The Rab GTPase-Activating Protein AS160 as a Common Regulator of Insulin- and Gαq-Mediated Intracellular GLUT4 Vesicle Distribution


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Abstract. Akt substrate of 160kDa (AS160) is a Rab GTPase activating protein (GAP) and was recently identified as a component of the insulin signaling pathway of glucose transporter type 4 (GLUT4) translocation. We and others previously reported that the activation of Gαq protein-coupled receptors (GαqPCRs) also stimulated GLUT4 translocation and glucose uptake in several cell lines. Here, we report that the activation of GαqPCRs also promoted phosphorylation of AS160 by the 5'-AMP activated protein kinase (AMPK). The suppression of AS160 phosphorylation by the siRNA mediated AMPKα1 subunit knockdown promoted GLUT4 vesicle retention in intracellular compartments. This suppression did not affect the ratio of non-induced cell surface GLUT4 to Gαq-induced it. Rat 3Y1 cells lacking AS160 did not show insulin-induced GLUT4 translocation. The cells stably expressing GLUT4 revealed GLUT4 vesicles that were mainly localized in the perinuclear region and less frequently on the cell surface. After expression of exogenous AS160, GLUT4 on the cell surface decreased and GLUT4 vesicles were redistributed throughout the cytoplasm. Although PMA-induced or sodium fluoride-induced GLUT4 translocation was significantly increased in these cells, insulin did not affect GLUT4 translocation. These results suggest that AS160 is a common regulator of insulin- and GαqPCR activation-mediated GLUT4 distribution in the cells.

Key words: AS160, Gαq protein-coupled receptor, AMPK, GLUT4

GLUCOSE metabolism is crucial throughout the body to facilitate glucose transport into the muscle and adipocytes by insulin [1]. Insulin binds to its own receptors on these cell surfaces, thereby activating their intrinsic tyrosine kinase, followed by the activation of phosphatidylinositol-3 kinase (PI3K) and Akt. Glucose transport is consequently stimulated, mainly due to translocation of glucose transporter type 4 (GLUT4) from an intracellular membrane compartment to the cell surface [2]. In addition, we and others reported that platelet-derived growth factor (PDGF) and epidermal growth factor, which can activate PI

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The abbreviations used are: AICAR, 5-aminoimidazol-4-carboxamide-1-b-D- ribofuranoside; AS160, Akt substrate of 160KDa; AMPK, 5'-AMP activated protein kinase; ACC, acetyl-CoA carboxylase; a1bAR, a1b adrenergic receptor; BK,R, B2 bradykinin receptor; CHO, Chinese hamster ovary; FBS, fetal bovine serum; GAP, GTPase-activating protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT4, glucose transporter type 4; GLUT4myc, myc tagged- glucose transporter type 4; KRHB, Krebs-Ringer-HEPES buffer containing 0.2% BSA; NaF, sodium fluoride; PBS, phosphate-buffer saline; siRNA, small interfering RNA.
3-K and Akt as well as insulin, also facilitate GLUT4 translocation and glucose transport in cultured cells [3-9] and skeletal muscles of transgenic mice expressing the PDGF receptor [10]. Moreover, it is also reported that atypical protein kinase C isoforms [11, 12] and TC10, a member of the Rho family of small GTPases [13], are implicated in insulin-induced GLUT4 translocation. Despite extensive studies, the signaling pathway involved in GLUT4 translocation, specifically in relation to the insulin signaling cascade and the vesicle trafficking machinery of GLUT4, is not fully elucidated.

Recently, Akt substrate of 160KDa (AS160) was identified as a key regulator of the signaling pathway involved in insulin-induced GLUT4 translocation in 3T3L1 adipocytes [14]. It was reported that AS160 showed GTPase-activating protein (GAP) activity toward Rabs 2A, 8A, 10, 11 or 14. Current evidence suggests that Akt phosphorylation of AS160 suppresses its GAP activity, and consequently increases the GTP-bound Rabs and triggers an increase in the rate of GLUT4 vesicle exocytosis [15-19]. Moreover, AS160 was shown to promote the retention of GLUT4 vesicles in intracellular components in 3T3L1 adipocytes [15, 20]. Furthermore, the GAP activity of AS160 was affected by its interaction with 14-3-3 [21] or Ca\(^{2+}\)/calmodulin [22]. AS160 was originally identified as a PI-3K/Akt mediator of insulin-induced GLUT4 translocation. However, recently several other factors such as muscle contraction/exercise, other growth factors such as platelet-derived growth factor (PDGF), insulin like growth factor-1 (IGF-1) and epidermal growth factor (EGF), or the Akt-independent signals such as chemically activating protein kinase Cs with PMA and AMPK with 5-aminoimidazol-4-carboxamide-1-b-D-ribofuranoside (AICAR) were also shown to increase AS160 phosphorylation in rodent skeletal muscle [23-26], human skeletal muscle [27] or cultured cells [28, 29].

