi Ando, Fumihiko Hakuno & Shin-Ichiro Takahashi

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Insulin receptor substrates (IRSs) are major targets of insulin receptor tyrosine kinases. Here we identified diacylglycerol kinase zeta (DGK ζ) as an IRS-1-associated protein, and examined roles of DGK ζ in glucose transporter 4 (GLUT4) translocation to the plasma membrane. When DGK ζ was knocked-down in 3T3-L1 adipocytes, insulin-induced GLUT4 translocation was inhibited without affecting other mediators of insulin-dependent signaling. Similarly, knockdown of phosphatidylinositol 4-phosphate 5-kinase 1 α (PIP5K1 α), which had been reported to interact with DGK ζ , also inhibited insulin-induced GLUT4 translocation. Moreover, DGK ζ interacted with IRS-1 without insulin stimulation, but insulin stimulation decreased this interaction. Over-expression of sDGK ζ (short-form DGK ζ), which competed out DGK ζ from IRS-1, enhanced GLUT4 translocation without insulin stimulation. Taking these results together with the data showing that cellular PIP5K activity was correlated with GLUT4 translocation ability, we concluded that IRS-1-associated DGK ζ prevents GLUT4 translocation in the absence of insulin and that the DGK ζ dissociated from IRS-1 by insulin stimulation enhances GLUT4 translocation through PIP5K1 α activity.

It is well known that insulin is a major anabolic hormone, which maintains glucose homeostasis by stimulating glucose uptake and utilization in muscle and adipose tissues and suppressing glucose production in liver. Glucose uptake in muscle and adipose tissues is induced by translocation of glucose transporter 4 (GLUT4) from multiple intracellular compartments to the plasma membrane (PM). In general, insulin, binding to its specific receptor on the plasma membrane, induces activation of intrinsic tyrosine kinase activity^{1,2}. Activated receptor phosphorylates several intracellular substrates including insulin receptor substrates (IRSs). Tyrosine phosphorylation of IRSs leads to their binding to several intermediate signaling molecules containing SH2 domains including a p85 phosphatidylinositol (PI) 3-kinase regulatory subunit and Grb2. These bindings allow activation of the distinct signaling pathways, PI 3-kinase cascade and Ras-MAPK cascade. In particular, activation of PI 3-kinase leads to activation of Ser/Thr kinase, Akt. Activated Akt kinase phosphorylates some substrates including AS160 which regulated the GLUT4 translocation from intracellular vesicles to the PM and subsequent glucose uptake³⁻⁸. Thus IRS-1 is an important mediator of insulin signal activation required for GLUT4 translocation and glucose uptake.

Recently, however, we found that IRS-1 formed high-molecular mass complexes in 3T3-L1 adipocytes not through recognition of tyrosine phosphorylation⁹. Furthermore, we and other groups have shown that some IRS-associated proteins (IRSAPs), Nexilin, PHIP, 53BP2S, HSP90, Nedd4 or GKAP42 modulated insulin/insulin-like growth factor (IGF)-dependent tyrosine phosphorylation of IRS-1 or IRS-2 in insulin/IGF targeted cells¹⁰⁻¹⁵. Further identification of other IRS-associated proteins and elucidation of their roles are essential prerequisites for understanding alternative mechanisms of modulation of insulin-mediated signals and bioactivities.

In this study, we identified diacylglycerol kinase (DGK) ζ as a novel IRS-1-associated protein. DGK ζ belongs to type IV among five DGK family groups^{16,17}. Each type of DGK has a unique character in terms of regulatory mechanism, binding protein, and subcellular localization. DGK had been reported to catalyze phosphorylation of diacylglycerol (DAG) to generate phosphatidic acid (PA) and to regulate various cellular responses by changing the balance between DAG and PA levels¹⁸⁻²⁰. DGK ζ contains two DAG binding domains (C1 domain), the myristoylated alanine-rich C kinase substrate (MARCKs) domain, catalytic domain and ankyrin repeat domain²¹. Recently it was reported that DGK ζ had roles in regulation of the transcription factors, p53 and NF- κ B²²⁻²⁴.

Departments of Animal Sciences and Applied Biological Chemistry, Graduate School of Agriculture and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, Japan. Correspondence and requests for materials should be addressed to F.H. (email: ahakuno@mail.ecc.u-tokyo.ac.jp) or S.-I.T. (email: atkshin@mail.ecc.u-tokyo.ac.jp)

DGK ζ had been reported to interact and form complexes with certain enzymes, which are activated by DAG (Ras-GRP, cPKC) or PA (PIP5K1 α), suggesting that DGK ζ complex formation might regulate the direction or the balance of signals activated by DAG or PA^{25–28}. In this study, the role of PIP5K1 α was also investigated.

Our present data indicated that both DGK ζ and PIP5K1 α regulated GLUT4 translocation to the PM by a novel mechanism. Interestingly, under basal conditions, DGK ζ and IRS-1 interacted with each other, and IRS-1-associated DGK ζ inhibited GLUT4 translocation. Once insulin signaling was activated, DGK ζ was dissociated from IRS-1 and this DGK ζ enhanced GLUT4 translocation through PIP5K1 α activity.

Results

We identified diacylglycerol kinase zeta (DGK ζ) as a novel IRS-1-associated protein. To identify proteins, which interact with IRS-1 and play some roles in GLUT4 translocation, we searched for IRS-1-associated proteins from a cDNA library of 3T3-L1 adipocytes by yeast two-hybrid screening utilizing full-length IRS-1 as a bait. Through this screening we identified 14-3-3 family proteins, μ chain of AP-1 complex protein and 53BP2S/ASPP2, which had been already reported^{11,12,29}. In addition to these proteins, we identified diacylglycerol kinase zeta (DGK ζ) as a novel IRS-1-associated protein. The interaction of DGK ζ with IRS-1 was confirmed by yeast two-hybrid assay (Fig. 1a). The interaction between DGK ζ and IRS-1 was detected in co-immunoprecipitation assays (Fig. 1b). Next, to confirm the interaction of endogenous IRS-1 and DGK ζ , cell lysates of fully differentiated 3T3-L1 adipocytes were immunoprecipitated with anti-DGK ζ or anti-IRS-1 antibody followed by immunoblotting analysis using anti-IRS-1 or anti-DGK ζ antibody. As shown in Fig. 1c, the interaction between endogenously expressed IRS-1 and DGK ζ was detected in 3T3-L1 adipocytes. Next, to define the regions of DGK ζ required for interaction with IRS-1, we made deletion constructs of DGK ζ and the interaction was examined by co-immunoprecipitation assay. Mutants which contained the C1 domain (full length, D2 and D3 mutant) could interact with IRS-1. But a mutant that lacks the C1 domain (D1 mutant) could not interact with IRS-1 (Fig. 1d). These data indicated that the N-terminal region containing the C1 domain is responsible for the interaction with IRS-1. Based on structural motifs, all DGK family proteins have C1 domains. Then we examined whether the other DGK family proteins could interact with IRS-1. Surprisingly, DGK α , DGK β , DGK γ , DGK δ , DGK ϵ , DGK η and DGK ι did not interact with IRS-1 even though these isoforms have the C1 domain. Only DGK ζ among various DGKs could interact with IRS-1 (Fig. 1e and Supplemental Fig. S1).

