Published in final edited form as:
Cell Metab. 2009 December ; 10(6): 516–523. doi:10.1016/j.cmet.2009.09.012.

A negative regulatory pathway of GLUT4 trafficking in adipocyte: new function of RIP140 in the cytoplasm via AS160

Ping-Chih Ho, **Yi-Wei Lin**, **Yao-Chen Tsui**, **Pawan Gupta**[#], and **Li-Na Wei**[*]
Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455, USA

SUMMARY

Receptor Interacting Protein 140 (RIP140), a nuclear receptor corepressor, is important for lipid and glucose metabolism. In adipocytes, RIP140 can be phosphorylated by protein kinase C epsilon (PKCε), followed by arginine methylation, and exported to the cytoplasm. This study demonstrates for the first time a cytoplasmic function for RIP140: to counteract insulin-stimulated glucose transporter 4 (GLUT4) membrane partitioning and glucose uptake in adipocytes. Cytoplasmic RIP140 interacts with the Akt substrate AS160, thereby impeding AS160 phosphorylation by Akt; this in turn reduces GLUT4 trafficking. This signal transduction pathway can be recapitulated in the epididymal adipocytes of diet-induced obese mice: nuclear PKCε is activated, cytoplasmic RIP140 increases, and GLUT4 trafficking and glucose uptake are reduced. The data reveal a new, cytoplasmic, function for RIP140 as a negative regulator of GLUT4 trafficking and glucose uptake, and shed insight into the regulation of basal and insulin-stimulated glucose disposal by a nuclear-initiated counteracting mechanism.

Keywords

GLUT4; cytoplasmic RIP140; adipocytes; AS160; Akt; post-translational modification

INTRODUCTION

Insulin sensitivity is key to the maintenance of systemic energy homeostasis, ensuring that metabolically healthy individuals can adapt to various fuel conditions by sensing blood glucose load and triggering its disposal (Herman and Kahn, 2006). The inability of an individual to respond to insulin signals to remove glucose, i.e., insulin resistance (IR), is the hallmark of Type II diabetes and can be caused by a variety of factors (Doria et al., 2008; Herman and Kahn, 2006; Schenk et al., 2008). Once IR is detected clinically, reversing the progression of the disease is often difficult. Early markers for IR, especially those involved in the regulation of insulin signaling and glucose disposal, are therefore important to further understand disease progression and develop therapeutic agents.

*Correspondence: Li-Na Wei, Department of Pharmacology, University of Minnesota Medical School, 6-120 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455-0217, USA, Tel: 612-625-9402, Fax: 612-625-8408, weixx009@umn.edu.
[#]Current address: Protein Science & Molecular Biology, Institute of Microbial Technology, Sector 39-A, Chandigarh 160036, India

The principal mechanism for glucose disposal, insulin-stimulated glucose uptake, mainly involves glucose transporter 4 (GLUT4) (Huang and Czech, 2007). Insulin activates a cascade of intracellular signaling events, including the mitogen-activated protein kinase pathway for growth and the phosphoinositide 3-kinase (PI3K)–Akt pathway for glycogen and protein synthesis and glucose uptake via GLUT4 (Herman and Kahn, 2006; Tilg and Moschen, 2008). IR is mainly attributed to defects in insulin signaling pathways (Guilherme et al., 2008; Muoio and Newgard, 2006; Schenk et al., 2008; Tilg and Moschen, 2008; Wellen and Hotamisligil, 2005), as well as GLUT4 synthesis, translocation, trafficking and down-regulation (Guilherme et al., 2008; Herman and Kahn, 2006; Huang and Czech, 2007). To this end, insulin stimulates the translocation of GLUT4 storage vesicles (GSVs) from intracellular compartments to the plasma membrane (PM), a key step regulating GLUT4 traffic.

Among the thirteen GLUTs, GLUT4 is the only isoform that is insulin-responsive, and it is expressed predominantly in muscle and adipose tissues (Huang and Czech, 2007). Insulin-stimulated GLUT4 partitioning to the PM is mediated primarily by PI3K-activated Akt which phosphorylates AS160, a Rab-GTPase-activating protein (Rab-GAP) (Hou and Pessin, 2007; Sakamoto and Holman, 2008). Phosphorylated AS160 (pAS160) is thought to become inactive, allowing more Rab-GTP to accumulate, which facilitates GSV trafficking to the PM (Larance et al., 2005; Ng et al., 2008; Sakamoto and Holman, 2008; Sano et al., 2003).

Receptor interacting protein 140 (RIP140), a widely known transcription corepressor (Christian et al., 2006; Wei, 2004), can be modified extensively by posttranslational modifications (PTMs) (Mostaqul Huq et al., 2008). Certain PTMs augment its nuclear activity (Gupta et al., 2005; Ho et al., 2008; Huq et al., 2009a, 2009b; Huq et al., 2007), whereas a specific cascade of sequential PTMs, initiated by nuclear PKCε phosphorylation of RIP140's Ser-102 and Ser-1003, triggers its export to the cytoplasm in adipocytes (Gupta et al., 2008; Mostaqul Huq et al., 2006). This report uncovers a new functional role for RIP140 in the adipocyte cytoplasm — to negatively regulate insulin-stimulated GLUT4 trafficking — and determines the mechanism of this action.

RESULTS

Increased cytoplasmic RIP140 reduces GLUT4 trafficking in 3T3-L1 adipocytes

We first found cytoplasmic RIP140 readily detectable in four-day differentiated cells, and robustly detected in eight-day differentiated cells (Figure 1A). Interestingly, 4-day cells were more responsive to insulin stimulation in glucose uptake than the 8-day cells (Figure 1B). Consistently, insulin dramatically stimulated GLUT4 partitioning into the PM of 4-day cells. The 8-day cells had higher basal glucose uptake and PM-associated GLUT4 levels, which could be stimulated by insulin (Figure 1C). Insulin-stimulated glucose uptake in these 3T3-L1 adipocytes seems lower than that of some reported studies, which might be due to difference in experimental procedures such as the differentiation cocktail (Romero et al., 2000).