In a previous study we reported that activation of \(G_{\alpha q}\) protein-coupled receptors (\(G_{\alpha q}\)PCRs), such as the \(\alpha_1b\) adrenergic receptor (\(\alpha_1bAR\)) or B2 bradykinin receptor (BK,R), also triggered stimulated glucose uptake in cultured cells [30, 31]. GLUT4 translocation and glucose uptake induced by \(G_{\alpha q}\)PCR activation is physiologically important in muscles and adipocytes [32-35]. The \(G_{\alpha q}\)PCR signaling pathways is independent on PI-3K and Akt activation, which is responsible for insulin-induced GLUT4 translocation. Furthermore, we and other groups reported that activation of \(G_{\alpha q}\)PCR induced AMPK activity [36-38]. Thus, we proposed that glucose transported by insulin or \(G_{\alpha q}\) activation has a distinct destination in cells; glucose transported into cells after \(G_{\alpha q}\) activation is immediately metabolized and consumed as an availability of cellular energy as well as ATP which the \(G_{\alpha q}\)-induced AMPK activation supplied through activation of ATP-generating pathways and the decrease of ATP expenditure by suppressing macromolecule synthesis [39]. In contrast, the glucose transported into cells by the insulin-induced Akt activation is mainly converted to glycogen, lipid or protein as cellular energy stores that will be consumed during starvation conditions [40].

In the current report, we show that activation of \(G_{\alpha q}\)PCR increased the phosphorylation level of AS160 via AMPK\(\alpha_1\), and AS160 regulated the \(G_{\alpha q}\)-mediated GLUT4 vesicle distribution. The suppression of AS160 expression in 3T3L1 adipocytes by siRNA elevated cell surface GLUT4, but did not affect the ratio of non-induced cell surface GLUT4 to insulin-induced it. In the present study we also report that AS160 regulates the intracellular distribution of GLUT4 vesicles in 3Y1 cells.

Materials and Methods

**Materials and Antibodies**

Rabbit polyclonal antibodies against AS160 (Rab-GAP) (#07-741), AMP-activated protein kinase-\(\alpha_1\) (#07-735) and \(a_2\) (#07-363), and insulin receptor substrate-1 (IRS-1) (#06-248) and 2 (IRS-2) (#06-506) were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibody against the insulin receptor \(\beta\) subunit (C-19) (SC-711) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit monoclonal antibodies against phospho-(Ser/Thr) Akt substrate (PAS) (110B7) (#9614), phospho-AMP-activated protein kinase-\(\alpha\) (Thr172) (40H9) (#2535) and rabbit polyclonal antibodies against phospho-Akt (Ser473) (#9271), phospho-acetyl-CoA carboxylase (Ser79) (#3661), acetyl-CoA carboxylase (#3622) and Akt (#9272) were purchased from Cell Signaling (Beverly, MA). Mouse monoclonal antibodies against \(\beta\)-tubulin (TUB 2.1) (T4026) were purchased from Sigma (St. Louis, MO). Mouse mono-
clonal antibody against myc was prepared from 9E10 cells. All other reagents used were of analytical grade.

**Cell Culture**

Chinese hamster ovary (CHO) cells were grown in Ham’s F-12 medium (Biological Industries Ltd, Israel) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). 3T3L1 fibroblasts, L6 myoblasts and 3Y1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Nissui, Tokyo) supplemented with 10% (v/v) FBS. L6 myoblasts were cultured to the myotube stage according to the method of Mitsumoto et al. [41] with slight modifications. In brief, when L6 myoblasts had reached confluence (day 0), medium was changed to DMEM supplemented with 2% (v/v) FBS and 10 ng/ml insulin. At day 2 and day 4 the medium was changed to differentiation medium lacking insulin. At day 4 or 5, L6 myotubes were used for experiments. 3T3L1 fibroblasts were cultured to the stage of adipocytes as previously described [30].