Knockdown or overexpression of DGK ζ modulates GLUT4 translocation. Because insulin-induced GLUT4 translocation is an important event, we next examined the effects of knockdown of DGK ζ on insulin-dependent GLUT4 translocation and signaling activation in 3T3-L1 adipocytes. As shown in Fig. 2a, insulin stimulation resulted in an approximately 6-fold increase in the control cells. However, DGK ζ knockdown significantly suppressed insulin-induced GLUT4 translocation to the PM. Interestingly, under basal conditions (without insulin stimulation), DGK ζ knockdown enhanced the level of GLUT4 translocation, suggesting that DGK ζ plays some roles in GLUT4 translocation even under basal conditions. Next, the effects of DGK ζ knockdown on the insulin signaling pathways were investigated. 3T3-L1 adipocytes were electroporated with siRNA against DGK ζ , resulting in a marked reduction in the DGK ζ protein level without any significant effect on the levels of other proteins (Fig. 2b). The total cell lysates from DGK ζ knockdown 3T3-L1 adipocytes were immunoprecipitated with anti-IRS-1 antibody, and assessed for insulin-induced IRS-1 tyrosine phosphorylation or binding of the p85 PI 3-kinase regulatory subunit to IRS-1. As shown in Fig. 2b, we found that DGK ζ knockdown did not affect insulin-induced IRS-1 tyrosine phosphorylation nor binding of the p85 PI 3-kinase regulatory subunit to IRS-1. Consistent with these effects of DGK ζ knockdown, the levels of insulin-induced Akt phosphorylation were also not affected (Fig. 2b). As shown in Fig. 2c, glucose uptake was significantly suppressed in DGK ζ knockdown adipocytes under both basal and insulin-stimulated conditions. We then examined the effects of DGK ζ overexpression on the insulin-induced GLUT4 translocation. As shown in Fig. 2d, DGK ζ overexpression significantly enhanced the insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. Furthermore, we measured the insulin signal activation in DGK ζ -overexpressed cells. Because of low transfection efficiency by electroporation into 3T3-L1 cells, we used CHO cells which have similar signal transduction to 3T3-L1, and are abundantly used for transfection^{30,31}. The insulin-induced IRS-1 tyrosine phosphorylation and association with p85 PI 3-kinase regulatory subunit and Akt phosphorylation were not affected in the DGK ζ -overexpressing cells (Fig. 2e).

Knockdown or overexpression of PIP5K1 α modulates the insulin-induced GLUT4 translocation. PIP5K1 α had been reported to interact with DGK ζ ²⁷. Moreover, PIP5K1 α was activated by PA, which is produced by DGK ζ activity²⁷. These results led us to assess the roles of PIP5K1 α in insulin-induced GLUT4 translocation to the PM. We knocked down PIP5K1 α in 3T3-L1 adipocytes and measured the GLUT4 translocation and signal activation. As shown in Fig. 3a, PIP5K1 α knockdown significantly suppressed insulin-induced GLUT4 translocation to PM. Under this condition, insulin-stimulated IRS-1 tyrosine phosphorylation and association with the p85 PI 3-kinase regulatory subunit were not affected. However, the Akt phosphorylation was significantly suppressed (Fig. 3b). As shown in Fig. 3c, insulin-induced glucose uptake was also significantly suppressed in PIP5K1 α knockdown adipocytes. We then examined the effects of PIP5K1 α overexpression on GLUT4 translocation using 3T3-L1 adipocytes transfected with a HA-PIP5K1 α expressing plasmid together with pGLUT4-myc-GFP plasmid. As shown in Fig. 3d, the PIP5K1 α overexpression enhanced GLUT4 translocation both under basal and insulin stimulated conditions. We then transfected CHO cells with HA-PIP5K1 α expressing plasmids and measured the insulin signal activation. The insulin-dependent IRS-1 tyrosine phosphorylation and association with p85 PI3K regulatory subunit and Akt phosphorylation were not changed in PIP5K1 α overexpressing cells (Fig. 3e).

