ClipR-59 plays a critical role in the regulation of body glucose homeostasis

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Abbreviations: ACC1, Acetyl-CoA carboxylase; adipQ, Adiponectin; CAP-Gly, Cytoskeleton associated protein-glycine rich; Clip, Cytoplasm linker protein; ClipR-59, Clip Related Protein 59kD; FASN, Fatty acid synthetase; GTT, Glucose tolerance assay; ITT, Insulin tolerance assay; TG, Transgenic mice; Trib3, Tribbles 3.

By regulating Akt membrane compartmentalization, ClipR-59 modulates adipocyte glucose transport. To elucidate the role of ClipR-59 in the regulation of whole body glucose homeostasis, we have generated adipose tissue specific transgenic mice and examined how forcing expression of ClipR-59 in adipose tissue affects body glucose homeostasis. We found that ClipR-59 adipose transgenic mice showed lower blood glucose level with increased glucose tolerance and enhanced insulin sensitivity. Moreover, ClipR-59 adipose transgenic mice were lean with reduced fat mass and against diet induced obesity. Finally, we examined the potential impact of ClipR-59 on adipose endocrine function and found that ClipR-59 expression enhanced adiponectin secretion in both 3T3-L1 adipocytes and adipose tissue, accompanied with increased circulating adiponectin and enhanced AMPK\(\alpha\) phosphorylation at Thr172 in adipose tissue and skeletal muscle. Overall, these studies demonstrate that ClipR-59 is likely an important regulator of body glucose homeostasis and adipocyte function.

Introduction

Adipose tissue is a vital organ in the regulation of body glucose and energy homeostasis. Impaired adipose function results in the development of metabolic syndrome and type II diabetes.1,2 The regulation of body glucose and energy homeostasis by adipose tissue depends on 2 distinct adipocyte functions: glucose uptake through insulin dependent Glut4 membrane translocation to modulates blood glucose levels3 and producing adipokines which act on other tissues to modulate peripheral insulin sensitivity and energy metabolism4,5. Insulin dependent glucose uptake and adipokine production in adipose tissue appears interlinked in adipose tissue. Glut4 specific knockout mice resulted in several insulin resistance, impaired adipokine production and glucose intolerance.6,7 On the other hand, adipokines, of particular adiponectin has been shown to regulate insulin sensitivity in adipocytes.8 It is therefore believed that the regulator of adipocyte glucose uptake will have profound impact on body glucose homeostasis as well as energy homeostasis.

ClipR-59 is a membrane associated protein characterized with 3 distinct structural domains including a Glu-Pro rich domain, 3 ankyrin repeats and 2 putative CAP-Gly domains.9 CAP-Gly domains are signature domain of ClipR-170 protein family that binds microtubule and regulates microtubule dynamics.10,11 In addition, ClipR-59 also undergoes palmitoylation at Cys534 and Cys535, which mediates the association of ClipR-59 with membrane.12,13 Interestingly, ClipR-59 CAP-Gly domains show no microtubule binding activity. Instead, it functions as protein-protein interaction module to interact with protein kinase Akt. As a result, ClipR-59, via its membrane association and Akt interaction, modulates Akt plasma membrane compartmentalization.14,15 In addition, ClipR-59 also interacts with AS160 and facilitates AS160 phosphorylation by Akt in adipocyte.14,15 Akt is the major downstream kinase and AS160 is well-known Akt substrate in insulin regulated Glut4 membrane translocation in insulin dependent Glut4 membrane translocation.16,17 Hence, by modulating Akt membrane compartmentalization and AS160 phosphorylation, ClipR-59 is involved in insulin dependent Glut4 membrane translocation in adipocyte.14,15

Given the critical role of insulin dependent Glut4 membrane translocation in the regulation of body glucose homeostasis, the regulation of Glut4 membrane translocation in adipocytes implies that ClipR-59 likely plays a role in the regulation of body glucose homeostasis. Here we report that we have generated adipose specific ClipR-59 transgenic mice and found that adipose ClipR-59 transgenic mice exhibited higher glucose tolerance and against diet induced obesity.