**Stable cell lines**

Chinese hamster ovary (CHO) cells stably expressing GLUT4myc (CHO-GLUT4myc cells) were constructed by inserting a human c-myc epitope (14 amino acids) into the first ectodomain of GLUT4 [42]. CHO-GLUT4myc cells stably expressing the human α1b-adrenergic receptors and the human bradykinin B2 receptors, were called CHO-GLUT4myc-α1bAR cells and CHO-GLUT4myc-BK2R cells respectively and were as previously described [30, 31]. L6-GLUT4myc cells were L6 cells stably expressing GLUT4myc and L6-GLUT4myc-BK2R cells were L6-GLUT4myc cells stably expressing the mouse bradykinin B2 receptors, as previously described [31]. 3T3L1-GLUT4myc cells were 3T3L1 cells stably expressing GLUT4myc as described previously [30] and 3Y1-GLUT4myc cells were 3Y1 cells stably expressing GLUT4myc as previously described [31]. 3Y1-GLUT4myc-AS160WT and 4P cells were established by cotransfecting the mammalian expression plasmids of human wild type or the 4P mutant of AS160 [14] into 3Y1-GLUT4myc cells with pSV2-bsr, a blasticidin S deaminase expression plasmid, and selected with blasticidin S hydrochloride (Funakoshi, Tokyo, Japan). Several independent clones expressing human wild type or the 4P mutant of AS160 were established.

**Immunoprecipitations and Western blot analysis**

Cell lysates were prepared with lysis buffer (1% (w/v) Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 140 mM NaCl, 10 mM EDTA, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 4 µg/ml apro- tinin, 4 µg/ml leupeptin and 4 µg/ml pepstatin A), and the precipitate was removed by centrifugation at 16,100 × g for 20 min at 4°C. Supernatant was collected and protein concentrations were determined using the Bradford method (PROTEIN ASSAY; Bio-Rad Laboratories, Richmond, CA) with BSA as a standard. Equal amounts of protein were subjected to immunoprecipitation with specific antibodies and Protein A-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden). The immunoprecipitates were washed three times with wash buffer (1% (w/v) Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 140 mM NaCl and 1 mM Dithiothreitol) and once with final wash buffer (0.02% (w/v) Nonidet P-40, 100 mM Tris-HCl, pH 7.4 and 100 mM NaCl). Extracts from the immunoprecipitates or equal amounts of cell lysates were resolved by SDS-polyacrylamide gel electrophoresis, transferred by electroblotting onto a nitrocellulose membrane and subsequently probed with specific antibodies. Proteins were visualized using enhanced chemiluminescence.

**Cell surface anti-c-myc antibody binding assay**

3T3L1-GLUT4myc adipocytes, L6-GLUT4myc myotubes or 3Y1-GLUT4myc cells cultured in 24-well plates were incubated in 1 ml Krebs-Ringer-HEPES buffer (pH 7.5, 136 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl₂, 1.25 mM MgSO₄ and 20 mM HEPES) containing 0.2% (w/v) BSA (fraction V, Sigma, St. Louis, MO), supplemented with 10 mM glucose for 60 min at 37°C, and then stimulated with indicated ligands for indicated period of time at 37°C. GLUT4myc translocation was measured as previously described [31, 43]. To evaluate the absolute amount of cell-surface GLUT4myc in 3Y1 cells, the nuclei of the cells were counter stained with DAPI (Sigma, St. Louis, MI) and the number of cells per well was calculated with the Infinite F500 platform (Tecan Group Ltd., Mannedorf, Switzerland), in addition the relative amount of cell-
surface GLUT4myc in each cell line were derived from the correction for the total GLUT4myc amount.

**Knockdown of AMPK or AS160 by siRNA**

siRNAs for the rat AMPKα1 subunit and control siRNA (Negative Control #1 siRNA) were synthesized and purchased from Ambion (Austin, TX). The siRNA sequences are as follows: AMPKα1 siRNA-1 5’-GCUUUCAGGGTTTCTCACGTTT-3’ and 5’-AUUUCAGGGGTTTCTCACATT-3’, AMPKα1 siRNA-2 5’-CTCGAGGTTCGTTTCTCACATT-3’ and 5’-UUCUCCAGGAUCUCCGAUU-3’. L6 myoblasts were seeded in 6 or 24 well plate and then differentiated into myotubes as described above. At day 3 (24 well plate) or 4 (6 well plate) the cells were transfected with the indicated siRNAs using Lipofectamine RNAiMAX (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer’s protocol. At 24 hours after transfection, L6 myotubes in 6 well plates were subjected to the assay to prepare cell lysates, and those in 24 well plates were subjected to the cell surface anti-c-myc antibody binding assay as described above. siRNAs for mouse AS160 and control siRNA (Negative Control #1 siRNA) were synthesized and purchased from Ambion. The siRNA sequences are as follows: mouse AS160 siRNA-1 5’-CCAAAGGGTTTTTCTCACATT-3’ and 5’-CAGAGGTTCGTTTCTCACATT-3’, AS160 siRNA-2 5’-AAUUCAGGGTTTCTCACATT-3’ and 5’-AAUUCAGGGTTTCTCACATT-3’. 3T3L1 adipocytes were seeded in 6 well collagen coated plates and differentiated into adipocytes as described above. At day 6, they were transfected with the indicated siRNAs using Lipofectamine RNAiMAX according to the manufacturer’s protocol. At 36 hours after transfection, L1 adipocytes in 24 well plates were subjected to the assay to prepare cell lysates or subjected to the cell surface anti-c-myc antibody binding assay as described above.