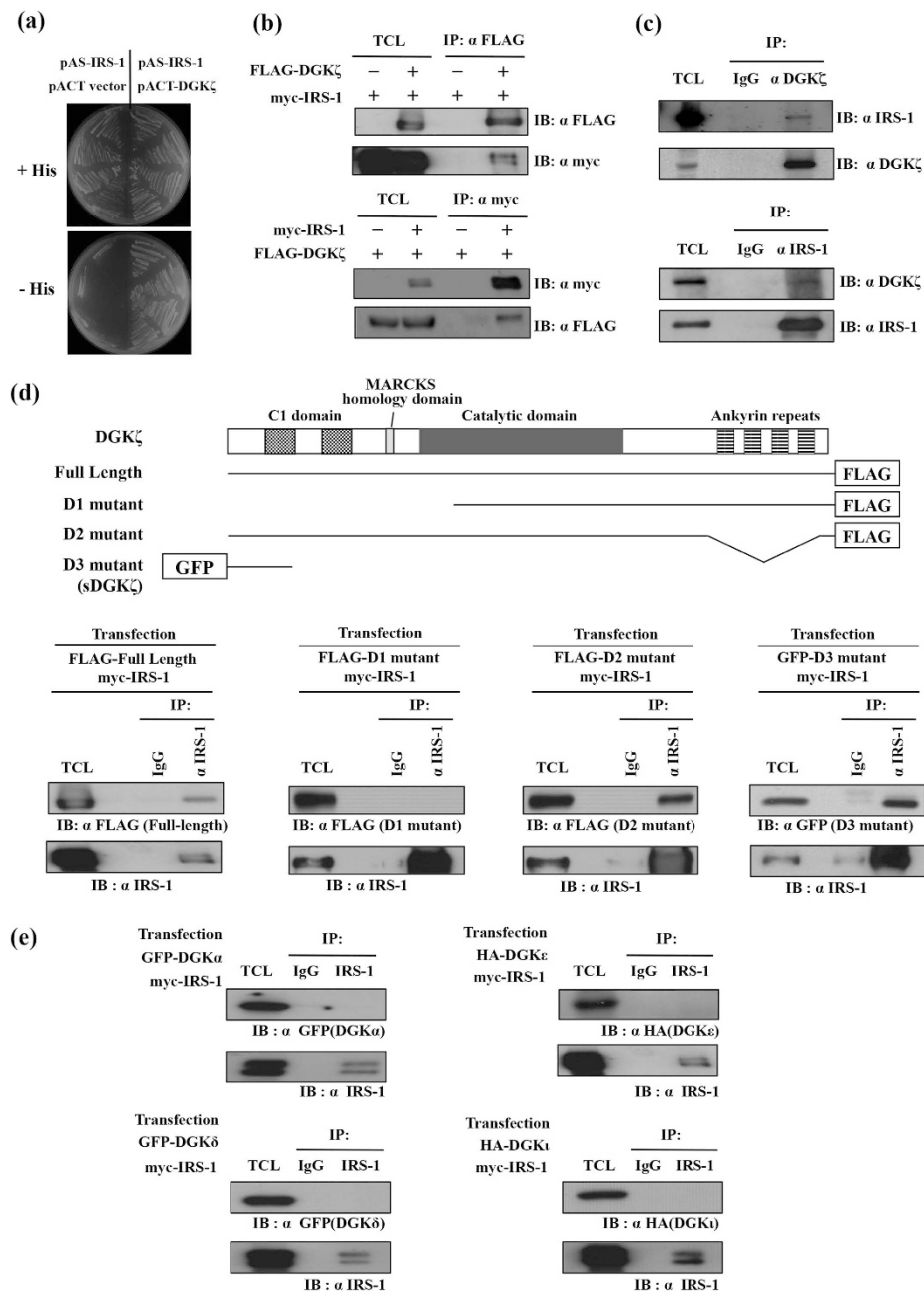


Figure 1. Interaction of DGK ζ with IRS-1. (a) pAS-IRS-1 which expresses full length of IRS-1 fused with Gal4-DNA binding domain was used as bait. pACT-DGK ζ which expresses N-terminal fragment of DGK ζ fused with Gal4-activation domain was used as prey. Yeast strain, AH109 was used for the two-hybrid assay. Yeast transformants were grown on the medium lacking leucine and tryptophan (SD + His) or the medium lacking leucine, tryptophan and histidine (SD-His). (b) HEK293T cells were transfected with pFLAG-DGK ζ (expressing FLAG-tagged DGK ζ) and pmyc-IRS-1 (expressing myc-tagged IRS-1). Cells were serum-starved for 4 h and cell lysates were prepared (TCL). Cell lysates were immunoprecipitated by FLAG-agarose beads or anti-myc antibody. TCL and immunoprecipitates¹⁴ were subjected to immunoblotting analysis with indicated antibodies. (c) Fully differentiated 3T3-L1 adipocytes were serum-starved for 4 h and TCL were prepared. Cell lysates were immunoprecipitated by anti-DGK ζ antibody, anti-IRS-1 antibody or control IgG respectively. TCL or IP were subjected to immunoblotting analysis using indicated antibody. (d) A schematic structure of DGK ζ protein is shown. Two C1 domains are shown in dotted boxes. Myristoylated alanine-rich C kinase substrate (MARCKS) domains indicated by the light grey box. The dark grey box indicates the catalytic domain and ankyrin repeat domain is shown in striped boxes. Full-length DGK ζ and three deletion constructs of DGK ζ (D1, D2 and D3) were tagged with FLAG or GFP. Each mutant and myc-IRS1 was co-expressed in HEK293T cells. Cell lysates were immunoprecipitated with anti-IRS-1 antibody or control IgG. IP or TCL were subjected to immunoblotting analysis using anti-FLAG or GFP antibody. (e) GFP-DGK α , GFP-DGK δ , HA-DGK ϵ or HA-DGK ι was co-expressed with myc-IRS-1 in HEK293T cells. Cell lysates were immunoprecipitated with anti-IRS-1 antibody and the interaction was detected with indicated antibody.

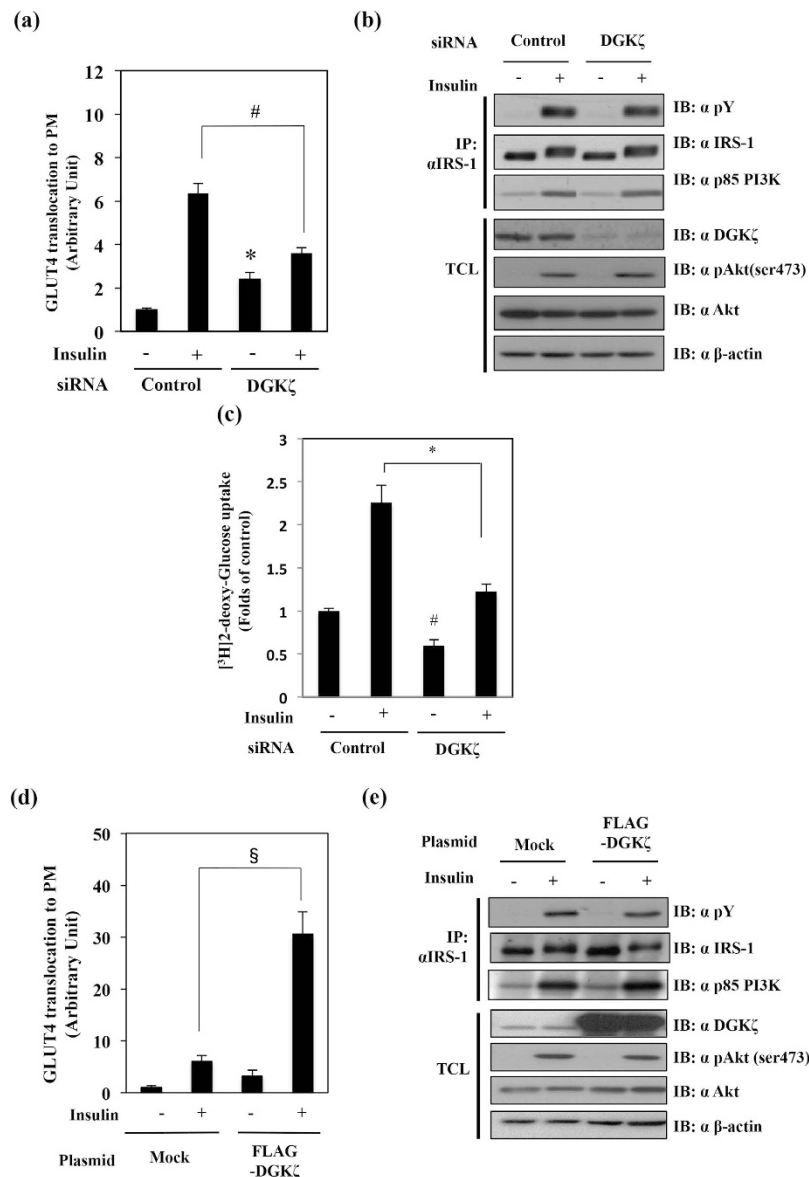


Figure 2. Effects of knockdown or overexpression of DGKζ on the GLUT4 translocation, glucose uptake and insulin signaling. (a) Fully differentiated 3T3-L1 adipocytes were electroporated with non-relevant control siRNA (control) or DGKζ siRNA together with the pGLUT4-myc-GFP plasmid. Beginning 24 h after the electroporation the cells were serum-starved for 4 h and treated with or without insulin (100 nM) for 20 min. Cells were fixed without permeabilization and stained with anti-myc antibody as described in the Materials and Methods. The ratio of (exofacial-exposed Myc epitope fluorescence/total GFP fluorescence) was quantified. The results are presented as the means ± S.E.M. of twenty cells. (b) Cell lysates were immunoprecipitated with the indicated antibodies, and IP or TCL were immunoblotted with the indicated antibodies. (c) 3T3-L1 adipocytes were electroporated with control siRNA or DGKζ siRNA were starved for 16–24 h, and followed by stimulation of 100 nM insulin for 20 min. Then 0.1 mM 2-deoxy-D-glucose containing 10 μCi/ml 2-deoxy-D-[2,6-³H] glucose was added, and cells were incubated for 4 min at 37 °C. These cells were harvested for glucose uptake as described in the methods. (d) 3T3-L1 adipocytes were electroporated with mock vector or pFLAG-DGKζ together with pGLUT4-myc-GFP plasmids. Cells were serum-starved for 4 h, followed by stimulation with insulin. GLUT4 translocation was then measured. (e) CHO cells were transfected with mock vector or pFLAG-DGKζ plasmid. TCL from transfectants were immunoprecipitated with the anti-IRS-1 antibody. IP or TCL were immunoblotted with the indicated antibodies. These are representative data from experiments independently performed at least three times. **p* < 0.05 as compared with control group. #*p* < 0.05 as compared with control plus insulin treatment group. §*p* < 0.05 as compared with the mock plus insulin treatment group.

