The Association of ClipR-59 Protein with AS160 Modulates AS160 Protein Phosphorylation and Adipocyte Glut4 Protein Membrane Translocation*

Wenying Ren, Sarwat Cheema, and Keyong Du

From the Molecular Oncology Research Institute, Tufts Medical Center, Boston, Massachusetts 02111

ClipR-59 is a membrane-associated protein and has been implicated in membrane signaling and vesicle trafficking. Recently, we have identified ClipR-59 as an Akt-interacting protein, and we have found that, by interacting with Akt, ClipR-59 modulates Akt subcellular compartmentalization and Akt substrate AS160 phosphorylation, thereby promoting Glut4 membrane translocation. Here, we have further investigated the regulatory effects of ClipR-59 on AS160 phosphorylation and subsequent adipocyte glucose transport. Our data showed that ClipR-59 interacted with AS160, which was mediated by the ankyrin repeats of ClipR-59 and regulated by insulin signaling. Moreover, the data also demonstrated that the interaction of ClipR-59 with AS160 was required for ClipR-59 to modulate Glut4 membrane translocation as ΔANK-ClipR-59, an AS160 interaction-defective mutant, failed to promote AS160 phosphorylation, Glut4 membrane translocation, and glucose transport induced by insulin in 3T3-L1 adipocytes. Because ClipR-59 also interacts with Akt and enhances the interaction between Akt and AS160, we suggest that ClipR-59 functions as a scaffold protein to facilitate Akt-mediated AS160 phosphorylation, thereby regulating glucose transport.

Background: The membrane-associated protein ClipR-59 is involved in vesicle trafficking and membrane signaling. Results: By interacting with AS160 and enhancing the association of AS160 with Akt, ClipR-59 promotes AS160 phosphorylation and Glut4 membrane translocation. Conclusion: ClipR-59 functions as a scaffold protein to facilitate AS160 phosphorylation by Akt. Significance: The interaction of ClipR-59 with AS160 emphasized the role of ClipR-59 in Glut4 membrane translocation.

Insulin is a primary metabolic hormone that regulates glucose homeostasis. Under physiological conditions, insulin binds to its receptor and induces insulin receptor intrinsic kinase activity, which set a motion to activate downstream Ser/Thr kinase Akt, the kinase that mediates the major metabolic effects, including promoting adipocyte glucose uptake and suppressing hepatic production (1). Adipose glucose transport induced by insulin occurs through redistribution of insulin-sensitive glucose transporter 4 (Glut4) from intracellular sites to the plasma membrane (PM).2 Under basal conditions, Glut4 is retained in distinct intracellular compartments or Glut4 storage vesicles localized in the trans-Golgi network, the late endosomes, and the recycling endosomes. After insulin stimulation, Glut4 is released from Glut4 storage vesicles and is tethered to the plasma membrane where Glut4 is docked and ultimately fused with the membrane (2). Akt participates in Glut4 membrane translocation through phosphorylation of its specific substrates (3). One of the Akt substrates is AS160 (also known as TBC1D4). AS160 is a membrane-associated Rab GTPase-activating protein (GAP) whose targets include Rab10 (4), Rab11 (5, 6), and Rab14 (7). AS160 plays a critical role in Glut4 membrane translocation, a process that is essential for insulin to maintain whole-body glucose homeostasis (3). Under basal conditions, AS160 acts as a brake on Glut4 translocation by maintaining one or more Rabs required for translocation in their inactive GDP-bound state. In response to insulin, Akt phosphorylates AS160 at Ser-318, Ser-570, Ser-588, Thr-642, and Thr-571 (8) and suppresses AS160 GAP activity (9). Consequently, the elevation of the GTP form of the Rabs occurs, leading to the increased docking and subsequent fusion of the Glut4 vesicles at the plasma membrane (10, 11). Because AS160 itself is associated with cellular membranes, it is believed that Akt, which is associated with the plasma membrane, is important for modulating Glut4 membrane translocation.

ClipR-59 (Clip-170-related protein 59 kDa, also termed as Clip3) was first recognized as a putative member of the Clip-170 protein family based on its two putative CAP-Gly domains (also known as microtubule binding domain) (12). The members of Clip-170 family bind to microtubules and regulate microtubule dynamics (13). However, ClipR-59 exhibits no association with microtubules in vivo. Instead, it is associated with cellular membranes, including plasma membrane, trans-Golgi network, and endosomes (14). The association of ClipR-59 with membrane suggests that ClipR-59 might be functionally important in the regulation of the membrane cellular events. Supporting this view, ClipR-59, when overexpression...
pressed, perturbed early/recycling endosome-trans-Golgi network dynamics (12). More recently, we isolated ClipR-59 as an Akt-interacting protein and found that ClipR-59 preferably interacted with active Akt and promoted Akt membrane association, thereby regulating Akt signaling compartmentalization (15). By modulating Akt cellular compartmentalization, ClipR-59 directly contributes to the regulation of Akt cellular homeostasis. Consistently, the augmentation of ClipR-59 expression increases the amount of Akt associated with the cellular membrane and concomitantly decreases that of Akt in other cellular compartments, thereby differentially regulating the phosphorylation of the Akt substrates. In adipocytes, one of the Akt substrates whose phosphorylation is influenced by ClipR-59 is AS160 (15).