Results

Generation of ClipR-59 adipocyte transgenic mice

ClipR-59 interacts with Akt and modulates Akt membrane localization and thereby adipocyte insulin dependent Glut4

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membrane translocation in adipocyte.\textsuperscript{14,15} Insulin dependent Glut4 membrane translocation is a basic mechanism to regulate body homeostasis.\textsuperscript{3} This implies that ClipR-59 play a role in the regulation of whole-body glucose homeostasis. To examine this, we generated adipose tissue-specific ClipR-59 transgenic mice with the aP2-ClipR-59 transgenic gene. Specifically, a Flag-tagged ClipR-59 cDNA was cloned into an adipose-specific aP2 promoter (Fig. 1a), and the ClipR-59 transgenic mouse was generated at the Tufts Transgenic Core Facility by pronuclear injection of the aP2-ClipR-59 transgenic gene into embryonic cells within the C6Black genetic background. We obtained a total of 8 pups of which 2 mice (no. 84 and 85) have the transgene judged by PCR analysis of individual mouse-tail DNA (Fig. 1b).

The ClipR-59 transgenic mice were inbred. The F1 mice appear normal and fertile, indicating that the expression of the ClipR-59 transgenic gene has no adverse effect on their development. Figure 1c shows a representative analysis of the genotype of the F1 generation. Of these 6 F1 littersmates, 3 have the ClipR-59 transgene, indicating that we have successfully generated ClipR-59 transgenic mice.

Next, we analyzed the expression of the ClipR-59 transgenic gene in several tissues, including fat, liver, and kidney. Shown in Figure 1d, the ClipR-59 transgenic gene is expressed in adipose tissue derived from mouse 129 but not from mouse 130. In addition, there is no detectable amount of ClipR-59 transgene expression in the liver and kidney of either mouse. From these data, we conclude that we have successfully generated ClipR-59 adipose tissue transgenic mice.

Glucose homeostasis of ClipR-59 adipose transgenic mice
To determine the impact of ClipR-59 adipose transgenic gene on mice, aP2-ClipR-59 transgenic mice were propagated from lines 131 and 134, respectively. First, we monitored growth and weight gain of these mice. We observed that they were about 10-20\% lighter than wildtype littermates through the first 4 months (Fig. 2a). Next, we measured the level of fasting blood glucose at age 3 months. The ClipR-59 transgenic mice (TG) had a slightly lower level of fasting blood glucose than the wildtype (WT) littermates (TG: 93 ± 3 mg/dl vs WT: 110 ± 5.8 mg/dl, n = 4) (Fig. 2b). The key parameter by which to determine how glucose homeostasis is affected is to measure how the mouse copes with blood glucose changes. Therefore, glucose tolerance tests were done to determine how aP2-ClipR-59 transgenic mice deal with changes of blood glucose (Fig. 2c). After glucose injection, aP2-ClipR-59 transgenic mice showed a higher rate of blood glucose disposal compared to wildtype mice, an indication that expression of ClipR-59 in adipose tissue increased glucose tolerance. Next, we performed an insulin tolerance assay to measure insulin activity. As shown in Figure 2d, ClipR-59 transgenic mice clearly showed higher insulin sensitivity.

The impact of ClipR-59 expression on adipose tissue
At 4 months of age, we scarified the mice and examined the impact of ClipR-59 expression on adipose tissue. A simple view of the dissected mice shows that transgenic mice had less fat mass (Fig. 3a). For a more accurate measure, we expressed the fat mass as a ratio of epididymal fat pad weight to total body weight. We found that the epididymal fat pad of induced ClipR-59 adipose mice (0.015 ± 0.005 fat mass/body-weight) was almost 60\% less than that of non-induced ClipR-59 mice (0.045 ± 0.007 fat mass/body-weight) (Fig. 3b). We also assessed the size of white adipocytes from wildtype and ClipR-59 transgenic mice through hematoxylin and eosin staining. In agreement with the finding that ClipR-59 transgenic mice were leaner, the adipocytes from ClipR-59 transgenic mice were smaller in size than those from wildtype littersmates (Fig. 3c). These data indicate that exogenous ClipR-59 expression in adipocytes has an impact on total fat mass.