Insulin stimulation dissociated DGKζ and PIP5K1α from IRS-1. To evaluate roles of the interaction between IRS-1 and DGKζ in GLUT4 translocation, the effect of insulin stimulation on the interaction was studied. Serum-starved CHO cells were stimulated with insulin for various times. Total cell lysates were

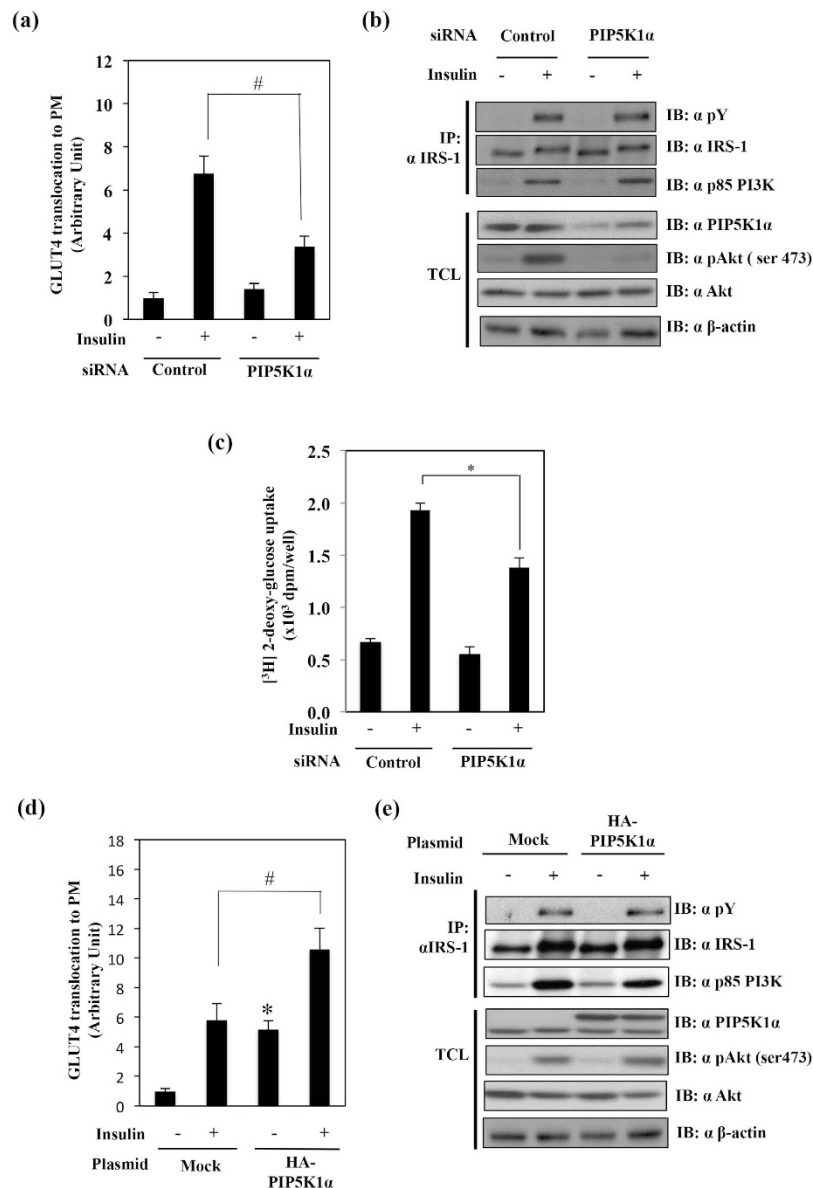


Figure 3. Effects of knockdown or overexpression of PIP5K1 α on GLUT4 translocation, glucose uptake and insulin signaling. (a) 3T3-L1 adipocytes were electroporated with non-relevant siRNA (control) or PIP5K1 α siRNA along with the pGLUT4-myc-GFP plasmid. GLUT4 translocation was measured as described in Fig. 2a. The results are presented as the means \pm S.E.M. of twenty cells. (b) Fully differentiated 3T3-L1 adipocytes were electroporated with control siRNA or PIP5K1 α siRNA. Activation of the canonical insulin signal pathway was assessed as described in Fig. 2b. (c) 3T3-L1 adipocytes were electroporated with control siRNA or PIP5K1 α siRNA were starved for 16–24 h, and followed by stimulation with insulin. These cells were harvested for glucose uptake as described in Fig. 2c. (d) 3T3-L1 adipocytes were electroporated with mock or pHA-PIP5K1 α together with pGLUT4-myc-GFP plasmids followed by insulin treatment. GLUT4 translocation was measured as described in Fig. 2a. (e) CHO cells were transfected with mock or pHA-PIP5K1 α plasmid. Activation of insulin signaling was examined as described in Fig. 2e. These are representative data from experiments independently performed at least three times. # $p < 0.05$ as compared with control plus insulin treatment group. * $p < 0.05$ as compared with control group.

immunoprecipitated by anti-IRS-1 antibody and immunoprecipitates were subjected to immunoblotting analysis with anti-DGK ζ antibody. We detected the interaction of IRS-1 with DGK ζ and PIP5K1 α in the absence of insulin stimulation (Fig. 4b). These data suggested that PIP5K1 α and DGK ζ formed a ternary complex with IRS-1 without insulin stimulation. However in the insulin stimulated cell lysates, the interaction of IRS-1 with DGK ζ or PIP5K1 α was not observed, indicating that insulin stimulation dissociated this complex (Fig. 4a).

Overexpression of sDGK ζ (short-form DGK ζ), which blocked the interaction of IRS-1 with DGK ζ , enhanced GLUT4 translocation. As shown in Fig. 1c, the N-terminal region of DGK ζ , which contained