Cytoplasmic RIP140 was evident in 4-day cells, and its levels increased by day 8; similarly, PKCε levels also increased during differentiation (Figure 1C). When RIP140 and PKCε were knocked down individually, both basal and insulin-stimulated PM-associated GLUT4 levels in 8-day cells increased significantly (Figure 1D), and basal and insulin-stimulated glucose uptake was also enhanced significantly (Figure 1E). While GLUT4 mRNA level was not altered by RIP140 or PKCε silencing (Figure 1D), total GLUT4 protein level was up-regulated (~2.5 folds) by RIP140 silencing (Figure S1), indicating additional functions, beyond affecting GLUT4 protein level, of RIP140 to modulate glucose uptake.

GLUT4 trafficking was assessed using G7 adipocytes which stably express myc-GLUT4-EGFP (Yu et al., 2007). Silencing RIP140 or PKCε increased, by approximately twofold, the

number of cells exhibiting surface-stained myc-GLUT4 (i.e., an intact myc rim stained with anti-myc) (Figure 1F); this was confirmed by immunoblotting (Figure S2). Myc-GLUT4 recycling was monitored by incubating G7 adipocytes with anti-myc for 60 min in the presence or absence of insulin, followed by fixation and permeabilization to stain internalized myc-GLUT4 with a secondary antibody. The internalized myc-GLUT4 signal was stronger in both RIP140- and PKCε-silenced cells (Figure 1G). Overall, these results suggest a functional role for both RIP140 and PKCε in negatively regulating GLUT4 trafficking to the PM.

We previously identified nuclear PKCε activation as an initial trigger to stimulate the export of nuclear RIP140. Two RIP140 mutants, CP (a phosphor-mimetic export enhancer) and CN (a phosphor-deficient mutant with reduced export), were used to rescue RIP140$^{-/-}$ adipocytes (Figure 2A). Re-expressing the wild type, or CP, but not CN, RIP140 reduced both basal and insulin-stimulated GLUT4 partitioning to the PM. Likewise, glucose uptake of the RIP140-null cells was significantly reduced by expressing Wt or CP RIP140, but unaffected by the CN mutant (Figure 2B). Taken together, these results indicate that cytoplasmic RIP140 negatively regulates basal and insulin-stimulated GLUT4 trafficking to the cell surface in these adipocytes. Furthermore, this effect can be initiated by PKCε-mediated phosphorylation of RIP140 in the nucleus.

Regulation of AS160 phosphorylation by RIP140

Several regulatory points of GLUT4 trafficking where RIP140 might play a role were examined. We found that RIP140 directly affected the phosphorylation of AS160 on Thr-642 by Akt (Figure 2C). Neither RIP140- nor PKCε-silencing affected the basal or insulin-activated Akt or ERK1/2 activation profiles, but both manipulations enhanced pAS160 levels (Figure 2D). AS160 protein, but not its mRNA, level was decreased slightly by silencing RIP140 or PKCε. Therefore, RIP140 silencing slightly decreases AS160 protein expression, but profoundly increases its phosphorylation. Treatment with PKC effectors indicated that PKC activation increased RIP140/AS160 complex formation (Figure 2E), suggesting that such complex formation might inhibit the phosphorylation of AS160.

RIP140 directly interacts with AS160 to impede Akt phosphorylation of AS160

The direct interaction and *in vivo* complex formation of RIP140 with AS160 was confirmed using glutathione *S*-transferase (GST) pulldown (Figure 3A) and coimmunoprecipitation (Figure 3B). The AS160-interaction domain of RIP140 was mapped to its amino (peptides 1–350) and carboxyl (peptides 717–end) termini. The interaction of RIP140 with AS160 interfered with the ability of AS160 to interact with Akt (Figure 3C). *In vitro* kinase assays confirmed that RIP140 reduced AS160 phosphorylation by approximately 50% (Figure 3D). Thus, RIP140 interacts directly with AS160, impeding its phosphorylation by Akt, and presumably maintaining the GAP activity of AS160. This would inactivate downstream targets such as Rab-GTPases, thereby reducing GSV trafficking.

High-fat diet activates nuclear PKCε and promotes cytoplasmic RIP140 accumulation

To determine if this signaling pathway could be recapitulated in animals under physiological or pathophysiological conditions, mice were fed a normal diet (ND) or a high-fat diet (HFD) for 5 weeks. Primary adipocytes from these mice were compared with respect to RIP140 subcellular distribution, nuclear PKCε activation and glucose uptake. Cytoplasmic RIP140 was clearly detected in the white adipocytes from epididymides of HFD-fed mice, but not in the ND controls (Figure 4A, upper). Indeed, the number of adipocytes expressing cytoplasmic RIP140 was nearly threefold higher in HFD mice than ND mice (Figure 4A, lower). (The specificity of anti-RIP140 was validated by immunohistochemistry and immunoblotting [Figures S3 and S4]). Likewise, the response of glucose uptake to insulin decreased from a nearly six-fold stimulation in ND mice to only about a 1.5-fold increase in HFD adipocytes

(Figure 4B), although basal glucose uptake in primary adipocytes was comparable between HFD and ND groups. This is different from the apparent elevation of basal glucose uptake levels in later (day 8) *in vitro* differentiated adipocytes (Figure 1), which might be due to factors varied between *in vivo* and *in vitro* conditions. RIP140 was located predominantly in the nuclei of ND adipocytes but appeared abundantly in the cytoplasm of HFD adipocytes (Figure 4C). Total RIP140 levels also increased in HFD adipocytes, which appeared to partially result from increased mRNA levels (Figure 4C, bottom). Finally, nuclear PKCε activity was also significantly (~fourfold) higher in HFD adipocytes (Figure 4D). Previous studies showed that long-term (>12 weeks) exposure to a HFD decreases insulin-stimulated signaling pathways, including phosphorylation of Akt, by regulating insulin receptor substrate's modifications (Schenk et al., 2008; Wellen and Hotamisligil, 2005). In the present study, feeding a HFD for only 5 weeks profoundly decreased insulin-stimulated AS160 phosphorylation, but not Akt activation (Figure 4E and Figure S5).