In this study, we have further examined the potential regulation of AS160 phosphorylation by ClipR-59. The results show that ClipR-59 is associated with AS160 via its ankyrin repeats and that the interaction of ClipR-59 with AS160 is required for ClipR-59 to modulate AS160 phosphorylation and subsequently glucose transport.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Insulin, dexamethasone, 3-isobutyl-1-methylxanthine, DAPI, rabbit anti-syntaxin 4 antibody and mouse monoclonal anti-FLAG antibody were from Sigma. Rabbit anti-Glut4 and anti-AS160 antibodies were from Millipore. Mouse monoclonal anti-HA antibody was from Covance. Mouse monoclonal anti-Glut4 antibody (1F8), rabbit monoclonal anti-Akt substrate antibody, Akt, and phospho-Akt antibodies were from Cell Signaling. Rabbit anti-ClipR-59 antibody has been described previously (15).

**Plasmids and Virus Production**—ClipR-59 and its mutants have been described (15). The expression vectors of Akt2, FLAG-AS160, and internal HA-tagged Glut4 were generous gifts from Drs. J. Q. Cheng (16), G. E. Lienhard (8), and Samuel Cushman (18), respectively. The adenoviruses were produced and purified by double CsCl gradient as described (19).

**Cell Culture and Transient Transfection**—HEK293 cells were grown in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). 3T3-L1 preadipocytes were grown in the same DMEM but supplemented with 10% bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). 3T3-L1 preadipocytes were cultured for 2 additional days after reaching 100% confluency and treated with differentiation medium (DMEM/high glucose containing 10% FBS, 2.5 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 2.5 μM dexamethasone, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) for 4 days. Then the medium was changed to regular medium. After 7 days of differentiation, the adipocytes were used for experiments. When the cells were infected with viruses, at least 75% of cells were transduced.

**Cell Imaging**—3T3-L1 adipocytes grown on coverslips were transduced with the indicated expression vectors. Then the cells were fixed and stained with the primary antibody followed by Cy3-conjugated goat anti-mouse secondary antibody. The fluorescence imaging is captured with confocal microscopy (Olympus).

**Immunoprecipitation Assays**—3T3-L1 adipocytes or transduced HEK293 cells were extracted with immunoprecipitation buffer (150 mM NaCl, 25 mM Tris, pH 7.6, 0.5 mM EDTA, 10% glycerol, 0.5% Nonidet P-40 plus a protease inhibitor mixture). 500 μg of total proteins from the cell lysates were subjected to immunoprecipitation with the corresponding antibodies.

**Subcellular Fractionation Assay**—3T3-L1 adipocytes with or without insulin treatment were suspended into HES I buffer (0.25 mM sucrose, 20 mM Tris, pH 7.6, 1 mM EDTA plus a protease inhibitor mixture). The cells were homogenized by passing through a 23-gauge needle 10 times, and then the homogenates were centrifuged at 19,000 × g for 20 min. To isolate the membrane fraction, the resultant pellets from the 19,000 × g centrifugation were layered on HES II buffer (1.12M sucrose, 20 mM Tris, pH 7.6, 1 mM EDTA) and centrifuged at 100,000 × g for 60 min. The resultant pellets were designated as the nuclear and mitochondrial fraction. The plasma membrane layers were removed from the sucrose cushion, suspended into HES I buffer, and centrifuged at 41,000 × g for 20 min. The resultant pellets were plasma membrane (PM). To isolate microsomes, the resultant supernatant from the 19,000 × g centrifugation was centrifuged at 175,000 × g for 75 min, and the pellets were collected as low density microsomes (LDM). The supernatant from the 175,000 × g centrifugation was saved and designated as cytosol.

**Fractionation of Intracellular Microsomes on Sucrose Gradient**—LDM from 3T3-L1 adipocytes were suspended in phosphate-buffered saline (PBS) and loaded onto a 4.6-ml continuous sucrose gradient (20–40% sucrose for velocity centrifugation) and centrifuged for 18 h in an SW 50.1 rotor at 48,000 rpm. The gradient was collected into 12 fractions (0.4 ml of each) starting from the top of the tube.

**GST Pulldown Assay**—GST-ClipR-59 fusion peptide was expressed in HEK293 cells from pEBG expression vector and purified with glutathione-Sepharose 4B according to the manufacturer’s instruction. The purified GST-ClipR-59 was then mixed with the 3T3-L1 adipocyte LDM fraction in immunoprecipitation buffer and incubated for 4 h. Then the beads were washed three times, and the proteins associated with GST beads were analyzed by Western blot with the corresponding antibodies. Also, before washing, a 2% volume of the mixtures was subjected to Western blot to determine the input level of each component.

**Glucose Uptake Assays**—3T3-L1 adipocytes were serum-deprived overnight and then were treated with or without insulin for 30 min. Then the cells were washed twice with Krebs buffer (50 mM HEPES, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO₄, 1.25 mM CaCl₂) and further incubated with Krebs buffer plus 100 mM 2-deoxyglucose, 1 μM 2-[³H]deoxyglucose (PerkinElmer Life Sciences). After 5 min of incubation, the cells were washed three times with PBS and lysed with 0.05 N NaOH. The amount of 2-[³H]deoxyglucose was counted and normalized to protein concentration in the lysates. In addition, the final results were also subtracted from the background glucose uptakes, which were determined by the amount of 2-[³H]de-
oxyglucose in the lysates when the cells were pretreated with 10 μM cytochalasin B (Sigma).

Western Blotting—After treatments, cells were washed twice with PBS and extracted with cell lysis buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, protease, and phosphatase inhibitors). For cellular fractionation experiments, the cellular fractions were directly dissolved into lysis buffer. Equal amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). After blocking in 5% dry milk, the membranes were incubated with each primary antibody, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. The protein bands were visualized using the ECL detection system (Pierce). The quantification of Western blot was determined with ImageJ software.

Statistical Analysis—Means ± S.D. were calculated, and statistically significant differences among groups were determined by one-way analysis of variance followed by post hoc comparisons or by two-tailed unpaired Student’s t test between two groups as appropriate, with significance at p < 0.05.