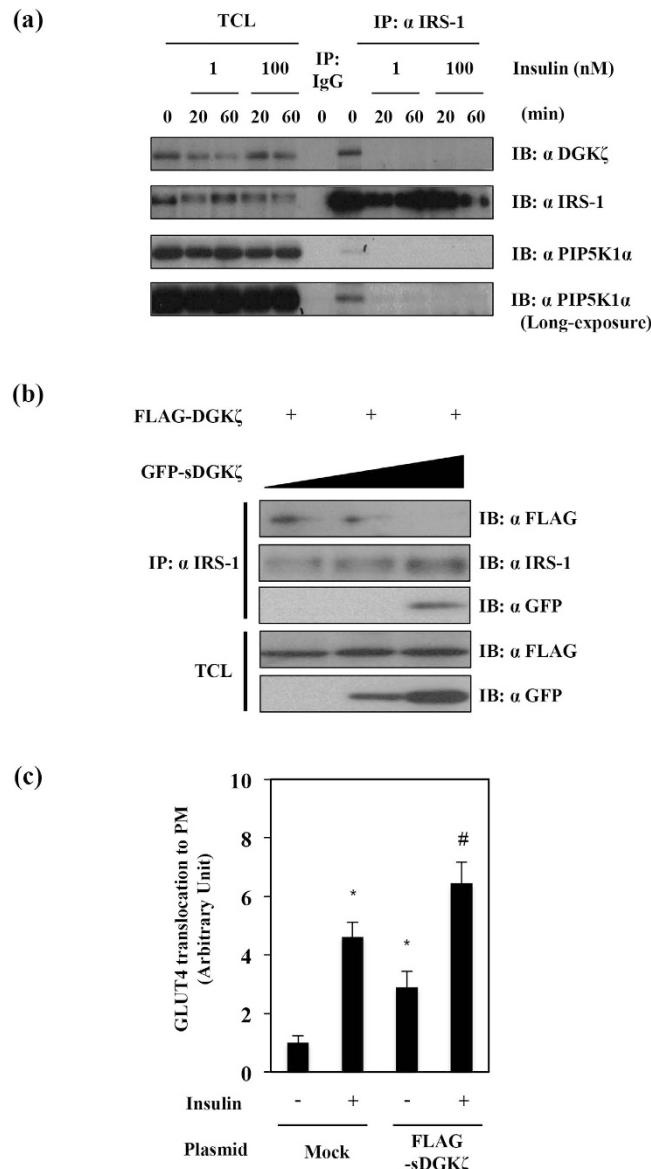


Figure 4. Effects of insulin treatment or sDGK ζ overexpression on interaction between DGK ζ and IRS-1 and GLUT4 translocation. (a) CHO cells were serum-starved for 18 h followed by treatment with insulin (1 nM or 100 nM) for 20 and 60 min, and then TCL were immunoprecipitated with anti-IRS-1 antibody or control IgG. TCL and IP were subjected to immunoblotting analysis with indicated antibodies. (b) HEK293T cells were transfected with pFLAG-DGK ζ plasmid and different amounts of pGFP-sDGK ζ plasmid. Cell lysates were prepared from each cell treatment and were immunoprecipitated with anti-IRS-1 antibody. TCL or IP were subjected to immunoblotting analysis with the indicated antibodies. (c) 3T3-L1 adipocytes were electroporated with mock vector and pFLAG-sDGK ζ together with pGLUT4-myc-GFP, and GLUT4 translocation ratio was measured in these cells. The results are presented as the means \pm S.E.M. of twenty cells. * $p < 0.05$ as compared with control group, # $p < 0.05$ as compared with mock group. These are representative data from experiments independently performed at least three times.

one C1 domain, was necessary and sufficient for the interaction with IRS-1. We prepared a construct expressing the GFP-tagged sDGK ζ , which contained only this C1 domain and the effect of the sDGK ζ overexpression on the interaction between DGK ζ and IRS-1 was examined. FLAG-tagged full length DGK ζ together with increasing amounts of GFP-sDGK ζ were expressed in CHO cells, and the interaction between IRS-1 and FLAG-DGK ζ was assessed. As shown in the left lane in Fig. 4b, we detected FLAG-DGK ζ in the immunoprecipitates using the anti-IRS-1 antibody. However, when GFP-sDGK ζ was over-expressed (Fig. 4b right lane), the interaction between IRS-1 and sDGK ζ was observed but the interaction between IRS-1 and FLAG-DGK ζ was inhibited. This indicated that GFP-sDGK ζ overexpression competitively inhibited the interaction between IRS-1 and DGK ζ . To evaluate the role of the interaction between IRS-1 and DGK ζ in GLUT4 translocation, we overexpressed sDGK ζ in 3T3-L1

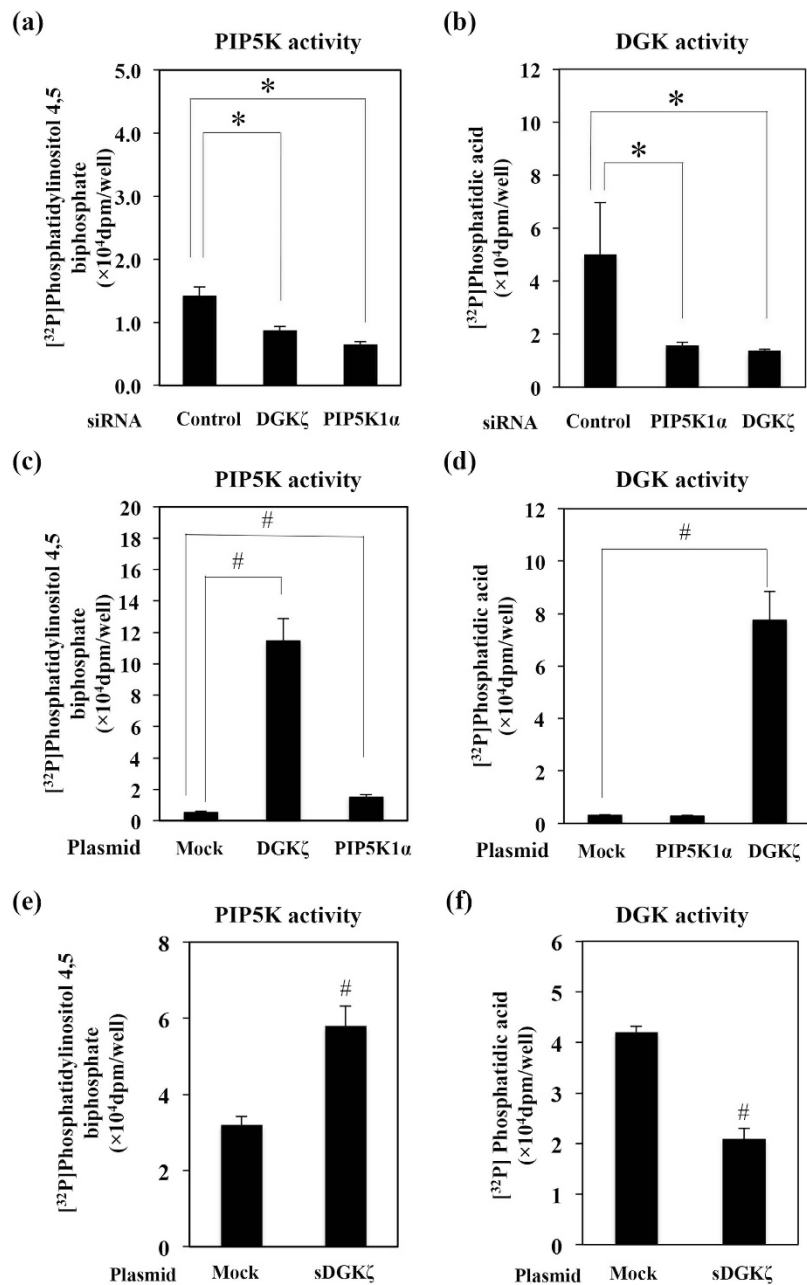


Figure 5. Effects of knockdown or overexpression of DGK ζ or PIP5K1 α on DGK or PIP5K activity. 3T3-L1 adipocytes were electroporated with non-relevant control siRNA (control), DGK ζ siRNA, or PIP5K1 α siRNA. Three days after electroporation, cell lysates were prepared to measure PIP5K activity (a) or DGK activity (b), respectively. Also, the CHO cells were transfected with mock vector or pFLAG-DGK ζ plasmid, or pHA-PIP5K1 α plasmid. Twenty four hours later, cell lysates were prepared to measure PIP5K activity (c) or DGK activity (d). CHO cells were transfected with mock vector or pGFP-sDGK ζ plasmid for measuring the PIP5K and DGK activities (e,f). The methods to measure PIP5K or DGK activity were described in “Materials and Methods”. The results are presented at the means \pm S.E.M. of three samples. These are representative data from experiments independently performed at least three times. The results are presented at the means \pm S.E.M. of five samples. * $p < 0.05$ as compared with control group, # $p < 0.05$ as compared with mock group.

adipocytes and GLUT4 translocation was measured in these cells. As shown in Fig. 4c, GLUT4 translocation was enhanced in sDGK ζ overexpressed 3T3-L1 adipocytes both with and without insulin stimulation.

DGK ζ and PIP5K1 α regulate each other's activity. We measured DGK or PIP5K activity in DGK ζ or PIP5K1 α knockdown cells. Fully differentiated 3T3-L1 adipocytes were electroporated with DGK ζ or PIP5K1 α siRNA and the electroporated cells were harvested to measure DGK or PIP5K activity. As shown in Fig. 5a, PIP5K activity was significantly suppressed in both DGK ζ knockdown and PIP5K1 α knockdown cells. In addition, DGK activity was also suppressed in both knockdown cells (Fig. 5b). Next, we overexpressed DGK ζ or PIP5K1 α in

CHO cells and DGK or PIP5K activity in these cell lysates was measured. As shown in Fig. 5c, PIP5K activity was enhanced in PIP5K1 α overexpressed CHO cells. Surprisingly, DGK ζ overexpression enhanced PIP5K activity more strongly than PIP5K1 α overexpression (Fig. 5c). On the other hand, DGK ζ overexpression enhanced DGK activity in CHO cells, but PIP5K1 α overexpression could not enhance DGK activity (Fig. 5d). Finally we measured DGK activity or PIP5K activity in the sDGK ζ -overexpressing CHO cells. sDGK ζ overexpression suppressed DGK activity whereas it significantly enhanced PIP5K activity (Fig. 5e,f).