The impact of adipose mass implies that ClipR-59 might affect the development of obesity. To determine how ClipR-59 transgenic mice copy with diet induced obesity, ClipR-59 transgenic mice and their wildtype littersmates were fed with regular chow or
high calorie diet (60%) up to 12 weeks. Wildtype mice become obese within 8 weeks on high calorie diet judged by more than 20% bodyweight gain. On the other hand, at this time, ClipR-59 transgenic mice showed no significant differences in bodyweight from wildtype mice on regular chow, suggesting that ClipR-59 transgenic mice were resistant against diet induced obesity (Fig. 3d).

In our early studies, we showed that ClipR-59, via recruiting Akt to membrane, regulates Glut4 membrane translocation.14,15 To determine whether there is a differ in adipose glucose membrane translocation between wildtype and ClipR-59 transgenic mice, the subcellular fractionation assay was performed in adipocytes from ClipR-59 transgenic mice and their wildtype littermates. As shown in Figure 4a and b, insulin promotes Glut4 membrane translocation and Akt activation as expected in wildtype adipocytes. In ClipR-59 transgenic adipocytes, the levels of Glut4 and phospho-Akt on PM were significantly higher than that of wildtype (panel i, and iii). In addition, we also examined membrane translocation of IRAP, the major cargo of Glut4 vesicle. Similarly, the levels of IRAP in PM were also significantly higher in ClipR-59 transgenic adipocytes than that in wildtype one. The difference in Glut4 in PM observed here is not because of sample variation as a comparable levels of syntaxin 4, a PM protein whose levels is not regulated by insulin were observed in each sample (panel iv). Since, there is no different between wildtype and ClipR-59 transgenic adipose tissue in the expression of Glut4, IRAP and overall Akt activation (panel v, iv and vii), these results further demonstrate that ClipR-59 in adipose tissue promotes Akt membrane association and Glut4 membrane translocation as we have previously reported.

ClipR-59 regulates adiponectin production

Glut4 transgenic mice show increase adipose mass and adiposity.18,19 The view thatClipR-59 transgenic mice exhibited increase Glut4 membrane translocation, yet with decreased fat mass implies that ClipR-59 could regulate other adipose function, i.e. adipokine production. Adipose tissue produces a number of adipokines include adiponectin, the most abundant adipokine to regulate energy metabolism.20 To examine this, adipose pad were isolated from 3 month wildtype and ClipR-59 transgenic mice and cultured in serum free medium for 6 hours. Then, the levels of adiponectin, in medium were determined. As shown Figure 5a, the amount of adiponectin in medium of ClipR-59 transgenic adipose tissue was about twice more than that of control adipose tissue (panel i and Fig. 5b for quantified result). In agreement with notion that ClipR-59 adipose transgenic mice expressed higher ClipR-59, the levels of ClipR-59 in ClipR-59 transgenic adipose tissue were higher than control adipose tissue (Fig. 5a, panel iii).

To further examine the regulation of adiponectin production by ClipR-59, we assessed the adiponectin production in 3T3-L1 adipocytes that were transduced with adenoviral vectors that expressed either ClipR-59 cDNA or GFP (served as a control). As shown in Figure 5c, the amount of adiponectin in medium from ClipR-59 overexpressing adipocytes was twice about that from control cells (panel i and the quantified result Fig. 5d). Correspondingly, the cellular levels of adiponectin were decreased about 50% in ClipR-59 overexpressing cells (panel ii). Next, we examined the impact of ClipR-59 knockdown on adiponectin production.
As shown in Figure 5e, the levels of adiponectin in cell culture medium decreased by more than 50% (panel i and Fig. 5f, the quantified result) with corresponding decreases in the cellular levels of adiponectin (panel ii). As expected, expression of ClipR-59 shRNA decreased the levels of ClipR-59 by more than 70% (panel iii).