**RT-PCR**

Total RNA was isolated from 3Y1 cells and L6 cells using the TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Total RNA (1μg) was subjected to reverse transcription using Superscript II reverse transcriptase (Invitrogen). Primers used in RT-PCR analysis were as follows: ratAS160 forward primer 5’-GCTCTCTGAGAGAATCGA-3’ and reverse primer 5’-GGATTTTGGTTGTAAGTTTCT-3’ (Genbank Accession No. XM_001074155); ratGAPDH forward primer 5’-CCACAGTTCCCATCACATG-3’ and reverse primer 5’-CCACACCTGTTGCTGAGGC-3’ (Genbank Accession No. AB017801). Amplification conditions for rat AS160 were pre-activation at 94°C for 1 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, and a final 7 min incubation at 72°C. Conditions for rat GAPDH were pre-activation at 98°C for 30 sec, 25 cycles of denaturation at 98°C for 10 s, annealing at 62°C for 30 s and extension at 72°C for 30 s; and a final 10 min incubation at 72°C.

**Immunofluorescence confocal microscopy**

3Y1-GLUT4myc cells were seeded on coverslips and wild type or the 4P mutant of AS160 were transiently expressed using FuGENE HD Transfection Reagent as described by the manufacturer (Roche Diagnostics, Mannheim, Germany). After 36 hrs of transient expression cells were fixed with 3% (w/v) paraformaldehyde for 20 min, and consequently permeabilized with 0.1% (w/v) Triton X-100 for 20 min, and consequently permeabilized with 0.1% (w/v) Triton X-100 for 4 min at room temperature. GLUT4 or Flag-tagged AS160 was detected by incubating cells with the anti-GLUT4 antibody [4] or anti-FLAG M2 antibody (Sigma) followed by Alexa 488-conjugated goat anti-rabbit IgG or Cy3-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR), respectively. Nuclei of cells were stained with DAPI (Sigma) and fluorescent images were obtained with a Zeiss LSM5 laser-scanning confocal microscope.

**Results**

AS160 in cultured cells was significantly phosphorylated in medium containing serum, and this was accompanied by phosphorylation of Akt and AMPK, both of which have been reported to be an upstream kinase of AS160 (Fig. 1). Since various growth factors, including insulin in serum, can activate Akt, which in turn can phosphorylate AS160, we exchanged normal medium supplemented with 10% FBS for Krebs-Ringer-HEPES buffer containing 0.2% BSA (KRHB buffer) to lower basal phosphorylation levels.
thought to be insulin target cells. We and other groups previously reported that stimulation of $G_\alpha q$PCRs activated AMPK using an in vitro kinase assay with the anti-AMPK antibody and a synthetic peptide substrate for AMPK [36-38]. In this report, we investigated activation of AMPK by immunoblot using anti-phospho-AMPK antibodies. As expected, norepinephrine and bradykinin increased the phosphorylation level of AMPK via the $\alpha_1b$AR and BK2R in CHO cells and L6 myotubes, respectively (Fig. 3a). Although the phosphorylation level of AMPK induced by bradykinin was low in L6 myotubes, we confirmed that acetyl-CoA carboxylase (ACC), which is a representative substrate of AMPK, is also phosphorylated by the stimulation of bradykinin (see Fig. 3c). In contrast, Akt, which has been originally reported as an AS160 kinase [45], was not phosphorylated by the stimulation of $G_\alpha q$PCRs (Fig. 3a). In addition, insulin reduced AMPK phosphorylation in L6 myotubes (Fig. 3a). This was consistent with the report of Yin and Birnbaum [46] that insulin decreased the basal phosphorylation level of AMPK through the reduction of intracellular cAMP levels in L1 adipocytes. Furthermore, we investigated whether