DISCUSSION

RIP140 knockout animals exhibit a phenotype mostly opposite to that of metabolic syndromes (Christian et al., 2006; White et al., 2008). At least one study has shown that silencing RIP140 improves insulin-stimulated glucose uptake in adipocytes (Powelka et al., 2006). These effects have been attributed to the nuclear actions of RIP140, largely because of its widely recognized function as a nuclear receptor coregulator (Christian et al., 2006; Mostaqul Huq et al., 2008; Puri et al., 2008). Here we report, for the first time, a new functional role for the cytoplasmic form of RIP140 as a negative regulator of GLUT4 trafficking. Cytoplasmic RIP140 interacts directly with AS160, preventing its phosphorylation by Akt, and thus, its inactivation. Maintaining AS160 in its active state preserves its ability to inactivate Rab-GTPase, thus reducing GLUT4 trafficking. The fact, that in mice exposure to a high-fat diet for 5 weeks readily elevates the level of RIP140 (particularly cytoplasmic RIP140) in epididymal adipose tissues, suggests a role for RIP140 in the progression of IR in diet-induced obesity. Interestingly, the mechanism of action appears to involve, at least in part, the activation of nuclear PKCε, which phosphorylates nuclear RIP140 and facilitates its subsequent export to the cytoplasm.

Most studies of IR focus on membrane-elicited insulin signal transduction pathways that stimulate GLUT4 trafficking. The nuclear-initiated signaling pathway identified here negatively regulates GLUT4 trafficking by increasing the export of RIP140. This conclusion is supported by the apparently effective rescue of RIP140-null cells by the CP, but not the CN, RIP140 mutant (Figure 2A, B). However, we have not completely ruled out contributions from the nuclear-localized RIP140. Rescuing RIP140 null adipocytes with RIP140 wild type or its cytoplasm-localized form (CP) slightly decreases GLUT4 protein level (Figure 2A), indicating certain unknown activity of cytoplasmic RIP140 that could modulate total GLUT4 protein level. Since manipulating the RIP140 level (or altering its PTMs) in 3T3-L1 adipocytes did not change GLUT4 mRNA levels (Figure 1D), the negative regulatory effect of RIP140 on GLUT4 trafficking does not involve its traditional function as a transcriptional corepressor. A recent study has reported that RIP140 is a key regulator in muscle metabolism (Seth et al., 2007). It would be interesting to determine if the mechanism described here for RIP140 function in adipocytes functions similarly in muscle cells.

Inflammation and ER stress in adipose tissue are critical events in diet-induced IR and subsequent diabetes (Guilherme et al., 2008; Schenk et al., 2008; Wellen and Hotamisligil, 2005). However, whether inflammation and ER stress represent early contributing events to IR has been debated (Schenk et al., 2008; Wellen and Hotamisligil, 2005). In fact, several studies showed that diet-induced inflammation and ER stress in adipose tissues are relatively late events (Duffaut et al., 2009; Ozcan et al., 2006). In this study, feeding mice a HFD for 5

weeks readily stimulated cytoplasmic RIP140 levels and reduced insulin stimulation of glucose uptake, without impairing Akt activation. This suggests that HFD-induced accumulation of cytoplasmic RIP140 could be an early event that dampens insulin sensitivity in adipocytes. Whether and how cytoplasmic RIP140 is related to inflammation or ER stress under prolonged feeding with a HFD needs to be evaluated.

PKCε activation in liver and muscle is an important factor for impaired insulin-stimulated glucose disposal (Laybutt et al., 1999; Samuel et al., 2007). Targeting PKCε in β-cells elevates insulin secretion, whereas PKCε deletion augments whole-body glucose disposal (Schmitz-Peiffer and Biden, 2008; Schmitz-Peiffer et al., 2007). This study shows that nuclear PKCε activity could also indicate a pathophysiological condition, at least with respect to the control of GLUT4 trafficking in adipocytes.

AS160 is a newly identified GAP that functions as a negative regulator of several Rab-GTPases involved in vesicular transport, docking and fusion (Hou and Pessin, 2007; Sakamoto and Holman, 2008; Sano et al., 2003). Our results show that RIP140 markedly down-regulates AS160 phosphorylation, with only a slight effect on AS160 protein expression and no effect on its mRNA levels. Thus, cytoplasmic RIP140 could also regulate AS160 protein levels via other, as yet unknown, posttranscriptional mechanisms. Whether RIP140 regulates trafficking of other vesicles remains to be determined.

EXPERIMENTAL PROCEDURES

Cell culture

COS-1 cells, 3T3-L1 fibroblasts, and RIP140$^{-/-}$ mouse embryonic fibroblasts (MEF) were maintained and differentiated as described (Ho et al., 2008). G7 fibroblasts were maintained and differentiated as described ((Yu et al., 2007). Transfection was conducted with Lipofectamine 2000 (Invitrogen) as described (Gupta et al., 2008) or Lipofectamine LTX according to the manufacturer's instructions. siRNA (QIAGEN) was introduced using a DeliverX Plus siRNA transfection kit (Panomics).

Myc-GLUT4-EGFP staining and GLUT4 internalization

G7 adipocytes were starved (3 hrs) in serum-free medium and treated with 100 nM insulin (20 min), cells were fixed and incubated with anti-myc (2 hrs, 4°C). After washing, cells were stained with Cy3-conjugated secondary antibody. Images were acquired on an Olympus FluoView1000 IX2 inverted confocal microscope. GLUT4 recycling was performed as described (Larance et al., 2005).

In vitro competition assays for protein interaction

In vitro protein interaction assay was performed as described (Ho et al., 2008) to examine competition between RIP140 and AS160. Flag-RIP140 was synthesized in a transcription translation–system (Promega) and incubated with GST-AS160 and an equal amount of Akt2 (Upstate) in coimmunoprecipitation buffer overnight at 4°C. Detail was described in the supplement. After washing, immunocomplexes and supernatants were analyzed with indicated antibodies on blots.