The notion that ClipR-59 expression regulates adiponectin production promotes us examining the circulating adiponectin in ClipR-59 transgenic mice. Toward this, we collected blood samples from both wildtype and aP-ClipR-59 transgenic mice and assessed the levels of adiponectin in these samples. As shown in Figure 6a and b, the circulating levels of adiponectin in ClipR-59 transgenic mice were twice more than that in wildtype mice. Next, we examined the circulating adiponectin under regular chow and high fat diet. As shown in Figure 6c and d, in agreement with the view that obesity reduces circulating adiponectin, the plasma levels of adiponectin were reduced by more than 60% under high calorie diet. Similarly, the plasma levels of adiponectin were high in ClipR-59 transgenic mice, which are only modestly reduced under high calorie diet.

In cells, adiponectin binds to its receptor and activates AMPK through induction of AMPKα subunit phosphorylation at Thr172 to regulate energy metabolism.21-23 With this mind, we examined phosphorylation levels of AMPKα at Thr172 in both adipose tissue and skeletal muscle from wildtype and ClipR-59 adipose transgenic mice. As shown in Figure 6e, the levels of phospho-AMPKα at Thr172 in ClipR-59 transgenic were about 2 fold higher than that in wildtype mice in both adipose tissue (panel I, Fig. 6f) and muscle (panel iii, Fig. 6g).

Taken together, these data demonstrate that ClipR-59 is involved in adipocyte adiponectin production which altered the activation of AMPKα.

One of adiponectin activities is to regulate lipid metabolism through modification of lipid metabolic genes such as PGC1α,24 Fatty acid synthetase (FASN)25 and SREBP-1c.26 To determine whether there are alterations in the expression of these genes in ClipR-59 adipose transgenic mice, we examined the expression of these in ClipR-59 transgenic adipose tissue by RT-PCR. We observed that the expression of PGC1α was increased whereas that of SREBP1c, FASN was decreased (Fig. 6h).

We also examined the expression of ACC1, Trib3 and ClipR-59 in ClipR-59 transgenic mice. No appreciable changes in the expression of ACC1 and Trib3 were observed. As expected, ClipR-59 expression was markedly higher in ClipR-59 transgenic mice. Taken together, we believe that ClipR-59 expression in adipose tissue altered the expression of lipid metabolic genes.

Discussion

To explore the role in ClipR-59 in metabolic regulation, we have generated ClipR-59 adipose transgenic mice. Based on our in vitro studies that ClipR-59 regulates insulin dependent
membrane translocation, we anticipate that ClipR-59 adipose transgenic mice will exhibit a lower blood glucose level and higher glucose tolerance, all of which were found to be true (Fig. 2), underscoring the role of ClipR-59 in the regulation of whole body glucose homeostasis.

Glut4 adipose transgenic mice showed increased glucose tolerance, but also increased adiposity. The view that ClipR-59 adipose tissue transgenic mice consist of less fat mass implies that ClipR-59 might potentially affect the other function of adipocytes. With this mind, we examined the possible effect of ClipR-59 on the secretion of adiponectin. Adiponectin is the most abundant adipokine produced from adipocyte and has been demonstrated to play a critical role in the regulation of glucose and energy metabolism. Our results showed that ClipR-59 modulates adiponectin production as forcing expression of ClipR-59 enhanced whereas knockdown of ClipR-59 decreased adiponectin production (Fig. 5). Moreover, we have observed that the circulating levels of adiponectin in ClipR-59 transgenic mice are higher than that in wildtype mice and (Fig. 6a–d) under both regular chow and high fat diet. Adiponectin is the most abundant adipokine and plays a critical role in the regulation of energy metabolism and peripheral insulin sensitivity. In cells, binding adiponectin to its receptor (AdipR) activates AMPK, which in turn regulates lipid and energy metabolism. Impaired Adiponectin signaling results in insulin resistance, obesity and type II diabetes. It is therefore plausible that regulation of adiponectin production by ClipR-59 in adipocytes would contribute to the phenotype observed in ClipR-59 adipose tissue transgenic mice, namely lean mice and enhances insulin sensitivity.

At present, how ClipR-59 regulates adiponectin production is not known. ClipR-59 is a palmitoylated protein which is associated with plasma membrane and trafficking vesicles cells and regulate vesicle trafficking and membrane dynamics. Thus, it is possible that the regulation of adiponectin production is because the function of ClipR-59 in plasma membrane. Further studies are required to elucidate the mechanism under which ClipR-59 to regulate adiponectin secretion.