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![Fig. 1. Phosphorylation level of AS160, AMPK and Akt during incubation with serum free medium in CHO cells.](image7)

CHO-G4m (GLUT4myc-$\alpha_1b$AR) cells were cultured in Ham’s F-12 medium supplemented with 10% FBS. Cell lysates were prepared at 0, 30 or 60 minutes after changing culture medium to Krebs-Ringer-HEPES buffer containing 0.2% BSA supplemented with 10 mM glucose. Equal amounts of cell lysates were subjected to immunoprecipitation with anti-AS160 antibody and then to Western blot analysis with anti-PAS antibody. Equal amounts of cell lysates were also subjected to Western blot with indicated antibodies.
Fig. 2. AS160 phosphorylation after stimulation of GαqPCRs

A, B: CHO-G4m (GLUT4myc)-α1bAR cells (A) or CHO-G4m (GLUT4myc)-BK2R (B) cells and their parental cells were preincubated with Krebs-Ringer-HEPES buffer containing 0.2% BSA supplemented with 10 mM glucose for 60 minutes, and then stimulated with control solution, 10^{-7} M insulin or 10^{-7} M norepinephrine (A) or 10^{-7} M bradykinin (B) for indicated periods of time. After stimulation, equal amounts of cell lysates were subjected to immunoprecipitation with anti AS160 antibody and then to Western blot analysis with anti PAS antibody. Representative experiments and quantitative analysis (National Institutes of Health Image Program) are shown. Values represent the means ± S.E.M. for n=3-6 per group. Data were analyzed with one-way ANOVA, and the multi comparison test was used with a significance level of 0.05, 0.01 or 0.001. *0.05; **0.01; ***0.001 versus control solution.

C: L6-G4m (GLUT4myc)-BK2R myotubes were preincubated with Krebs-Ringer-HEPES buffer containing 0.2% BSA supplemented with 10 mM glucose for 60 minutes, and then stimulated with control solution, 10^{-7} M insulin or 10^{-7} M bradykinin for 10 minutes. After stimulation, equal amounts of cell lysates were subjected to immunoprecipitation with anti AS160 antibody and then to Western blot analysis with anti PAS antibody.
Fig. 3. Role of AMPK on AS160 phosphorylation after stimulation of GqPCRAs

A. CHO-G4m (GLUT4myc)-a1bAR cells and L6-G4m (GLUT4myc)-BK,R myotubes were preincubated with Krebs-Ringer-HEPES buffer containing 0.2% BSA supplemented with 10 mM glucose for 60 minutes, and then stimulated with control solution, 10^{-6} M norepinephrine (CHO), 10^{-7} M bradykinin (L6), 10^{-7} M insulin or 2 mM AICAR for 10 minutes. After stimulation, equal amounts of cell lysates were subjected to Western blot analysis with indicated antibodies.

B. L6-G4m (GLUT4myc)-BK,R cells were seeded in 24 well plate, and differentiated into myotubes. At day 4, they were transfected with the indicated amounts of either siRNA-1 or -2 for rat AMPKα1 subunit (1 nM; 0.6 pmol/well, 10 nM; 6 pmol/well, 30 nM; 18 pmol/well). At 24 hours after transfection, L6 myotubes were lysed and cell lysates were subjected to western blot analysis with the anti AMPKα1 antibody or anti Akt antibody.

C, D. L6-G4m-BK,R cells were seeded in 6 well plates and differentiated into myotubes. At day 4, they were transfected with either 50nM (150pmol/well) control siRNA, siRNA-1 or -2 for rat AMPKα1 subunit. At 24 hours after transfection, L6 myotubes were preincubated with Krebs-Ringer-HEPES buffer containing 0.2% BSA supplemented with 10 mM glucose for 60 minutes, and then stimulated with control solution or 10^{-7} M bradykinin for 10 minutes. After stimulation, L6 myotubes were lysed and cell lysates were subjected to Western blot analysis with the indicated antibodies (C) or to immunoprecipitation with anti-AS160 antibody and then to Western blot analysis with anti-PAS antibody and anti-AS160 antibody (D). Representative experiments and quantitative analysis (National Institutes of Health Image Program) are shown. Values represent the means ± S.E.M. for n=3-7 per group. Data were analyzed with one-way ANOVA and the multi comparison test was used with a significance level of 0.05 or 0.01, *0.05; **0.01 versus control siRNA.
phosphorylation of AS160 by the induction of Gαq mediated signals depends on the activation of AMPK. Since AMPK α2 is not expressed in L6 myotubes (see Discussion), we transfected siRNA for the α1 catalytic subunit of rat AMPK (AMPK α1) into L6 myotubes with liposome, as described in the Materials and Methods section. As shown in Fig. 3B, two siRNA sequences targeting AMPKα1 decreased the total amount of AMPK in a dose dependent manner, in contrast, the amount of Akt did not change. This result in a decrease in phosphorylated AMPK through activation of GαqPCRs. Consequently the phosphorylation of acetyl-CoA carboxylase (ACC), which is a representative substrate of AMPK, was also significantly suppressed (Fig. 3C), indicating that the decrease in AMPK α1 is enough to suppress the kinase activity of AMPK in L6 myotubes. Therefore, since knockdown of AMPKα1 resulted in 40% reduction in AMPK protein (Fig. 3C), the phosphorylation level of AS160 also decreased approximately 40% compared to control siRNA (Fig. 3D). These results suggest that the stimulation of Gαq phosphorylates, at least in part, AS160 through AMPKα1.