PKCε kinase activity assay

Nuclear PKCε activity was assayed using 200 g of immunoprecipitated nuclear extract with a radiometric kit (Upstate) according to the manufacturer's instructions.

Statistical analysis

Experiments were performed at least twice and that results were the same. Results are presented as means ± SD. Comparisons between groups were made by unpaired two-tailed Student's *t*-tests. *P* values < 0.05 were considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank J. Bogan for G7 cells, N-P Tsai for discussions on the manuscript, M. Parker for RIP140-null MEF, S G Ha, S C Chan and Y-S Chuang for technical help. This study was supported in part by NIH grants DK54733, DK60521, DA11190 and K02-DA13926 to L.-N.W.

References

Christian M, White R, Parker MG. Metabolic regulation by the nuclear receptor corepressor RIP140. Trends Endocrinol Metab 2006;17:243–250. [PubMed: 16815031]

Doria A, Patti ME, Kahn CR. The emerging genetic architecture of type 2 diabetes. Cell Metab 2008;8:186–200. [PubMed: 18762020]

Duffaut C, Galitzky J, Lafontan M, Bouloumie A. Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity. Biochem Biophys Res Commun 2009;384:482–485. [PubMed: 19422792]

Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol 2008;9:367–377. [PubMed: 18401346]

Gupta P, Ho PC, Huq MD, Khan AA, Tsai NP, Wei LN. PKCepsilon stimulated arginine methylation of RIP140 for its nuclear-cytoplasmic export in adipocyte differentiation. PLoS ONE 2008;3:e2658. [PubMed: 18628823]

Gupta P, Huq MD, Khan SA, Tsai NP, Wei LN. Regulation of co-repressive activity of and HDAC recruitment to RIP140 by site-specific phosphorylation. Mol Cell Proteomics 2005;4:1776–1784. [PubMed: 16093479]

Herman MA, Kahn BB. Glucose transport and sensing in the maintenance of glucose homeostasis and metabolic harmony. J Clin Invest 2006;116:1767–1775. [PubMed: 16823474]

Ho PC, Gupta P, Tsui YC, Ha SG, Huq M, Wei LN. Modulation of lysine acetylation-stimulated repressive activity by Erk2-mediated phosphorylation of RIP140 in adipocyte differentiation. Cell Signal 2008;20:1911–1919. [PubMed: 18655826]

Hou JC, Pessin JE. Ins (endocytosis) and outs (exocytosis) of GLUT4 trafficking. Curr Opin Cell Biol 2007;19:466–473. [PubMed: 17644329]

Huang S, Czech MP. The GLUT4 glucose transporter. Cell Metab 2007;5:237–252. [PubMed: 17403369]

Huq MD, Ha SG, Barcelona H, Wei LN. Lysine Methylation of Nuclear Co-Repressor Receptor Interacting Protein 140. J Proteome Res. 2009a

Huq MD, Ha SG, Barcelona H, Wei LN. Lysine methylation of nuclear co-repressor receptor interacting protein 140. J Proteome Res 2009b;8:1156–1167. [PubMed: 19216533]

Huq MD, Tsai NP, Lin YP, Higgins L, Wei LN. Vitamin B6 conjugation to nuclear corepressor RIP140 and its role in gene regulation. Nat Chem Biol 2007;3:161–165. [PubMed: 17277785]

Larance M, Ramm G, Stockli J, van Dam EM, Winata S, Wasinger V, Simpson F, Graham M, Junutula JR, Guilhaus M, James DE. Characterization of the role of the Rab GTPase-activating protein AS160 in insulin-regulated GLUT4 trafficking. J Biol Chem 2005;280:37803–37813. [PubMed: 16154996]

Laybutt DR, Schmitz-Peiffer C, Saha AK, Ruderman NB, Biden TJ, Kraegen EW. Muscle lipid accumulation and protein kinase C activation in the insulin-resistant chronically glucose-infused rat. Am J Physiol 1999;277:E1070–1076. [PubMed: 10600797]

Mostaqul Huq MD, Gupta P, Tsai NP, White R, Parker MG, Wei LN. Suppression of receptor interacting protein 140 repressive activity by protein arginine methylation. Embo J 2006;25:5094–5104. [PubMed: 17053781]

Mostaqul Huq MD, Gupta P, Wei LN. Post-translational modifications of nuclear co-repressor RIP140: a therapeutic target for metabolic diseases. Curr Med Chem 2008;15:386–392. [PubMed: 18288993]

Muoio DM, Newgard CB. Obesity-related derangements in metabolic regulation. Annu Rev Biochem 2006;75:367–401. [PubMed: 16756496]

Ng Y, Ramm G, Lopez JA, James DE. Rapid activation of Akt2 is sufficient to stimulate GLUT4 translocation in 3T3-L1 adipocytes. Cell Metab 2008;7:348–356. [PubMed: 18396141]

Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Gorgun CZ, Hotamisligil GS. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science 2006;313:1137–1140. [PubMed: 16931765]

Powelka AM, Seth A, Virbasius JV, Kiskinis E, Nicoloro SM, Guilherme A, Tang X, Straubhaar J, Cherniack AD, Parker MG, Czech MP. Suppression of oxidative metabolism and mitochondrial biogenesis by the transcriptional corepressor RIP140 in mouse adipocytes. J Clin Invest 2006;116:125–136. [PubMed: 16374519]

Puri V, Virbasius JV, Guilherme A, Czech MP. RNAi screens reveal novel metabolic regulators: RIP140, MAP4k4 and the lipid droplet associated fat specific protein (FSP) 27. Acta Physiol (Oxf) 2008;192:103–115. [PubMed: 18171433]

Romero R, Casanova B, Pulido N, Suarez AI, Rodriguez E, Rovira A. Stimulation of glucose transport by thyroid hormone in 3T3-L1 adipocytes: increased abundance of GLUT1 and GLUT4 glucose transporter proteins. J Endocrinol 2000;164:187–195. [PubMed: 10657854]