Discussion

IRSs are major substrates of insulin receptor tyrosine kinases. Phosphorylated tyrosine residues of IRSs bind to specific signaling molecules including the p85 PI 3-kinase regulatory subunit, resulting in activation of the downstream signals. Thus the interaction of IRSs with signaling molecules through recognition of tyrosine phosphorylation is believed to play critical roles in activation of the downstream signaling pathways. However, recently, we have reported that IRSs interact with various proteins (IRS-associated proteins; IRSAPs) even without insulin stimulation and form a high-molecular-mass complex (IRSome)³². We have already succeeded in identifying some IRSAPs, and we and others showed that IRSAPs have roles in modulation of insulin-induced IRS tyrosine phosphorylation (Nexillin, PHIP, 53BP2S, HSP90, Nedd4 and GKAP42)^{10–15}, regulation of IRS-1 localization (AP-1)²⁹ and regulation of RNA metabolism (PABPC1)³³. In addition to these IRSAPs, in this paper, we identified DGK ζ as a novel IRSAP by using yeast two-hybrid screening, and examined roles of DGK ζ in GLUT4 translocation. DGK ζ belongs to type IV of the DGK family of proteins and was reported to interact and form complexes with some enzymes, which are activated by DAG (Ras-GRP, cPKC) or PA (PIP5K1 α)^{25–27}. The function of PIP5K1 α , one of the DGK ζ binding proteins activated by PA in GLUT4 translocation, was also investigated.

The knockdown of DGK ζ or PIP5K1 α reduced insulin-induced GLUT4 translocation to the PM. Overexpression of DGK ζ or PIP5K1 α enhanced it. These results indicated that both DGK ζ and PIP5K1 α positively regulate the insulin-induced GLUT4 translocation. In addition, DGK ζ knockdown, DGK ζ overexpression or PIP5K1 α overexpression did not affect the insulin canonical signals of the PI 3-kinase pathway (Supplemental Fig. S2), which are required for GLUT4 translocation, suggesting the existence of a novel pathway to regulate GLUT4 translocation. However, in PIP5K1 α knockdown cells, Akt activation was obviously suppressed (Supplemental Fig. S2). This suppression might be caused by a shortage of a PI 3-kinase substrate, PI 4,5 P2 (PIP2), the product of PIP5K1 α . Suppression of GLUT4 translocation in PIP5K1 α knockdown cells might be also explained by the suppression of Akt phosphorylation. In contrast, PIP5K1 α overexpression enhanced GLUT4 translocation in the absence of insulin. These data suggested that PIP5K1 α has a function to enhance GLUT4 translocation in a PI 3-kinase independent pathway. Thus we concluded that DGK ζ and PIP5K1 α positively regulate GLUT4 translocation possibly through non-canonical insulin signaling.

What is the role of the interaction between IRS-1 and DGK ζ ? Both DGK ζ and PIP5K1 α could be detected in the immunoprecipitates with anti-IRS-1 antibody, suggesting that IRS-1, DGK ζ and PIP5K1 α formed a ternary complex under basal conditions (without insulin stimulation). However, when the insulin signaling pathway was activated, the signals of DGK ζ and PIP5K1 α on the immunoblots disappeared, suggesting that DGK ζ and PIP5K1 α were released from IRS-1 by insulin stimulation (Fig. 4a). Overexpression of sDGK ζ , which can competitively dissociate DGK ζ from IRS-1 (Fig. 4b), enhanced GLUT4 translocation in 3T3-L1 adipocytes even in the absence of insulin (Fig. 4c), indicating that IRS-1-associated DGK ζ inhibited GLUT4 translocation. The data showing that DGK ζ knockdown enhanced GLUT4 translocation under basal conditions also supported the concept that IRS-1-associated DGK ζ inhibited GLUT4 translocation without insulin stimulation (Fig. 6). It is possible that IRS-1-associated DGK ζ inhibited GLUT4 translocation through DGK activity. On the contrary, PIP5K1 α knockdown did not enhance GLUT4 translocation under basal conditions, suggesting that IRS-1-associated PIP5K1 α did not function to inhibit GLUT4 translocation.

In DGK ζ knockdown cells, under basal conditions (without insulin stimulation), GLUT4 translocation was enhanced but glucose uptake was suppressed (Fig. 2a,c). In addition, the fold stimulation of glucose uptake by insulin in DGK ζ knockdown cells (2.258 ± 0.198) was almost comparable to that in control cells (2.056 ± 0.145). This shows uncoupling of GLUT4 translocation and glucose uptake. We have reported that chronic GH (growth hormone)¹¹ pretreatment in 3T3-L1 adipocytes suppressed glucose uptake without affecting GLUT4 translocation to PM³⁴. Other studies also have reported that a PI3K inhibitor (wortmannin) caused significant inhibition of insulin-stimulated glucose uptake, which did not prevent GLUT4 translocation in muscle cell and adipocytes³¹. From these data, others and we proposed the concept that GLUT4 translocation and fusion with PM are not sufficient to enhance glucose uptake *per se*, but that additional activation steps are required. This concept could explain an apparent uncoupling of GLUT4 translocation and glucose uptake in DGK ζ knockdown cells. We also will in future experiments measure the glucose uptake in sDGK ζ -overexpressing cells to show the uncoupling of GLUT4 translocation and glucose uptake in those cells. We attempted to overexpress sDGK ζ by infection with sDGK ζ -expressing lentivirus. However, due to the low level expression of exogenous sDGK ζ , we could not detect the enhancement of glucose uptake in sDGK ζ -expressing 3T3-L1 adipocytes. Much higher expression of sDGK ζ might be required to enhance the glucose uptake. Further analysis is required to demonstrate the enhancement of glucose uptake by inhibiting IRS-1 and DGK ζ interaction.

What is the role of the DGK ζ or PIP5K1 α dissociated from IRS-1? Since sDGK ζ overexpression or insulin stimulation under which conditions DGK ζ and PIP5K1 α complex were dissociated from IRS-1, enhanced GLUT4 translocation, the DGK ζ and PIP5K1 α released from IRS-1 might function to enhance GLUT4 translocation to PM (Fig. 6). Overexpression of DGK ζ , PIP5K1 α or sDGK ζ enhanced insulin-stimulated GLUT4 translocation. In all these cells, PIP5K activity was significantly activated. On the contrary, DGK activity was enhanced both in DGK ζ and PIP5K1 α overexpressing cells but not in sDGK ζ overexpressing cells (Fig. 5). These data suggested that PIP5K