Furthermore, we examined whether the suppression of the AS160 signal affected GLUT4 vesicle trafficking. First, we investigated the influence of AS160 knockdown on insulin-induced GLUT4 vesicle trafficking in 3T3L1 adipocytes by cell-surface anti-c-myc antibody binding assay. The suppression of AS160 expression using siRNA targeting mouse AS160 (as160 4P), in which four of the six predicted Akt phosphorylation sites (ser318, ser588, Thr642 and ser751) were changed to alanine [14], to investigate whether insulin does not promote GLUT4 translocation in rat 3Y1 cells, suggesting that 3Y1 cells might lack the molecule(s) that play a critical role in insulin-induced GLUT4 translocation, because GLUT4 translocation is activated by PMA and sodium fluoride (NaF) in 3Y1 cells [43]. We previously reported that the insulin receptor and insulin receptor substrate-1 were lacking in 3Y1 cells because they could not be detected by Western blot analysis in previous experiments [43]. However, this report used different antibodies against the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) (see Materials and Methods section) and small amounts of each protein were detected (Fig. 5A). Akt phosphorylation by insulin was detected with the anti phospho-Akt antibody in 3Y1 cells that did not express exogenous IR and IRS-1 (Fig. 5C). As shown in Fig. 5A, we found that AS160mRNA and protein could not be detected in rat 3Y1 cells, although both 3Y1 fibroblasts and L6 myotubes are derived from rat tissue. Therefore, we constructed 3Y1 cells stably expressing wild type AS160 (AS160WT) or the 4P mutant of AS160 (AS160 4P), in which four of the six predicted Akt phosphorylation sites (Ser318, Ser588, Thr642 and Ser751) were changed to alanine [14], to investigate the role of AS160 on GLUT4 vesicle trafficking.

First, we evaluated the absolute amount of GLUT4myc on the cell-surface in 3Y1 cells stably expressing AS160WT or AS160 4P. We established and investigated the two 3Y1-G4m cell lines stably expressing wild type AS160 (3Y1-G4m-AS160WT1 and 3Y1-G4m-AS160WT2). As shown in Fig. 5B, although these two clones had the different expression levels of wild type AS160, the basal amount of cell-surface GLUT4myc corrected by the total number of cells and the total amount of GLUT4myc decreased in parallel with the level of exogenous AS160 protein. These results suggest that AS160 has a role in the promotion of GLUT4 vesicle distribution in the intracellular membrane compartment (retention).

Secondary, we investigated whether insulin phosphorylated exogenous AS160 in 3Y1 cells. As shown
Fig. 4. Effect of knockdown of AS160 or the AMPKα1 subunit on basal cell surface GLUT4 and GLUT4 translocation by insulin or GαqPCRs stimulation

A. 3T3L1-G4m (GLUT4myc) fibroblasts were seeded in 24 well collagen coated plates and differentiated into adipocytes. At day 6, cells were transfected with either 10 nM (6 pmol/well) control siRNA, siRNA-1 or -2 for mouse AS160. At 36 hours after transfection, cells were preincubated with Krebs-Ringer-HEPES buffer containing 0.2% BSA supplemented with 10 mM glucose for 60 minutes, and stimulated with control solution or 10^{-7} M insulin for 30 minutes at 37°C. 3T3L1-G4m adipocytes were lysed and cell lysate were subjected to Western Blot analysis with anti-AS160, anti-β-tubulin or anti-myc antibodies and GLUT4myc translocation was measured. Values represent the means ± S.E.M. for n=6 per group. Data were analyzed with one-way ANOVA and the multi comparison test was used with a significance level of 0.05.