As endocrine organ, adipose tissue produces a number of adipokines such as leptin, etc, which play critical role in energy metabolism. In current studies, we have not examined whether ClipR-59 is involved in the production of other adipokines. However, it will be great interesting to examine this in future.

In summary, we have examined the potential role of ClipR-59 in the regulation of glucose homeostasis in vivo and found that ClipR-59 potentially regulates tow important functions of adipocyte, Glut4 membrane translocation and adiponectin production, thereby, glucose homeostasis. To further establish the role of ClipR-59 in adipocyte function, ClipR-59 adipose tissue conditional knockout mice, which is currently in the production, is required.

Figure 4. Subcellular fractionation assays of adipocytes from wildtype and ClipR-59 transgenic mice. (a) Adipocytes were prepared from epidydymal pads from either wildtype or ClipR-59 transgenic mice. Then, the adipocytes were treated with or without 10nM insulin and plasma membrane were prepared as described in Methods and Materials. The plasma membrane fractions were analyzed in Western Blot with Anti-Glut4, anti-IRAP, anti-phospho-Akt, anti-Akt and anti-syntaxin 4 (stx4) antibodies, respective as indicated. PM: plasma membrane. Tcl: total cell lysates. (b) Quantification of Glut4 membrane translocation in (a). The Glut4 levels in PM in wildtype adipocytes without insulin simulation were set as 1 after normalized to the levels of syntaxin 4 on PM. Bar graphs show means ± STDV, n = 3.

Methods and Materials

Reagents

Anti-Flag antibody (F3165) was from Sigma. All common used chemicals from Thermos Scientific. Anti-adiponectin (2789), anti-phospho-AMPKα at Thr172 (Cat# 2535S), anti-AMPKα (Cat# 5831S), anti-IRAP (Cat# 6918), anti-Akt (4685) and anti-phospho-Akt (Cat# 8200S) antibodies were from Cell Signaling. anti-Glut4 (Cat# SC-53566), Anti-β-tubulin (SC-23950), anti-syntaxin 4 (Cat# SC-101301) were from Santa Cruz. aP2 promoter construct (Cat# 11424) was from Addgene. High calorie diet (Cat# F3282) was from Bio-serv laboratory. Anti-ClipR-59 antibody has been described.

Generation of ClipR-59 transgenic mice

Flag-tagged ClipR-59 cDNA, which consists of CREB 5’UTR was cloned into pBS-aP2 between Smal and Not I site. Then, ClipR-59 transgenic gene were released by XhoI site. The resulted transgenic gene was injected to C6Black mouse embryo in Tufts Medical Center transgenic core facility.

Genotyping and PCR

Mouse Genotyping were carried out by genomic PCR. Specifically, mouse genomic DNA were prepared with DNaseasy kit (Qiagen Cat# 69506). Then, PCR were carried with Forward primer corresponding CREB UTR of transgene and Reverse primer corresponding ClipR-59 transgene: forward
5'-GGAGAAGCCGAGTGTTGGTG-3 (within CREB UTR) and reverse 5'-GGTGCACAACAGGCTTCTG-3 within ClipR-59. The cycles were 94°C, 30" 56°C 30" and 72°C 30" for total of 25 cycles. The PCR products were analyzed on 1.5% agarose gel. Since there primers are specifically to the transgene, transgene is the only one that will be amplified in PCR. In addition, the amplified transgene consists of an EcoRI site which will facilitate to confirm the amplified transgene. All works with mice were performed according to approved mouse protocol by DLAM of Tufts Medical Center.

mRNA and RT-PCR

Tissue RNAs were prepared with RNeasy kit (Qiagen Cat# 74106). The cDNA generation was carried out with RT kit from Ambion (10928-034) with above primers. The primers for endogenous ClipR-59 are the following: forward 5'- ATGACTAAGACGGATCCTG-3 and reverse 5'-GGTGCACAACAGGCTTCTG-3. The primers for PGC1α, ACC1, SREBP1c, FASN and Trib3 and the procedure for quantitative PCR have been described30. The realtime PCR was carried out as the following: 94°C; 45" 56°C 45" and 72°C 30" for total of 29 cycles.
Isolation of primary adipocytes and subcellular fractionation assay