Sakamoto K, Holman GD. Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. Am J Physiol Endocrinol Metab 2008;295:E29–37. [PubMed: 18477703]

Samuel VT, Liu ZX, Wang A, Beddow SA, Geisler JG, Kahn M, Zhang XM, Monia BP, Bhanot S, Shulman GI. Inhibition of protein kinase Cepsilon prevents hepatic insulin resistance in nonalcoholic fatty liver disease. J Clin Invest 2007;117:739–745. [PubMed: 17318260]

Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, Lienhard GE. Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. J Biol Chem 2003;278:14599–14602. [PubMed: 12637568]

Schenk S, Saberi M, Olefsky JM. Insulin sensitivity: modulation by nutrients and inflammation. J Clin Invest 2008;118:2992–3002. [PubMed: 18769626]

Schmitz-Peiffer C, Biden TJ. Protein kinase C function in muscle, liver, and beta-cells and its therapeutic implications for type 2 diabetes. Diabetes 2008;57:1774–1783. [PubMed: 18586909]

Schmitz-Peiffer C, Laybutt DR, Burchfield JG, Gurisik E, Narasimhan S, Mitchell CJ, Pedersen DJ, Braun U, Cooney GJ, Leitges M, Biden TJ. Inhibition of PKCepsilon improves glucose-stimulated insulin secretion and reduces insulin clearance. Cell Metab 2007;6:320–328. [PubMed: 17908560]

Seth A, Steel JH, Nichol D, Pocock V, Kumaran MK, Fritah A, Mobberley M, Ryder TA, Rowlerson A, Scott J, Poutanen M, White R, Parker M. The transcriptional corepressor RIP140 regulates oxidative metabolism in skeletal muscle. Cell Metab 2007;6:236–245. [PubMed: 17767910]

Tilg H, Moschen AR. Insulin resistance, inflammation, and non-alcoholic fatty liver disease. Trends Endocrinol Metab 2008;19:371–379. [PubMed: 18929493]

Wei LN. Retinoids and receptor interacting protein 140 (RIP140) in gene regulation. Curr Med Chem 2004;11:1527–1532. [PubMed: 15180561]

Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest 2005;115:1111–1119. [PubMed: 15864338]

White R, Morganstein D, Christian M, Seth A, Herzog B, Parker MG. Role of RIP140 in metabolic tissues: connections to disease. FEBS Lett 2008;582:39–45. [PubMed: 18023280]

Yu C, Cresswell J, Loffler MG, Bogan JS. The glucose transporter 4-regulating protein TUG is essential for highly insulin-responsive glucose uptake in 3T3-L1 adipocytes. J Biol Chem 2007;282:7710–7722. [PubMed: 17202135]

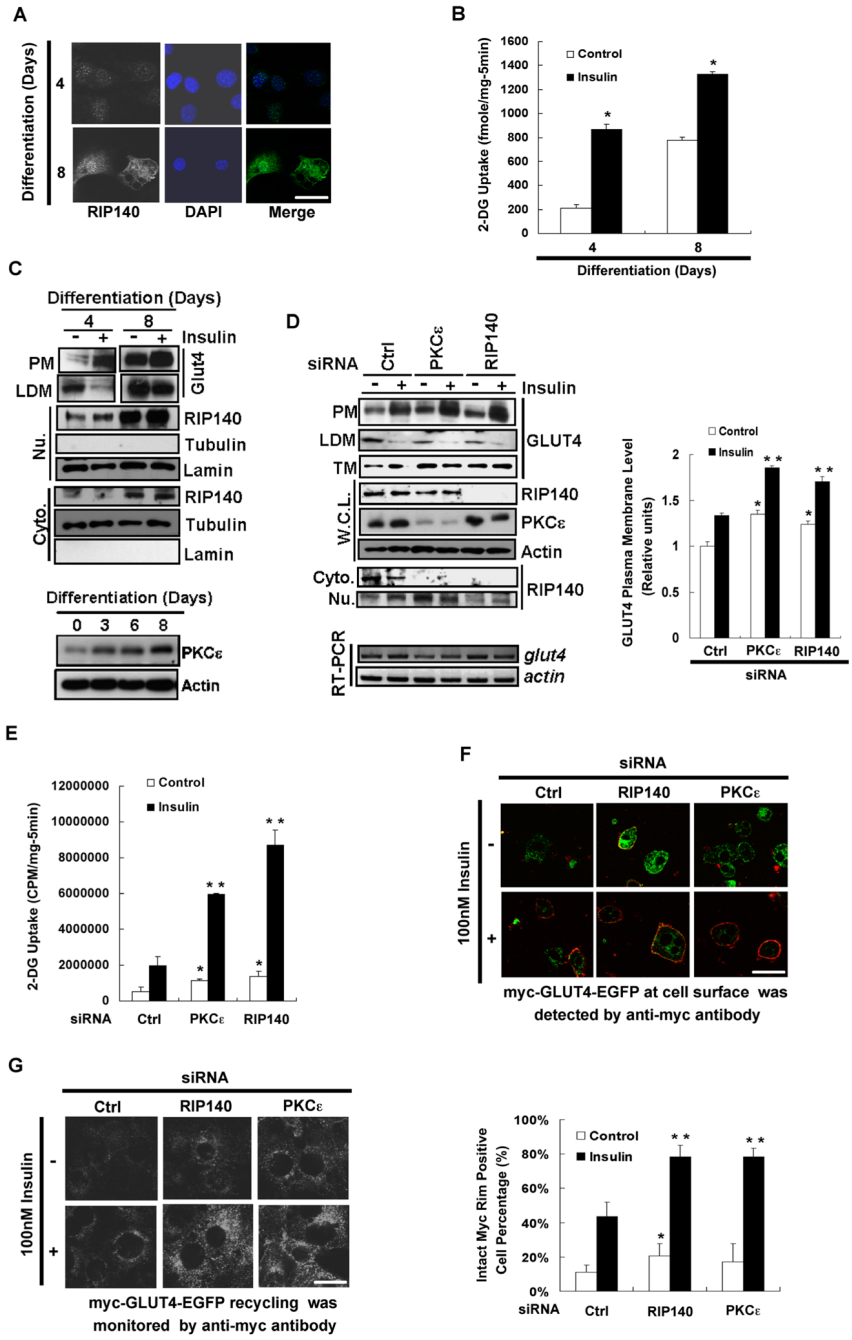

Figure 1. Increase of basal and insulin-stimulated GLUT4 trafficking and glucose uptake in adipocytes by knockdown of RIP140 or PKCε