B. L6-G4m (GLUT4myc)-BK,R cells were seeded in 24 well plates, and differentiated into myotubes. At day 3, cells were transfected with either 50 nM (30 pmol/well) control siRNA, siRNA-1 or -2 for the rat AMPKα1 subunit. At 24 hours after transfection, cells were preincubated with Krebs-Ringer-HEPES buffer containing 0.2% BSA supplemented with 10 mM glucose for 60 minutes, stimulated with control solution or 10^{-7} M bradykinin for 5 minutes at 37°C. L6-G4m-BK,R myotubes were lysed and cell lysate were subjected to Western Blot analysis with anti-myc antibodies and GLUT4myc translocation was measured. Values represent the means ± S.E.M. for n=9-15 per group. Data were analyzed with one-way ANOVA, and the multi comparison test was used with a significance level of 0.01 or 0.001.

C. L6-G4m-BK,R cells were seeded in 24 well plates and differentiated into myotubes. At day 3, they were transfected with 50 nM (30 pmol/well) control siRNA or siRNA-1 for rat AMPKα1. At 24 hours after transfection cells were stimulated with 10^{-7} M bradykinin for 0, 10, 30 or 60 minutes at 37°C and GLUT4myc translocation was measured. Values represent the means ± S.E.M. for n=6 per group.
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fit the minimal PKC consensus phosphorylation motif [28]. On the other hand, NaF has been reported as an inhibitor of protein phosphatase 2a (PP2a) [47] that was shown to inhibit akt [48], and protein phosphatase 2c (PP2c) that was shown to inactivate AMPK [49]. Therefore, it is likely that NaF inhibited the dephosphorylation of akt via PP2a, and AMPK in Fig 5c, exogenous AS160 was clearly phosphorylated by insulin and this phosphorylation was accompanied by activation of Akt. PMA and NaF also phosphorylated AS160 in 3Y1 cells. It has been reported that conventional and novel protein kinase Cs activated by PMA can phosphorylate AS160 in CHO cells because most Akt phosphorylation sites on AS160

Fig. 5. Effect of wild type or the 4P mutant of AS160 expressed in 3Y1 cells on GLUT4 vesicle distribution.
A. Equal amounts of CHO, 3Y1, or rat skeletal muscle cell lysate were prepared and subjected to Western blot analysis with the indicated antibodies. Total RNA of 3Y1 cells and L6 myotubes was subjected to RT-PCR analysis using rat AS160 or rat GAPDH probes as a control. After PCR, an aliquot of the reaction mixture was resolved on a 1% agarose gel.
B. 3Y1-G4m (GLUT4-myc)-cells stably expressing wild type (AS160WT) or the 4P mutant (AS160 4P) of AS160 and their parental cells were seeded in 24 well plates and subjected to cell surface anti-c-myc antibody binding assay. The basals amounts of cell-surface GLUT4-myc corrected by the total number of cells (described in Materials and Methods) and the total amount of GLUT4-myc (referred to [43]) on wild type and the 4P mutant of AS160 are represented as the ratio to the amount on the parental cells. Values represent the means ± S.E.M. for n = 3 per group. Data were analyzed with one-way ANOVA and the multi comparison test was used with a significance level of 0.001. **0.001. The amounts of exogenous AS160s, β-tubulin or GLUT4-myc were indicated in the lower panels.
C. Equal amounts of lysates from 3Y1-G4m cells stably expressing AS160WT or AS160 4P were prepared and subjected to immunoprecipitation with the anti-AS160 antibody and then to Western blot analysis with anti-AS160 antibody (upper panel). Equal amounts of cell lysates of 3Y1-G4m cells were subjected to Western blot with the indicated antibodies (lower panel).
D. 3Y1-G4m cells stably expressing AS160WT or AS160 4P and their parental cells were seeded in 24 well plates and preincubated with Krebs-Ringer-HEPES buffer containing 0.2% BSA supplemented with 10 mM glucose for 60 minutes, followed by stimulation with control solution, 10^{-7} M Insulin, 10^{-6} M PMA or 10^{-2} M NaF for 30 minutes at 37°C and GLUT4-myc translocation was measured. Relative values to each basal represent the means ± S.E.M. for n = 10-19 per group. Data were analyzed with one-way ANOVA and the multi comparison test was used with a significance level of 0.05 or 0.001. *0.05; ***0.001; N.S. not significant.
E. 3Y1-G4m cells were transiently expressed with wild type AS160 (AS160WT) or the 4P mutant of AS160 (AS160 4P). After transient expression for 36 hrs, cells were fixed with PFA and permeabilized with Triton X-100. GLUT4 (green), chromatin (blue) or flag-tagged AS160 (red) were stained with anti-GLUT4 antibodies, DAPI or anti-FLAG antibodies, respectively.
ROLE OF AS160 IN GLUT4 DISTRIBUTION