The isolation of primary adipocytes from fat pad and subcellular fractionation assay have been described. Briefly, the epididymal fat pads were taken from mice and minced. Then, the minced adipose tissue were digested with collagenase type IIa (Sigma, Cat# C6885) in TESCA buffer (50 mM TES, 0.36 mM Calcium chloride, pH 7.4) at 37°C for 2 hours. After digestion, the digested tissues were filtered and cultured in DMEM. For subcellular fractionation assay, the primary adipocytes were serum-derived from 6 hours and following treated with or without 100nM insulin for 30 mins. For examining adiponectin secretion, the culture medium were taken after 6 hours incubation.

Cell culture

3T3-L1 preadipocytes (ATCC Cat#CL’183,) were cultured in DMEM supplemented with 10% bovine serum and 1x antibiotic-antimycotic. HEK293 Cells were cultured in DMEM (Cat# 11995073, Life technologies) supplemented with 10% FBS (Cat# 26140079, Life technologies) and 1x antibiotic-antimycotic (Cat#15240112, Life technologies). The differentiation of 3T3-L1 pre-adipocytes were carried out as previously described. Briefly, after grown to confluence, 3T3-L1 preadipocytes were cultured for 2 more days. Then, the medium was switched to differentiation medium (DMED supplemented with 10% FBS, 1uM Dexamethesone. 1mM IBMX and 2ug insulin/ml. Four days later, the medium regular medium supplemented with 1ug/ml insulin. The cells were used for experiments. The adenoviral expression vectors that express either ClipR-59 cDNA or ClipR-59 shRNA have been described, and purified thorough double CsCl banding as described. To transduce 3T3-L1 adipocytes, the adenoviruses were incubated with 3T3-L1 adipocytes for overnight. The amount of viruses used is to ensure that more than 75% cells were transduced based on the GPF positive cells. The adenoviral vectors consist of a GFP gene which expressed separately from targeted gene. For adiponectin secretion studies, the 3T3-L1 adipocytes were incubated in serum-free medium for 4-6 hours. Then, the cell culture medium and total cell lysates were prepared for Western blot.

The blood sample and tissue extract preparation

Blood specimens (usually 50 ul) were collected from mice. Then, the specimens were centrifuged for 3000 rpm for 15 min. The supernatants (plasma sera were collected for Western blot. To obtain tissue extract, the individual tissue were taken from mice and minced in a tissue culture plate. Then, the tissues were homogenized in SDS-UREA buffer (8M Urea, 1xPBS, 1 mM NaF, 1mM NaVO, 3.5 mM β-glycerophosphate and 5% Glycerol). The protein concentration was determine with BCA kit (Thomas Fisher Scientific).
Glucose measurement, Glucose tolerance assay and insulin tolerance assay
Age matched wildtype and transgenic mice (6 per group) were fasting for 6 hours. Then mice will be weighed, fasting blood glucose was measured, and the mice will be injected intraperitoneally with a 20% D-glucose solution (2 grams D-glucose/kg body weight). After 15, 30, 60, and 120 minutes, blood will be sampled from the tail and glucose levels measured as previously described. For insulin tolerance assay, baseline glucose level will be determined as described above. Subsequently, the mice will be injected intraperitoneally with human insulin (Lily, U-500) (0.75 units per kg body weight). After 30, 60, and 120 minutes, blood will be sampled from the tail, and glucose levels will be measured as described.  

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Adipocyte

H&E Staining
Mouse adipose tissues were fixed in 4% formalin solution. The tissue slides prepared from Tuft Pathology laboratory and stained with Hematoxylin and Eosin as described.  

Intraperitoneal glucose tolerance test
Age matched wildtype and transgenic mice (6 per group) were fasted for 6 hours before a 20% D-glucose solution (2 grams D-glucose/kg body weight) was administered. Blood glucose was measured before and after glucose administration. Blood glucose was measured at 15, 30, 60, and 120 minutes after glucose administration and the area under the curve (AUC) was calculated. No potential conflicts of interest were disclosed.

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