(A) Cytoplasmic RIP140 in 3T3-L1 adipocytes (on day 4 and day 8 of differentiation). Scale bar = 40 μM. (B) Glucose uptake in 3T3-L1 adipocytes which were serum starved (3 hr) and stimulated with or without insulin (100nM) for 20 min. (C) Membrane partitioning of GLUT4; distribution and expression of RIP140 in differentiated 3T3-L1 adipocytes in the absence or presence of 170nM insulin for 20 min. Levels of PKCε and other marker proteins were also monitored. PM: plasma membrane; LDM: low-density membrane; Nu: nuclear fraction; Cyto: cytoplasmic fraction. (D) Basal and insulin-stimulated GLUT4 partitioning in 3T3-L1 adipocytes. Knockdowns were conducted on day 5 of differentiation; insulin (170nM)

stimulation for 20 min was performed on day 8. Left: Distributions of GLUT4, RIP140, and marker proteins. GLUT4 mRNA levels were monitored by RT-PCR. TM: total membrane fraction; W.C.L.: whole-cell lysate. Right: Relative GLUT4 levels in PM, normalized to the non-insulin-treated control. *: $p < 0.05$ vs. control; **: $p < 0.05$ vs. insulin treatment (n = 3). (E) Glucose uptake in differentiated 3T3-L1 adipocytes (Day 8). (F) Myc-GLUT4-GFP labeling in G7 adipocytes. Upper: Membrane-localized GLUT4 was detected with anti-myc. Bar = 30 μM. Lower: Quantification by scoring the percentage of cells with an intact myc rim on the surface. *: $p < 0.05$ vs. control; **: $p < 0.05$ vs. insulin treatment (n = 3). (G) Immunohistochemical staining of recycled myc-GLUT4 in G7 adipocytes. Bar = 40 μM. **Data are presented as means ± SD.**

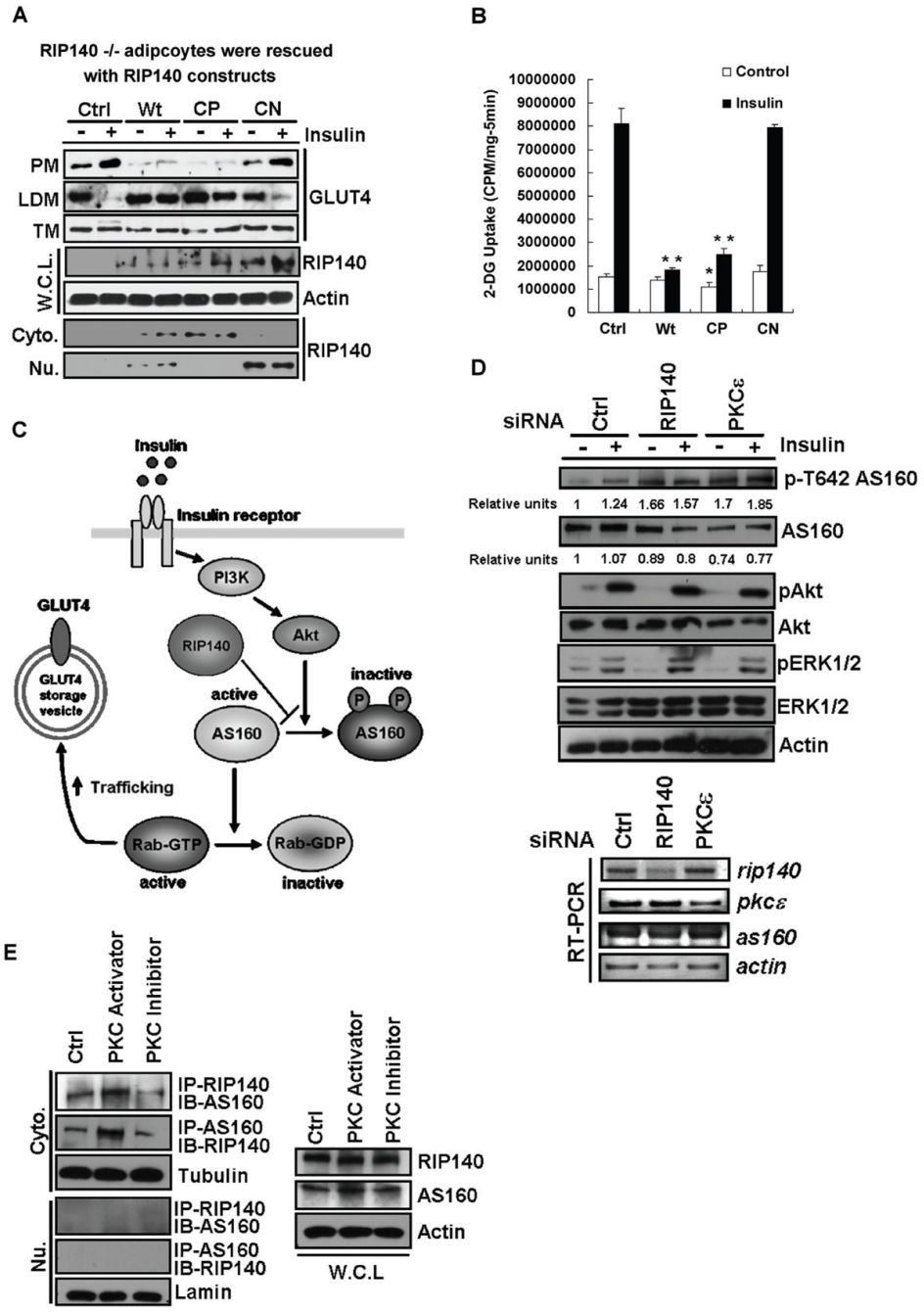

Figure 2. Cytoplasmic RIP140 modulates AS160 phosphorylation and negatively regulates GLUT4 trafficking