RESULTS

ClipR-59 and AS160 Are in the Same Complex—Biochemical characterization of the ClipR-59 cellular localization revealed that ClipR-59 was associated with PM, LDM, and high density microsomes in adipocytes (15). Adipocyte LDM consists of numerous factors, including Glut4 and AS160, that are critical for insulin-regulated glucose transport (3). Because ClipR-59 also regulated adipocyte glucose transport, we sought to examine the potential localization relationship among AS160, ClipR-59, and Glut4 in LDM. To this end, we fractionated the LDM of 3T3-L1 adipocytes with a continuous sucrose gradient (20–40%) and assessed the presence of ClipR-59, Glut4, and AS160 in each fraction by Western blot with antibodies specific to each protein. As shown in Fig. 1A, both ClipR-59 and Glut4 were present in fractions 5–9 with the peak around fraction 7 (panels ii and iii, respectively). AS160 was also found in fractions 5–9 with the peak around 7. However, unlike ClipR-59 and Glut4, AS160 was also present in the rest of the fractions with an additional peak at fraction 10 (Fig. 1A, panel i). Thus, the data here indicate that ClipR-59, AS160, and Glut4 exist in a subpopulation of LDM. As a control, we examined the distribution of EEA1 (20), an early endosomal and a de facto LDM marker, through the sucrose gradient. We observed that EEA1 was present in most fractions with an increment change following sucrose gradient (Fig. 1A, panel iv).

Because ClipR-59, AS160, and Glut4 were concomitantly presented in the same fraction in continuous sucrose gradient, we considered the possibility that these proteins would be in the same complex. To test this, the GST pulldown assay was carried out with a GST-ClipR-59 fusion protein and LDM fraction of 3T3-L1 adipocytes. As shown in Fig. 1B, both AS160 and Glut4 were retained on GST-ClipR-59 beads but not GST beads (compare lanes 1 and 2 in panels i and ii). Given that the comparable levels of AS160 and Glut4 were detected in the whole LDM (Fig. 1B, panels iii and iv), these data suggest ClipR-59 and AS160 may be in the same complex.

ClipR-59 Interacts with AS160, Which Is Negatively Regulated by Insulin Signaling—To further examine the association between AS160 and ClipR-59, we carried out co-immunoprecipitation (co-IP) assay with lysates of HEK293 cells transiently expressing HA-tagged ClipR-59 and FLAG-tagged AS160 using an anti-HA antibody. As shown in Fig. 2A, FLAG-AS160 was recovered from the anti-HA immunoprecipitates of cell lysates with HA-ClipR-59 expression but not that without HA-ClipR-59 expression (Fig. 2A, top panel, compare lanes 1 and 2), demonstrating the association of ClipR-59 with AS160. In these
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FIGURE 2. ClipR-59 is associated with AS160, and their interaction is negatively regulated by insulin signaling. A, co-immunoprecipitation assay of HEK293 cell lysates expressing FLAG-tagged AS160 or AS160–4P and HA-tagged ClipR-59 with anti-HA antibody. HEK293 cells were transfected with FLAG-tagged AS160 (wild type and 4P) and HA-tagged ClipR-59 expression vectors. Thirty-six hours post-transfection, the total cell lysates were prepared for immunoprecipitation (IP) assay with the anti-HA antibody. Top, FLAG-tagged AS160 in HA immunoprecipitates. Middle, input of AS160. Bottom, input of HA-tagged ClipR-59. B, GST pulldown assay of lysates from HEK293 cells transfected with GST-ClipR-59. HEK293 cells were transiently transfected with pEBG or pEBG-ClipR-59 expression vector. The total cell lysates were prepared after the cells were serum-starved overnight and pretreated with DMSO (vehicle (Veh)), PI3K kinase inhibitor LY294002 (LY, 10 μM), and Akt inhibitor VIII (A8, 2.5 μM) followed by insulin and incubated with GST beads. After collection, the GST beads were subjected to Western blot with the anti-AS160 antibody (panel i). The expression levels of AS160, phospho-Akt, Akt, and GST-ClipR-59 are shown in panels ii–v, respectively. Inh, inhibitor. IB, immunoblot. C, co-immunoprecipitation of 3T3-L1 adipocyte lysates with anti-ClipR-59 antibody. The lysates from 3T3-L1 adipocytes with or without insulin (Ins) treatment were immunoprecipitated using the rabbit anti-ClipR-59 antibody and then the immunoprecipitates were analyzed in Western blot with the anti-AS160 antibody (top). Middle, input of AS160. Bottom, input of ClipR-59. All of these experiments were repeated twice with similar results.

experiments, we also assessed the association of ClipR-59 with phosphorylation-defective AS160 mutant 4P-AS160 in which Ser-318, Ser-570, Ser-588, and Thr-642 are substituted with alanine (8). We observed more 4P-AS160 recovered from the HA-ClipR-59 immuno-

precipitates than that from the wild type AS160 (Fig. 2A, top panel, compare lanes 2 and 3). The expression of ClipR-59 had no impact on AS160 expression as a comparable input level of each protein was observed in each sample (Fig. 2A, middle and bottom panels).