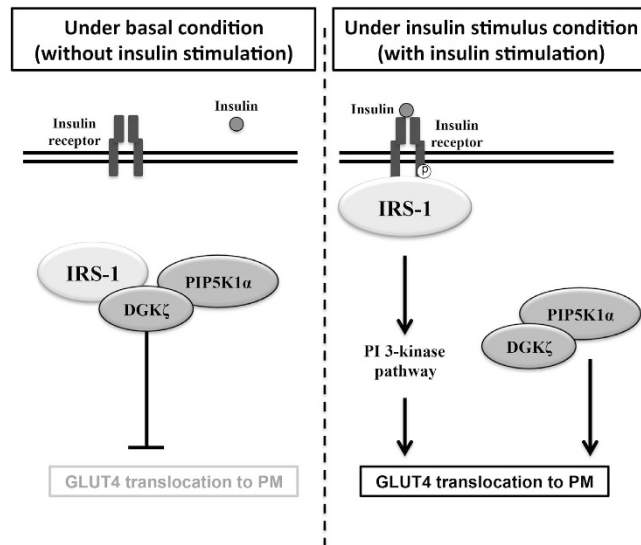


Figure 6. Working hypothesis of a novel regulatory mechanism of GLUT4 translocation by IRS-1-DGK ζ -PIP5K1 α complex. Our working hypothesis of regulatory mechanism of GLUT4 translocation to the PM is shown. In the absence of insulin (without insulin stimulation), insulin signal is not activated, and IRS-1 forms a ternary complex with DGK ζ and PIP5K1 α . The IRS-1-associated DGK ζ inhibited GLUT4 translocation to the PM. In the presence of insulin, IRS-1 is tyrosine-phosphorylated by activated insulin receptor tyrosine kinases, and the DGK ζ -PIP5K1 α complex is dissociated from IRS-1. Then, IRS-1 can activate the canonical insulin signal, the PI 3-kinase pathway. In addition, the released DGK ζ -PIP5K1 α complex enhances GLUT4 translocation independently of PI 3-kinase pathway activation. In this paper, we show that both pathways are required for the GLUT4 translocation to the PM.

activity in cells contributes to enhancement of GLUT4 translocation. We hypothesized that the dissociated DGK ζ activates PIP5K1 α , and PIP5K1 α activity plays important roles in insulin-induced GLUT4 translocation.

In the present study, knockdown or overexpression of DGK ζ decreased or enhanced PIP5K (Fig. 5a,c) and DGK activity (Fig. 5b,d), respectively. In addition, overexpression of PIP5K1 α enhanced the PIP5K activity (Fig. 5c), but not the DGK activity (Fig. 5d). These results suggested that DGK ζ may affect the PIP5K activity by PA generation, but PIP5K1 α may not affect the DGK ζ activity by PIP2 generation, consistent with a previous study²⁷. In this study, we have shown that overexpression of sDGK ζ inhibited the DGK activity but enhanced PIP5K activity (Fig. 5e). Because sDGK ζ has a C1 domain, it is possible that sDGK ζ competes with DGK ζ for DAG. And sDGK ζ enhances the PIP5K activity through an unknown mechanism that needs further investigation. Taken together, these findings raise the possibility of a direct linkage among DGK ζ and PIP5K1 α activation, and GLUT4 translocation via a PI-3 kinase-independent pathway.

How do DGK ζ and PIP5K1 α regulate GLUT4 translocation to PM independent of canonical insulin signaling? Many reports support the concept of an insulin stimulated cytoskeleton rearrangement via an actin-dependent pathway, not PI 3-kinase dependent pathway and this rearrangement could play an important role in GLUT4 translocation to the PM^{35,36}. In addition, increased PIP2 due to overexpression of PIP5K1 α promotes actin polymerization on the membrane-bound vesicles to and from motile actin comets, which inhibit the endocytosis of GLUT4³⁷. Moreover, PIP2 regulated the GLUT4 exocytosis/endocytosis ratio via coating with filamentous-actin, neural Wiskott–Aldrich syndrome protein (N-WASP), dynamin, cortactin and caveolin on the GLUT4-containing vesicles. These findings suggested that PIP2 has a marked effect on the GLUT4 endocytosis, and intracellular vesicle traffic due to the change in actin dynamics^{38–40}.

Recently there were some reports showing that diacylglycerol kinase proteins or activity are involved in the regulation of insulin bioactivity in different tissues. For example, knockdown of type I DGK (α , β , γ) proteins in pancreatic β -cells from male mice results in impairment of insulin secretion⁴¹. The reduced DGK δ protein expression is accompanied by the increased DAG level and elevated PKC activity, resulting in the impairment of IRS-1 tyrosine phosphorylation in skeletal muscle^{42–44}. On the other hand, in the present study, we have shown that DGK ζ regulates the GLUT4 translocation in 3T3-L1 adipocytes. Actually only DGK ζ could interact with IRS-1 (Fig. 1d). In addition, DGK ζ knockdown in 3T3-L1 adipocytes almost completely suppressed the DGK activity (Fig. 5b). Therefore, the dynamic regulatory mechanism of GLUT4 translocation through the IRS-1-DGK ζ -PIP5K1 α complex in 3T3-L1 adipocytes might be the unique role of DGK ζ . It is possible that another DGK isoform has a special function to regulate the insulin signals and bioactivities in different tissues.

In summary, we found that DGK ζ and PIP5K1 α interact with IRS-1 without insulin stimulation. Under basal conditions, IRS-1-associated DGK ζ inhibited GLUT4 translocation to the PM. Insulin stimulation dissociated the DGK ζ and PIP5K1 α from IRS-1, leading to enhancement of insulin-induced GLUT4 translocation through PIP5K activity (Fig. 6).

Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), and Hanks' buffered salt solution were purchased from Nissui Pharmaceutical CO., (Tokyo, Japan). Calf serum (CS), fetal bovine serum (FBS) and bovine insulin were obtained from Sigma Aldrich (St. Louis, MO, USA). Penicillin and streptomycin were obtained from Banyu Pharmaceutical CO., (Ibaraki, Japan). Polyclonal anti-IRS-1 antibody was raised in rabbits as described previously⁴⁵. All animal care and experiments conformed to the Guidelines for Animal Experiments of The University of Tokyo, and were approved by the Animal Research Committee of The University of Tokyo. Polyclonal anti-DGK ζ antibody and anti-PIP5K1 α antibody were kindly provided by Dr. MK Topham (University of Utah, Salt Lake, USA)²⁷. Anti-IR β antibody and anti- β -actin antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA USA). Anti-PI 3-kinase p85 subunit antibody, anti-Myc antibody (9E10) and anti-phosphotyrosine antibody (clone 4G10) were obtained from Millipore (Billerica, MA, USA). Anti-phospho-Akt (Ser-473) antibody and anti-Akt antibody were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-FLAG antibody and anti-FLAG antibody-conjugated agarose beads were obtained from Sigma Aldrich. Horseradish peroxidase (HRP)-conjugated secondary anti-rabbit and anti-mouse IgG antibody were obtained from GE Healthcare (Pittsburgh, PA, USA). Enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Science (Boston, MA, USA). Alexa Fluor 594-conjugated secondary anti-mouse IgG antibody was obtained from Invitrogen (Carlsbad, CA, USA). Protein A-Sepharose was purchased from PerkinElmer (Waltham, MA, USA). Control, DGK ζ and PIP5K1 α specific siRNAs were purchased from RNAi CO. (Tokyo, Japan). The sequence of the DGK ζ siRNA used was 5'-CCA ACG UGU CCG GUG ACU UCU-3'. The sequence of the PIP5K1 α siRNA used was 5'-CUU GCC UCG GUC AGU CAA AAU-3'. The nonrelevant control siRNA sequence was 5'-GUA CCG CAC GUC AUU CGU AUC-3'. Other chemicals were of the reagent grade available commercially.