(A) GLUT4 trafficking. RIP140-null adipocytes were differentiated and transfected with wild type (Wt) RIP140, a PKCε phosphormimetic mutant (CP), or a constitutive negative mutant (CN) on day 5. Cells were starved for 3 hr and then treated with or without insulin for 20 min and lysates were prepared on day 8. (B) Glucose uptake in RIP140-null and rescued adipocytes. **Data are presented as means ± SD.** *: $p < 0.05$ vs. control treatment; **: $p < 0.05$ vs. insulin treatment in control vector-transfected cells (n = 3). (C) Summary of GLUT4 trafficking regulated by AS160, with the potential point of RIP140 intervention indicated. (D) Upper: Phosphorylation of AS160 and related kinases in G7 adipocytes, with and without insulin

(170nM) stimulation for 20 min. Lower: mRNA levels monitored by RT-PCR. (E) RIP140/AS160 complex formation in 3T3-L1 adipocytes. Day-8 differentiated cells were treated with control vehicle (Ctrl), 4 μM of phorbol 12-myristate 13-acetate (PKC activator) or 50 μM of calphastin (PKC inhibitor) for 3 hrs. Left: Nuclear and cytoplasmic fractions were subjected to reciprocal immunoprecipitation (IP) and immunoblotting (IB) as indicated. Right: Total protein levels.

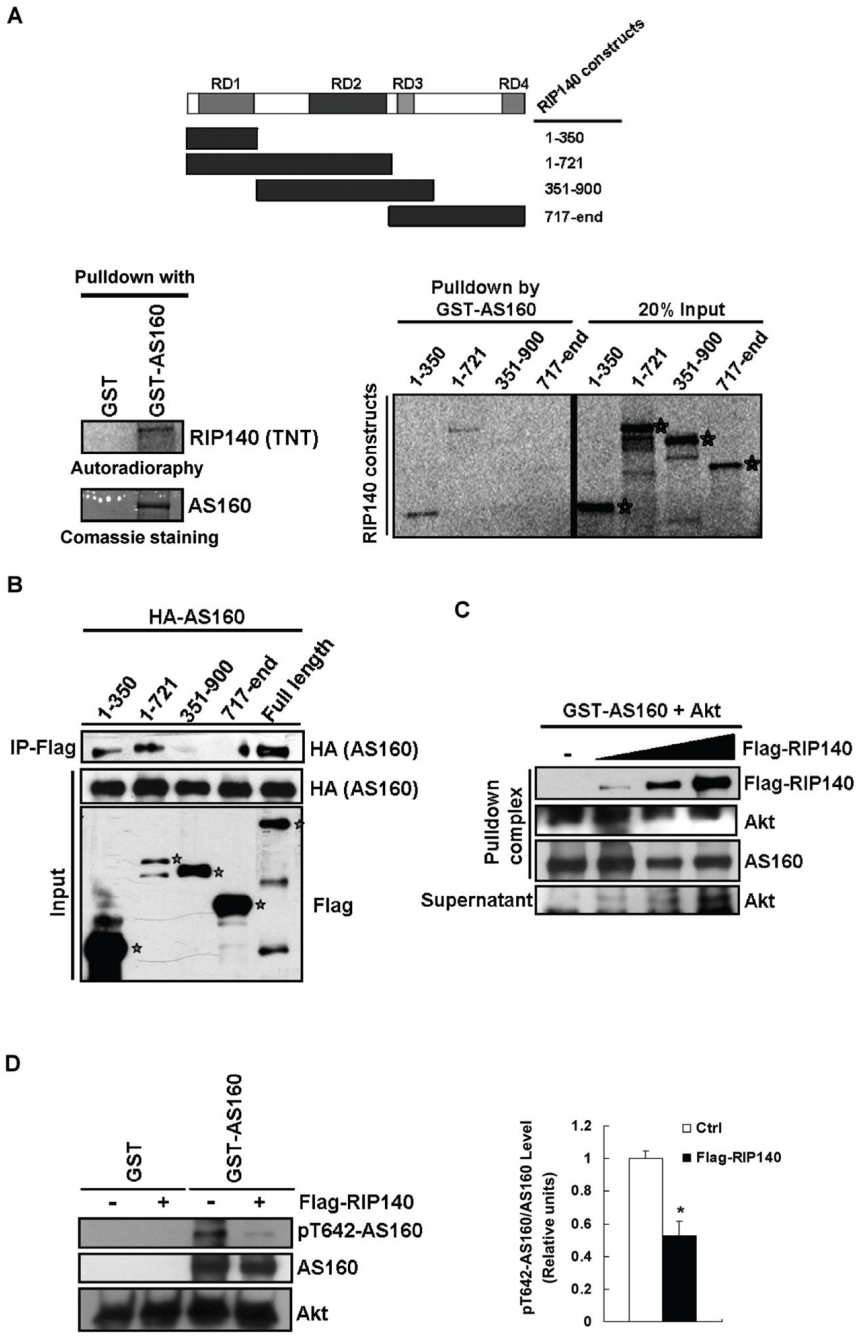

Figure 3. Direct interaction of AS160 with RIP140 maintains AS160 activity by impeding Akt-mediated phosphorylation

(A) GST pulldown assay. Upper: Map of RIP140 constructs. Lower left: Interaction of isotope-labeled full-length RIP140 with full-length GST-AS160. Lower right: Interaction of AS160 with various RIP140 segments. (B) *In vivo* complex formation of RIP140 with AS160 in COS-1 cells: immunoprecipitation with anti-Flag (for Flag-RIP140) antibodies and immunoblotting with anti-HA (for HA-AS160). Specific Flag-RIP140 fragments are indicated (*). (C) Competition between RIP140 and Akt to form complexes with AS160. GST-AS160 (~5 μg) was incubated overnight with 0.25 μg active Akt2 and increasing amounts of Flag-RIP140 in a 0.5ml Co-IP buffer. Pulldown complexes and supernatants were immunoblotted as indicated.