The higher interaction affinity of 4P-AS160 with ClipR-59 suggests that the association between AS160 and ClipR-59 might be regulated by insulin. To test this, GST pulldown assay was performed with lysates from HEK293 cells transfected with GST-ClipR-59 and treated with 100 nM insulin for 30 min. Because HEK293 cells endogenously express AS160, this assay was used to examine the interaction between the exogenously expressed GST-ClipR-59 and the endogenous AS160. As shown in Fig. 2B, AS160 was recovered from GST-ClipR-59 beads (panel i, lane 1), in agreement with the view that AS160 is associated with ClipR-59. Treatment of cells with insulin led to a marked decrease in the amount of AS160 recovered from the GST-ClipR-59 beads (Fig. 2B, compare lanes 1 and 2). In these experiments, HEK293 cells transiently expressing GST-ClipR-59 were also treated with PI3K inhibitor LY294002 or Akt inhibitor VIII for 45 min prior to insulin treatment. Pretreatment of cells with either LY294002 or Akt inhibitor VIII not only strengthened the association of ClipR-59 with AS160 (Fig. 2B, compare lanes 1, 3, and 5, panel i) but also abolished the inhibitory effects of insulin on the association of ClipR-59 with AS160 (compare lanes 2, 4 and 6, panel i), without altering the cellular levels of AS160 and GST-ClipR-59 (panels ii and v). We also examined Akt phosphorylation, and we observed that Akt phosphorylation at Ser-473 was robustly elevated following insulin treatment (Fig. 2B, compare lanes 1 and 2, panel iii), but it was completely abolished by LY294002 and Akt inhibitor VIII (compare lanes 2, 4, and 6, panel iii) without affecting the total cellular level of Akt (panel iv), in line with the view that insulin promotes whereas both LY294002 and Akt inhibitor VIII suppress Akt phosphorylation induced by insulin. As an additional examination of the association between AS160 and ClipR-59, co-IP assay was also carried out with the lysates from 3T3-L1 adipocytes treated with 100 nM insulin for 30 min. Insulin stimulation decreased the amount of AS160 recovered from the anti-ClipR-59 immunoprecipitates by more than 70% (Fig. 2C, compare lanes 2 and 3). Collectively, these data demonstrate that ClipR-59 is associated with AS160, and their association is negatively regulated by insulin signaling.

Sequence of AS160 That Mediates the Interaction between ClipR-59 and AS160—Following demonstration of ClipR-59 interacting with AS160, we set to determine the domain responsible for this interaction in AS160. To this end, we generated the truncated forms of AS160, including ΔC-AS160 and ΔN-AS160 (Fig. 3A), and we assessed their abilities to interact with ClipR-59 when they were transiently expressed in HEK293 cells with HA-tagged ClipR-59 by co-IP assay. ClipR-59 was recovered from both wild type and ΔC-AS160 immunoprecipitates but not ΔN-AS160 immunoprecipitates (Fig. 3B), suggesting that the amino-terminal sequence of AS160 may mediate the interaction between ClipR-59 and AS160. To recapitulate these results, we also performed reciprocal immunoprecipitation assay using an anti-HA antibody. Consistently, wild type AS160 and ΔC-AS160, but not ΔN-AS160, were recovered from the HA-ClipR-59 immunoprecipitates (Fig. 3C).

Having identified the amino terminus of AS160 that mediates the interaction between ClipR-59 and AS160, we further analyzed this region. As shown in Fig. 3C, removal of proximal
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384 amino acids from ΔC-AS160 construct had no impact on the AS160 and ClipR-59 interaction, indicating that the region of AS160 from amino acids 385 to 720 is responsible for AS160 interaction with ClipR-59. The region of AS160 from amino acids 385 to 720 consists of three Akt phosphorylation sites, and hence mediating the interaction between AS160 and ClipR-59 by this region is compatible with the idea that the association between AS160 and ClipR-59 is regulated by Akt-induced AS160 phosphorylation.

Ankyrin Repeats of ClipR-59 Mediate the Interaction between ClipR-59 and AS160—After identifying the domain in AS160 that mediates the AS160 and ClipR-59 interaction, we next examined the domain(s) in ClipR-59 that is important for their interaction. There are two major functional domains in ClipR-59 as follows: ankyrin repeats and CAP-Gly domains. Thus, our studies were focused on these two domains. To determine the role of each domain in the interaction of ClipR-59 with AS160, we generated two ClipR-59 mutants, ΔANK-ClipR-59 and Δ2CAP-ClipR-59, in which the ankyrin repeats and CAP-Gly domains were internally deleted, respectively (Fig. 4A). These two constructs were transiently co-expressed with FLAG-tagged AS160 in HEK293 cells, respectively. The FLAG-tagged AS160 was immunoprecipitated, and the presence of HA-ClipR-59 in the FLAG immunoprecipitates was assayed by Western blot. As shown in Fig. 4B, HA-ClipR-59 was recovered from the FLAG-AS160 immunoprecipitates of HEK293 cell lysates expressing HA-ClipR-59 and FLAG-tagged AS160 but not from that of control cell lysates expressing HA-tagged ClipR-59 alone (top panel, compares lanes 1 and 2). Removal of the ankyrin repeats dramatically diminished the amount of HA-ClipR-59 associated with the FLAG-AS160 immunoprecipitates (Fig. 4B, top panel, compare lanes 2 and 3). We also examined the interaction between 4P-AS160 and these ClipR-59 mutants. In agreement with the view that phosphorylation of AS160 negatively regulates the interaction between ClipR-59 and AS160 (Fig. 2B), the amount of HA-ClipR-59 recovered from the FLAG-4P-AS160 immunoprecipitates was 5-fold more than that from the FLAG-AS160 immunoprecipitates (compare lanes 2 and 6). Similarly, removal of the ankyrin repeats abolished the interaction between ClipR-59 and 4P-AS160. These data indicate that the ankyrin repeats mediate the interaction between ClipR-59 and AS160.