Plasmids. pAS-IRS-1 was prepared as described previously¹¹ and used for two-hybrid screening as bait. The FLAG tagged full-length DGK ζ , HA tagged PIP5K1 α , GFP tagged DGK α , GFP tagged DGK γ , HA tagged DGK ϵ , HA tagged DGK β , GFP tagged DGK δ , HA tagged DGK ι and deletion series of DGK ζ expressing plasmids were a gift from Dr. MK Topham. The short-form DGK ζ (sDGK ζ), which contains C1 domain, and was constructed as follows. The C1 domain of DGK ζ were generated by PCR using FLAG-DGK ζ as a template. Amplified fragments were digested by EcoR1 and BglII and inserted into pCMV-FLAG or pEGFP-C1 vector in-frame. The exofacial myc-tagged GLUT4-EGFP was kindly provided by Dr. J. E. Pessin³⁴.

Yeast two-hybrid screening. Yeast two-hybrid screening was performed as described previously¹¹. Yeast strain AH109 was purchased from TaKaRa Bio Company (Japan).

Cell cultures. HEK293T cells and CHO cells were cultured as described previously¹¹. Murine 3T3-L1 preadipocytes were purchased from the American Type Tissue Culture Collection. 3T3-L1 preadipocytes were cultured in DMEM containing 10% calf serum at 37 °C in 5% CO₂ atmosphere and induced to differentiate into adipocytes as described¹¹.

Transient transfection of HEK293T cells, CHO cells or 3T3-L1 adipocytes. HEK293T cells or CHO cells were transiently transfected with expression plasmids by lipofectamine 2000 according to the manufacture protocol (Invitrogen, USA) or by the polyethyleneimine method as described before²⁹. Transient transfection of 3T3-L1 adipocytes was described previously¹⁵. In some experiments, the electroporated adipocytes were seeded on coverslips.

GLUT4 translocation assay. Differentiated 3T3-L1 adipocytes electroporated with siRNA or plasmid along with pGLUT4-myc-green fluorescent protein (GFP) were grown on coverslips. Twenty-four hours after electroporation, cells were serum-starved for 4 h and treated with or without insulin for 20 min. Cells were then fixed using 4% paraformaldehyde for 10 min without permeabilization, blocked with 3% bovine serum albumin for 1 h at room temperature. Coverslips were immunostained with anti-Myc antibody for 1 h at 37 °C. After incubation, the coverslips were washed three times with PBS solution, followed by incubation with Alexa Fluor 594-labeled goat anti-mouse IgG for 1 h at 37 °C. Coverslips were again washed three times with PBS and mounted in VECTASHIELD medium. PM localization was determined by using confocal fluorescence microscopy (OLYMPUS, Tokyo, Japan) to score 20 representative cells per condition for the appearance of a PM ring of GLUT4. The ratio of GLUT4 translocation was calculated by myc fluorescence on PM/GFP fluorescence in whole cells.

Glucose uptake assay. Differentiated 3T3-L1 adipocytes were incubated with the indicated concentration of insulin in Krebs-Ringer phosphate (KRP) buffer (20 mM Hepes, 140 mM NaCl, 5 mM KCl, 2.5 mM MgSO₄, 1 mM CaCl₂, 1% BSA, pH7.4) for 20 min at 37 °C. Then 0.1 mM 2-deoxy-D-glucose containing 10 μ Ci/ml 2-deoxy-D-[2,6-³H] glucose was added, and cells were incubated for 4 min at 37 °C. The reaction was terminated by addition of ice-cold PBS containing 10 mM D-glucose. Cells were lysed with 0.1 N NaOH, and radioactivity taken up by cells was measured by a liquid scintillation counter.

Immunoprecipitation followed by immunoblotting. Cells were lysed at 4 °C with ice-cold Tris/TritonX-100 lysis buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 20 μ g/ml phenylmethylsulfonylfluoride (PMSF), 5 μ g/ml pepstatin, 10 μ g/ml leupeptin, 100 KIU/ml aprotinin, 1 mM Na₃VO₄, and 10 mg/ml *p*-nitrophenylphosphate (PNPP)]. Insoluble materials were removed by centrifugation at 15,000 \times g for 10 min at 4 °C and supernatant was prepared as total cell lysates. For immunoprecipitation, 1 mg protein of total cell lysates were incubated with the indicated antibody for 2 h at 4 °C and the immunocomplexes were precipitated with 10 μ l of protein A-Sepharose for polyclonal antibody. These precipitates were washed

3 times with ice-cold lysis buffer. These precipitates or total cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with indicated antibody.

DGK activity assay. DGK activity was measured in the whole cell lysates by octylglucoside mixed micelle assay²⁵. The cells were lysed with lysis buffer (20 mM Tris-HCl pH7.4, 0.25 M sucrose, 1 mM DTT), and then the cells were sonicated for 5–10 sec. The cell lysates were immunoprecipitated with indicated antibody. Immunoprecipitates were washed once with lysis buffer, LiCl buffer (100 mM Tris-HCl, pH 7.4, and 500 mM LiCl), distilled water, and TNE buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM EDTA) and finally resuspended in 40 μ l of reaction buffer (50 mM MOPS, pH7.2, 20 mM NaF, 1 mM DTT). A kinase reaction was initiated by incubation of the reaction mixture (50 μ l total) containing 1 mM 1,2 diacyl-*sn*-glycerol-3-phospho-L-serine, 50 mM MOPS, 20 mM NaF, 1 mM DTT, 50 mM octylglucoside in the presences of 1 mM [γ -³²P]ATP at 25 °C for 20 min. The reaction was stopped by adding 1 M HCl. The 10 μ l PA solution (2.5 mg/mL) and 250 μ l elution solution [CHCl₃/MeOH (1:1)] were added into the reaction mixture. After the centrifugation, the lower layer of solution was taken for measuring DGK activity. A lipid product was spotted onto a silica gel plate, and developed with ethyl acetate/2.2.4.-trimethyl pentane/acetic acid/H₂O (45:25:10:5). The DGK activity was visualized by autoradiography (FLA-5000 image system, Fuji Photo Film Co., Ltd.). Also, the images were quantified using the Image Reader and Image Gauge (Fuji Photo Film Co., Ltd.).

PIP5K activity assay. PIP5K assays were performed in 50 μ l reaction mixtures, containing 50 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EGTA, 1 mg/mL PI(4)P (phosphatidylinositol-4-phosphate/CHCl₃) and 50 μ M [γ -³²P]ATP (1 μ Ci/assay) (27). The reaction mixture was preincubated at 25 °C for 5 min, and the reactions were initiated by the addition of the 10 μ l of cell lysate. After 20 min incubation, the reaction was stopped by adding 100 μ l of 1 M HCl. The samples were centrifuged for 2 min, and then the lower layer of each sample was taken to examine the PIP5K activity. A lipid product was spotted onto a silica gel plate, and developed with ethyl acetate/2.2.4.-trimethyl pentane/acetic acid/H₂O (45: 25:10:5). The PIP5K activity was visualized by autoradiography. Also, the images were quantified using the Image Reader and Image Gauge.

Statistical analysis. Data are expressed as mean \pm S.E.M. Comparisons between two groups were performed using Student's t-test, whereas comparisons among more than two groups were analyzed by one-way or two-way ANOVA and the Tukey post hoc test. Values of $P < 0.05$ were considered statistically significant.

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Author Contributions

T.Y.L., M.K., H.F., B.Y., Y.A., F.H. and S.-I.T. designed research. T.Y.L., M.K., H.F., B.Y., Y.A. and F.H. acquired, analyzed and interpreted data. T.Y.L., B.Y., F.H. and S.-I.T. drafted this paper and H.F. revised it. All authors approved the final manuscript, and agreed to be accountable for all aspects of this work.

Additional Information

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