(D) Akt-mediated AS160 phosphorylation *in vitro*. GST or GST-AS160 (~5 μg) were incubated overnight with or without *in vitro*-synthesized Flag-RIP140 (i.e., the highest amount used in panel C). After washing, 0.5 μg active Akt was added to the pulldown complexes in Akt kinase buffer for kinase reaction. pAS160 (pT642-AS160), total AS160 and Akt were determined by immunoblotting. Right: quantitative result of kinase assay for AS160 from two independent experiments. Data are presented as means ± SD. *: $p < 0.05$ vs. control.

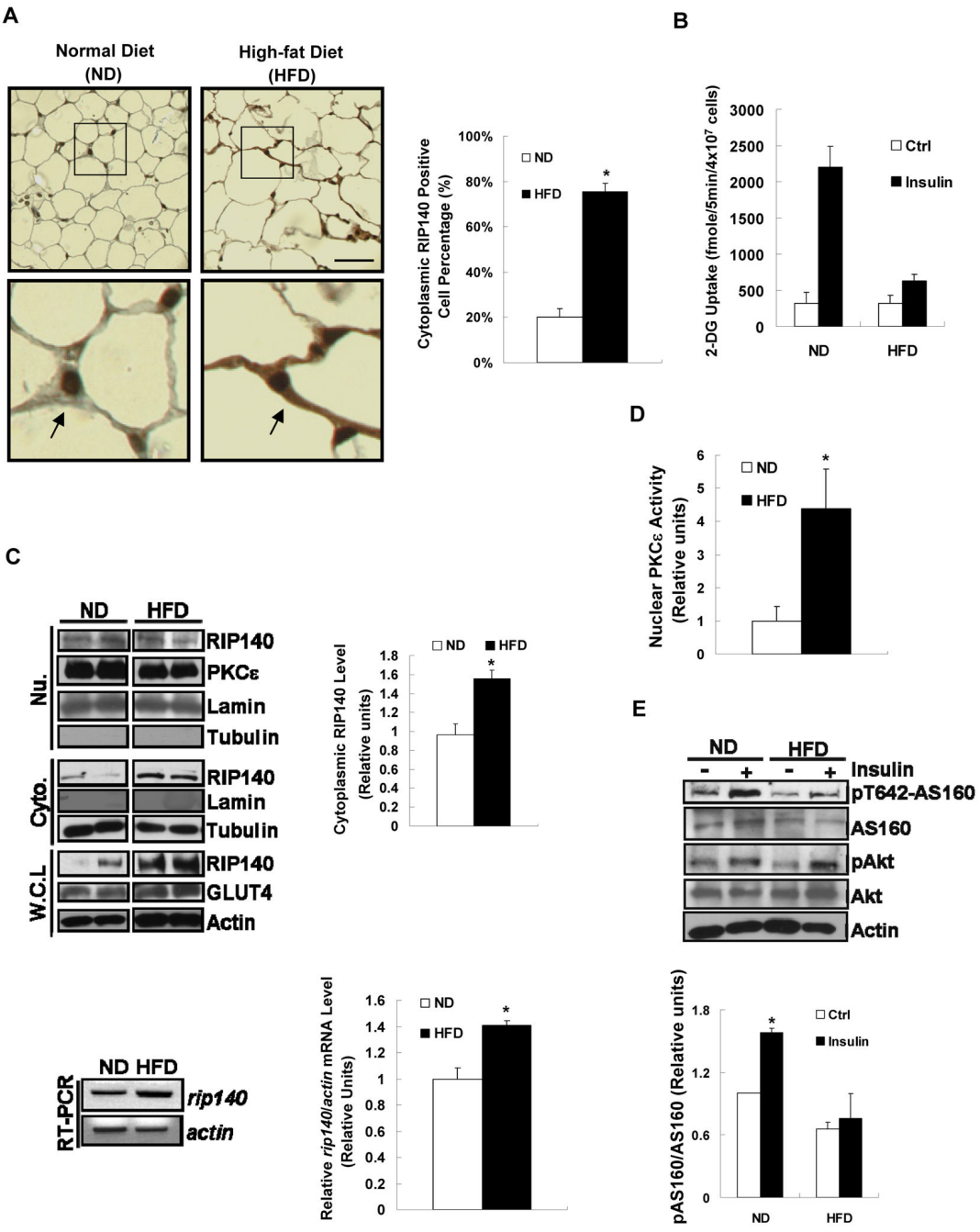

Figure 4. Increases in cytoplasmic RIP140 and nuclear PKCε activity in epididymal white adipose tissues from obese mice

(A) Endogenous RIP140 in epididymal adipose tissues from mice fed ND or HFD for 5 weeks. Arrows in enlarged images (lower) point to the cytoplasmic signals of RIP140. Scale bar = 25 μM. Right: Effect of HFD on the percentage of cytoplasmic RIP140-positive cells. (B) Glucose uptake in epididymal adipocytes (ND: 24 mice; HFD: 16 mice). (C) Upper left: Immunoblots of nuclear, cytoplasmic, and whole-cell RIP140 in epididymal adipocytes; each lane shows samples pooled from five mice. Relevant protein markers were monitored. Upper right: Quantification of cytoplasmic RIP140, normalized against that in ND-fed mice. Lower left: The mRNA levels determined by RT-PCR. Lower right: Quantification of RIP140 mRNA,

normalized against actin. (D) Nuclear PKCε activity (ND: 18 mice; HFD: 12 mice). The average PKCε activity in ND group was set as 1. *: $p < 0.05$ vs. normal diet. (E) Phosphorylation of AS160 and relevant kinases in adipocytes, with or without insulin treatment (170nM, 20 min). Lower panel: ratio of pT642-AS160/total AS160. Data are presented as means ± SD. *: $p < 0.05$ vs. control set.