It is noteworthy that although the removal of ankyrin repeats from ClipR-59 abrogated the interaction between ClipR-59 and AS160, that of CAP-Gly domains enhanced the interaction between ClipR-59 and AS160 (Fig. 4B, top panel, compare lanes 2 and 3, the number below each lane represents the ratio of the amount of ClipR-59 recovered from anti-FLAG immunoprecipitates and that of input, middle panel). It is also noted that although Δ2CAP-ClipR-59 exhibited a higher affinity to interact with AS160 compared with wild type ClipR-59, there was no difference observed between Δ2CAP-ClipR-59 and wild type ClipR-59 to interact with 4P-AS160 (Fig. 4B, compare lanes 6 and 9). In the preceding experiments (Fig. 2), we showed that Akt-induced AS160 phosphorylation decreased the affinity of AS160 to interact with ClipR-59. In our earlier studies, we have...
shown that ClipR-59 interacts with active Akt via its CAP-Gly domain (15). Therefore, the higher affinity of \( \Delta 2\text{CAP-ClipR-59} \) with AS160 but not 4P-AS160 further emphasizes the notion that the interaction between AS160 and ClipR-59 is regulated by Akt via phosphorylation of AS160.

**ClipR-59 Enhances the Association of AS160 with Akt**—ClipR-59 interacts with active Akt and promotes Akt-mediated AS160 phosphorylation (15). Because ClipR-59 interacts with AS160 (Fig. 2), we wondered whether ClipR-59 expression could enhance the association of AS160 with Akt, and thereby AS160 phosphorylation. To address this question, co-IP assay was carried out with the total cell lysates from HEK293 cells transiently expressing HA-Akt2 and FLAG-AS160 together with or without HA-ClipR-59. Akt2 was used because ClipR-59 preferably binds activated Akt (15). As shown in Fig. 5A, both HA-Akt2 and HA-ClipR-59 were recovered from the anti-FLAG immunoprecipitates of the cell lysates expressing FLAG-AS160 and HA-Akt (top panel, lane 2), FLAG-AS160 and HA-ClipR-59 (top panel, lane 3), and FLAG-AS160, HA-Akt2, and HA-ClipR-59 (top panel, lane 4), respectively, but not the one without FLAG-AS160 expression (top panel, lane 1). The presence of Akt and ClipR-59 in the anti-FLAG-AS160 immunoprecipitates argues that AS160 interacts with both Akt and ClipR-59 simultaneously. Unexpectedly, we did not see an appreciable change in the level of Akt associated with FLAG-AS160 in ClipR-59-expressed cells. These observations are not due to differential expression of FLAG-AS160, HA-Akt, and HA-ClipR-59, as all of these proteins are comparably expressed in the transiently transfected cells (Fig. 5A, middle and bottom panels).

In Fig. 2, we showed that the association of ClipR-59 with AS160 was attenuated following AS160 phosphorylation. Because ClipR-59 interacts with active Akt (15), it is possible that once ClipR-59 brings Akt to the complex, AS160 is promptly phosphorylated, leading to the dissociation of AS160 from the complex. As a result, the impact of ClipR-59, if there is one, on the association of AS160 with Akt would be difficult to detect. To circumvent this, we then proceeded with additional co-IP studies where FLAG-4P-AS160 and HA-E299K-Akt2 were used instead of wild type AS160 and Akt2. Because 4P-AS160 is a phosphorylation-defective form of AS160 and E299K-Akt2 is a kinase-inactive form of Akt2, we reasoned that the association of 4P-AS160 and ClipR-59 would not be impacted by the expression of E299K-Akt2. In addition, the cells were also treated with or without 100 nM insulin for 30 min after they were serum-starved overnight to accommodate the notion that ClipR-59 preferentially binds activated Akt (15). As shown in Fig. 5B, although HA-E229K-Akt2 was recovered from the FLAG-4P-AS160 immunoprecipitates, no appreciable effect of ClipR-59 on the association of AS160 with E299K-Akt2 was observed under basal conditions (top panel, compare lanes 1 and 2). In sharp contrast, more than a 2-fold increase in the amount of E299K-Akt2 was recovered from the FLAG-4P-AS160 immunoprecipitates of ClipR-59 expressed cells following insulin stimulation compared with that without ClipR-59 expression (Fig. 5B, compare lanes 3 and 4), arguing that ClipR-59 enhances the association of AS160 with active Akt. We also assessed the expression of HA-ClipR-59, HA-E229K-Akt2, and FLAG-4P-AS160, and Akt phosphorylation as well. We observed that all of these proteins were expressed accord-

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**FIGURE 5.** **ClipR-59 enhances the interaction between AS160 and Akt.** A, co-immunoprecipitation assay shows that ClipR-59 interacts with both Akt and AS160. The total cell lysates from HEK293 cells transiently expressing FLAG-AS160 and HA-Akt2, FLAG-AS160 and HA-ClipR-59, and FLAG-AS160, HA-Akt2, and HA-ClipR-59 were immunoprecipitated (IP) with anti-FLAG antibody. The cell lysates with HA-Akt2 and HA-ClipR-59 but not FLAG-AS160 expression serve as a control. The FLAG immunoprecipitates were probed with the anti-HA antibody (top). Middle, input levels of HA-Akt and HA-ClipR-59. Bottom, FLAG-AS160 in anti-FLAG immunoprecipitates. B, similar to A but FLAG-4P-AS160 and HA-E299K-Akt2 were used, and the cells were stimulated with (+) or without (−) insulin. Top, HA-E299K-Akt2 and HA-ClipR-59 in anti-FLAG-AS160 immunoprecipitates. Middle, input level of HA-E299K-Akt2 and HA-ClipR-59. Bottom, FLAG-AS160 in anti-FLAG-AS160 immunoprecipitates. Bottom, input of phospho-Akt. C, co-immunoprecipitation assay of the total cell lysates from fully differentiated 3T3-L1 adipocytes that were transduced with GFP or FLAG-ClipR-59 adenoviral expression vector. Adenoviral transduced 3T3-L1 adipocytes were serum-deprived overnight and treated with (+) or without (−) 100 nM insulin for 30 min. Then total cell lysates were prepared for co-immunoprecipitation assay with the anti-phospho-Akt S473 antibody. The anti-phospho-Akt immunoprecipitates were probed in Western blot with the anti-AS160 (panel l) and anti-Akt antibodies (panel iii). The cellular levels of AS160, Akt, and FLAG-ClipR-59 are shown in panels ii–v, respectively. IB, immunoblot.
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ingly in each sample (Fig. 5B, middle panels), and Akt was effectively activated as insulin treatment led to Akt phosphorylation (bottom panel, compare lanes 1 and 2 with 3 and 4). Moreover, in agreement with the notion that ClipR-59 has no impact on the total cellular Akt activation, comparable levels of Akt phosphorylation were detected regardless of the ClipR-59 expression (Fig. 5B, bottom, compare lanes 3 and 4).

To further examine the impact of ClipR-59 on the interaction of Akt and AS160, we next carried out a co-IP assay with the total cell lysates from differentiated 3T3-L1 adipocytes that were transduced with an adenoviral expression vector expressing GFP (control) or FLAG-ClipR-59 and treated with or without 100 nM insulin for 30 min, using an anti-phospho-Akt (Ser-473) antibody that recognizes both phosphorylated Akt1 and Akt2. Anti-phospho-Akt antibody was used because it specifically recognizes activated Akt, the form of Akt that interacts with ClipR-59 (15). The anti-phospho-Akt immunoprecipitates were probed in Western blot with an AS160 antibody. As shown in Fig. 5C, the amount of AS160 associated with the phospho-Akt immunoprecipitates of the cell lysates under basal condition and in the absence of exogenously expressed ClipR-59 cells was almost undetectable (panel i, compare lanes 1–3). In marked contrast, AS160 was readily detected in the phospho-Akt immunoprecipitates of ClipR-59–expressed cell lysates with insulin treatment compared with that of the control cells (Fig. 5C, compare lanes 3 and 4). In addition, we have also examined the expression levels of AS160 (Fig. 5C, panel ii) and FLAG-ClipR-59 (panel v) in total cell lysates and that of Akt in both pAkt immunoprecipitates (panel iii) and total cell lysates (panel iv). We observed the comparable levels of Akt and AS160 in each sample and the expression of FLAG-ClipR-59 in transduced adipocytes. Finally, Akt was only present in the phospho-Akt immunoprecipitates from insulin-treated cell lysates (Fig. 5C, panel iii, compare lanes 1 and 2 with 3 and 4), demonstrating the specificity of anti-phospho-Akt to immunoprecipitate phosphorylated Akt. Collectively, these results reveal that ClipR-59, Akt, and AS160 are present in the same complex and that ClipR-59 enhances the interaction between Akt and AS160.

Association of ClipR-59 with AS160 Is Required for ClipR-59 to Promote Glut4 Membrane Translocation—Both AS160 and ClipR-59 participate in regulating Glut4 membrane localization (15, 21). We wondered whether the interaction between ClipR-59 and AS160 could impact Glut4 cellular localization. To this end, 3T3-L1 adipocytes were initially transduced with an adenoviral vector that expressed GFP–ClipR-59 fusion peptide, which indicates the cells expressing ClipR-59. Forty eight hours post-transduction, the cells were stained with an anti-Glut4 mouse monoclonal antibody (1F8) or an anti-caveolin antibody. As shown in Fig. 6A, GFP–ClipR-59 expressed plasma membrane localization in agreement with the view that ClipR-59 is a membrane-associated protein. Without GFP–ClipR-59 expression, Glut4 was largely localized inside the cells without apparent membrane staining (Fig. 6A, panel ii). With GFP–ClipR-59 expression, Glut4 markedly showed membrane staining. These results are consistent with our previous studies in which forcing expression of ClipR-59 in adipocytes promotes Glut4 membrane translocation (Fig. 6A, panel ii, bottom). As a control, 3T3-L1 adipocytes were also transduced with an adenoviral vector that expresses GFP alone. No appreciable membrane staining was observed in the control cells (Fig. 6A, panel i). The effect of ClipR-59 on Glut4 localization was specific as ClipR-59 has no appreciable impact on the cellular localization of caveolin, a membrane-associated protein in adipocytes (Fig. 6A, panels iii and iv). Thus, these data demonstrate that ClipR-59 expression alone promotes Glut4 membrane translocation as described previously (15).

Next, we co-transduced differentiated 3T3-L1 adipocytes with an adenoviral expression vector that expresses a HA-tagged Glut4 and the ones that express GFP (control), ClipR-59, and ΔANK–ClipR-59, respectively. To ensure that HA-Glut4 was sufficiently expressed in co-transduced cells, the ratio of adenoviral vector expressing HA–Glut4 to that of ClipR-59 was 5:1. After these cells were treated with or without 100 nM insulin for 30 min, they were stained with anti-HA antibody without permeabilization. In HA-Glut4, the HA epitope is inserted into the first exofacial loop of Glut4 (22). When the cells expressing HA–Glut4 are stained with anti-HA antibody under nonpermeabilized conditions, membrane-localized HA–Glut4 is specifically detected, thereby measuring the level of Glut4 on the membrane. It is noted that because this staining only detects membrane-associated Glut4, the resulting fluorescence exhibits a ring. As presented in Fig. 6B, without insulin treatment, little HA staining was detected, which increased following stimulation (panel i-a, compare left and right). In the presence of ClipR-59, a significant membrane staining was observed (Fig. 6B, panel ii-a, right), indicative of Glut4 membrane translocation (panel ii, left). Insulin stimulation further increased the level of HA staining, which is about two times more than control cells (Fig. 6B, compare panel i-a, left, and panel ii-a, left). In the ΔANK–ClipR-59–expressed adipocytes, a somehow decreased level of Glut4 membrane staining was observed compared (Fig. 6B, panel iii-a, right) with that in the control cells (panel iii), suggesting that the expression of AS160 interaction-defective ClipR-59 may have a negative impact on Glut4 membrane translocation. It is noted that all of adenoviral vectors express GFP, which marked the transduced cells and the level of adenoviral vectors in each cell. As shown in Fig. 6B, all of the cells expressed comparable levels of GFP, an indication that these cells were equally transduced with adenoviruses (panels i-b, ii-b and iii-b).

To further demonstrate that the interaction of ClipR-59 with AS160 is required for ClipR-59 to promote Glut4 membrane localization, we next carried out subcellular fractionation assays in 3T3-L1 adipocytes expressing either wild type or ΔANK–ClipR-59. Specifically, 3T3-L1 adipocytes were transduced with GFP–, ClipR-59–, and ΔANK–ClipR-59–expressing adenoviruses, respectively. Twenty four hours post-transduction, the cells were serum-starved overnight and treated with 100 nM insulin for 30 min, and the PM and LDM fractions were prepared for Western blot assay. We first assessed Glut4 membrane localization. As presented in Fig. 6C, forcing expression of ClipR-59 increased the level of Glut4 in a membrane fraction, whereas that of ΔANK–ClipR-59 did not (panel i, compare lanes 2, 4 and 6), providing the additional evidence that the interaction of ClipR-59 with AS160 is required for ClipR-59
In agreement with the previous findings that insulin and the anti-phospho-Akt substrate antibodies as described AS160 immunoprecipitates were analyzed by Western blot from LDM with an anti-AS160-specific antibody, and the anti-AS160 expression adenovirus expressing HA-Glut4, and ClipR-59 promote AS160 phosphorylation (15), insulin-stimulated AS160 phosphorylation and ClipR-59 overexpression further enhanced the phosphorylation of AS160 associated with LDM (Fig. 6D, panel iv, compare lanes 1 and 2 with 3 and 4). However, ΔANK-ClipR-59 expression had no impact on the phosphorylation of AS160 associated with LDM (Fig. 6C, panel iv, compare lanes 1 and 2 with 5 and 6). We also examined the level of AS160 in LDM and total lysates. Insulin treatment decreased the level of AS160 associated with LDM (Fig. 6C, panel v, compare lane 1 with 2) as reported previously (11). Forcing expression of wild type ClipR-59 reduced the level of AS160 associated with LDM under basal conditions, which was further decreased under insulin stimulation condition (Fig. 6C, panel vi, compare lanes 1–4). Conversely, the expression of ΔANK-ClipR-59 did not lead to a decrease in AS160 associated with LDM under either of the two conditions (Fig. 6C, panel vi, compare lanes 1, 2, 5, and 6). Of note, the changes in the level of AS160 were not due to the differential expression of AS160 as a comparable level of AS160 was detected in total cell lysates independent of ClipR-59 expression (Fig. 6C, panel vii).

Interaction of ClipR-59 with AS160 Is Required for ClipR-59 to Promote Glucose Transport—Next, we carried out glucose transport assay in 3T3-L1 adipocytes under the expression of GFP, ClipR-59, and ΔANK-ClipR-59, respectively (Fig. 6D). In
agreement with the notion that insulin promotes glucose transport (23), insulin treatment of 3T3-L1 adipocytes led to a 5-fold increase in glucose uptake. Overexpression of ClipR-59 increased glucose uptake in basal and insulin-stimulated 3T3-L1 adipocytes as shown previously (15), whereas expression of ΔANK-ClipR-59 had no enhancement on insulin-elicited glucose transport. Instead, it appeared to reduce the ability of insulin to promote glucose transport. These data indicate that the interaction of ClipR-59 with AS160 is required for ClipR-59 to promote glucose transport.

DISCUSSION

ClipR-59 is gaining attention for its potential role in membrane signaling and subcellular trafficking (14, 15). Currently, the knowledge of how ClipR-59 regulates cellular pathways is still limited. In our preceding paper (15), we showed that ClipR-59 regulates Akt cellular compartmentalization and adipocyte glucose transport by interacting with Akt. We also showed that ClipR-59 up-regulated AS160 phosphorylation, a well-studied regulator of the specialized Glut4 trafficking pathway (11). To better understand the role of ClipR-59 in the regulation of signaling pathways responsible for adipocyte glucose transport, we have now further investigated the regulation of AS160 phosphorylation by ClipR-59.

In our sucrose gradient studies, we found that AS160, Glut4, and ClipR-59 are in the same fractions (Fig. 1). These experiments reveal two important features of ClipR-59. 1) ClipR-59 is likely associated with Glut4 vesicles as evidenced by the fact that ClipR-59 exhibits a similar localization as Glut4 in LDM and that Glut4 can be pulled down by GST-ClipR-59. 2) ClipR-59 is associated with AS160 (Figs. 1 and 2), which is known as a component of Glut4 vesicles (5). Although ClipR-59 could interact with either Glut4 or AS160 or both, we have specifically focused on the potential interaction between AS160 and ClipR-59 because of the regulatory role of AS160 in Glut4 membrane translocation. Consistently, we found that not only ClipR-59 interacts with AS160 but also that this interaction is regulated by insulin as insulin treatment abrogated the interaction, and the phosphorylation-defective AS160 showed a higher affinity to interact with ClipR-59 (Fig. 2).

To further substantiate the interaction of ClipR-59 with AS160, we have characterized the domains in ClipR-59 that are important for the interaction between AS160 and ClipR-59. These studies demonstrated that the ankyrin repeats of ClipR-59 mediated the interaction between AS160 and ClipR-59, as removal of ankyrin repeats completely abolished their interaction (Fig. 4B). In these experiments, we also noticed that Δ2CAP-ClipR-59, in which CAP-Gly domains are removed, showed higher affinity to interact with AS160. ClipR-59 interacts with Akt by which ClipR-59 promotes AS160 phosphorylation. Because AS160 phosphorylation negatively modulates the interaction between AS160 and ClipR-59, the increased interaction between AS160 and Δ2CAP-ClipR-59 reflects the notion that Δ2CAP-ClipR-59 does not promote AS160 phosphorylation, thereby exhibiting a higher affinity to bind AS160. This not only provides further evidence that phosphorylation of AS160 negatively regulates the interaction between AS160 and ClipR-59, it also implies that the interaction between AS160 and ClipR-59 as detected by co-IP under-represents the interaction between AS160 and ClipR-59.

Because ClipR-59 interacts with active Akt (15) and because ClipR-59 interacts with AS160 and promotes AS160 phosphorylation, we have investigated the potentiality that ClipR-59, AS160, and Akt are in the same complex and that ClipR-59 enhances the association of AS160 with Akt. We first examined this with wild type AS160, Akt, and ClipR-59 transiently expressed in HEK293 cells. In these experiments, we did find that both ClipR-59 and Akt were associated with AS160 (Fig. 5A). However, on the contrary, we did not observe any impact of forcing ClipR-59 expression on the association of AS160 and Akt (Fig. 5A). This likely reflects the view that ClipR-59 interacts with active Akt, which results in the phosphorylation of AS160 and dissociation of AS160 from the complex. Indeed, when 4P-AS160, a phosphorylation-defective AS160 mutant, and kinase-inactive E299K-Akt2 were used in the co-IP experiments, the augmentation of the association between AS160 and Akt by ClipR-59 became apparent (Fig. 5B). In addition, we also used the phospho-Akt antibody for our co-IP experiments in fully differentiated 3T3-L1 adipocytes. From these experiments (Fig. 5C), we found that exogenous expression of ClipR-59 specifically enhanced the association of AS160 with phospho-Akt. Overall, these studies not only demonstrate that ClipR-59 functions as the bona fide scaffold protein for AS160 and Akt, it further emphasizes that ClipR-59 interacts with active Akt (15), and the phosphorylation of AS160 negatively regulates the interaction between AS160 and ClipR-59 (Fig. 2).

The phosphorylation of AS160, which suppresses AS160 GAP activity, triggers the movement of vesicles containing Glut4 and the fusion of these vesicles with the plasma membrane in adipocytes. To determine how the interaction of ClipR-59 with AS160 affects the ability of ClipR-59 to modulate Glut4 membrane translocation, we examined whether AS160 interaction-defective ClipR-59 ΔANK-ClipR-59 could modulate Glut4 membrane translocation. In both cellular immunostaining and subcellular fractionation assays, we showed that ΔANK-ClipR-59 lacked the capability to promote Glut4 membrane translocation (Fig. 6, B and C). Furthermore, when expressed in adipocytes, ΔANK-ClipR-59 also failed to promote Glut4 transport (Fig. 6D), demonstrating that the interaction of ClipR-59 with AS160 is required for ClipR-59 to promote Glut4 membrane translocation.

In adipocytes, LDM consists of many factors important for Glut4 membrane translocation (24) and is the major fraction in which AS160 resides, and the phosphorylation of AS160 by Akt results in the release of AS160 from the membrane into the cytosol (25). In agreement with this view, we observed that insulin treatment of adipocytes decreased the amount of AS160 associated with LDM. ClipR-59 expression further decreased the level of AS160 in LDM under basal and insulin-induced conditions. This is consistent with the results that ClipR-59 increased AS160 phosphorylation in both cases. Notably, although the expression of wild type ClipR-59 decreased the LDM-associated AS160, the expression of ΔANK-ClipR-59 had no such effect. The observed effects of ClipR-59 but not of ΔANK-ClipR-59 on the diminishment of AS160 associated with LDM are in the line with
the view that the interaction between AS160 and ClipR-59 enhances AS160 phosphorylation.

Recent studies on AS160 phosphorylation induced by insulin show that AS160 is differentially phosphorylated in different cellular compartments and that AS160 is highly phosphorylated in plasma membrane fractions (26). This indicates that recruitment of AS160 into the membrane is a critical step for AS160 phosphorylation. Thus, promotion of AS160 phosphorylation by the membrane-associated ClipR-59 is consistent with this view. It is noteworthy that even though AS160 is mainly characterized in adipocytes, it was also implicated in bacteria trafficking in eukaryotic cells, the critical step for bacteria survival in eukaryotic cells (27). Therefore, it would be interesting to determine whether ClipR-59 also regulates other vesicle trafficking in the future.

In summary, we have demonstrated in this study that ClipR-59 interacts with AS160 and that this interaction is critical for ClipR-59 to regulate adipocyte glucose transport. Together with our previous data that ClipR-59 interacts with Akt, we suggest that ClipR-59 functions as a scaffold protein to facilitate binding Akt to AS160 and subsequently phosphorylating AS160 by Akt (Fig. 7). The interaction of ClipR-59 with AS160 in the regulation of Glut4 membrane translocation is significant. Current studies have established that insulin-dependent Glut4 membrane translocation in adipocyte and muscle cells is essential for the regulation of blood glucose levels (28), and the impairment of this process is directly related to the development of type II diabetes (17, 29). In this regard, ClipR-59 may also play a role in the regulation of glucose homeostasis, which is currently under investigation with ClipR-59 adipose-specific transgenic mice.

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