3Y1-G4m-AS160WT1 3Y1-G4m-AS160 4P
IP: anti-AS160 IB: anti-PAS
IP: anti-AS160 IB: anti-AS160
IB: anti-AMPK IB: anti-phospho-Akt
IB: anti-β-tubulin

3Y1-G4m
Ins. PMA NaF
IB: anti-AMPK
IB: anti-phospho-Akt
IB: anti-β-tubulin

5C

via PP2C. This would be consistent with the finding that phosphorylation of AS160 by NaF was concomitant with phosphorylation of Akt and AMPK (Fig. 5C). In addition, phosphorylation of the 4P mutants could not be detected by the phospho- (Ser/Thr) Akt substrate (PAS) antibody, suggesting that the PAS antibody recognizes the four main Akt phosphorylation sites (Ser318, Ser588, Thr642 and Ser751) of AS160.

Thirdly, we investigated whether the expression of AS160 could induce GLUT4 translocation by insulin in 3Y1 cells. Even though AS160 was fully phosphorylated by insulin in 3Y1 cells stably expressing AS160WT (Fig. 5C), insulin did not promote GLUT4 translocation (Fig. 5D). However, GLUT4 translocation was significantly increased by PMA or NaF in 3Y1 cells stably expressing AS160WT (Fig. 5D).

Finally, we investigated the effect of AS160 expression on GLUT4 vesicle localization by GLUT4 immunostaining using anti GLUT4 antibody in 3Y1-G4m cells transiently expressing wild type and the 4P mutant of AS160. As shown in Fig. 5E, 3Y1 cells expressing AS160WT or AS160 4P (red in lower panel) exhibited a distinguishable cytoplasmic GLUT4 redistribution (green in upper panel), whereas parental cells not expressing wild type and the 4P mutant of AS160 distributed GLUT4 mainly at a perinuclear region.

Discussion

Our findings in the current study indicate that the AMPK/AS160 signaling pathway regulates basal distribution of GLUT4 on the cell surface (Fig. 4B), however it is not likely that this signal affects a Goq-induced increase of GLUT4 translocation (Fig. 4C). Therefore, these results indicate the presence of other signaling pathways, other than AMPK and AS160, to regulate the Goq-induced GLUT4 translocation. However, there is still a possibility that a residual AMPK phosphorylation of AS160 is sufficient
enough for a Gaq-induced increase of GLUT4 translocation, because it was recently reported that a minimal Akt phosphorylation is sufficient for insulin-induced GLUT4 translocation in L6 myotubes [50]. Furthermore, we found that the siRNA knockdown of AS160 significantly increased cell-surface GLUT4myc at the basal state, but it did not affect the amount of insulin-mediated GLUT4myc on the cell surface of 3T3L1 adipocytes (Fig. 4A). This indicates that it is highly likely that AS160 is implicated in the GLUT4 vesicle trafficking, but is not involved in regulating the absolute amount of insulin-mediated GLUT4 on the cell surface. These results are identical to the findings for AMPK/AS160 signaling pathway about the Gaq-induced GLUT4 translocation as described above. However, it has been shown that the knockdown of AS160 decreases the absolute amount of insulin-mediated GLUT4 on the cell surface in the previous report [20]. The precise cause for this discrepancy is unknown, however, in this report we used siRNA and not shRNA to knockdown AS160 and this method might not be efficient enough to suppress insulin-induced GLUT4myc translocation. Regarding the knockdown of AS160 in 3T3L1 adipocytes, the cell-surface GLUT4myc increased to the maximum at 36 hours after siRNA transfection. Moreover, the transfection efficacy of siRNA was not high enough to suppress the expression of AS160 at a late stage of adipogenesis, therefore we examined the effect of AS160 knockdown on GLUT4 vesicle trafficking at an earlier stage (see Materials and Methods section). In addition, the treatment of a transfection reagent for siRNA seemed to decrease the fold increments due to unidentified reasons (data not shown). These experimental conditions likely resulted in a small increase in GLUT4myc translocation stimulated by insulin even with control siRNA in 3T3L1 adipocytes (Fig. 4A).

The basal distribution of GLUT4myc on the cell surface was apparently decreased by suppression of AMPKα1 activity (Fig. 4B), indicating that AMPK regulates basal GLUT4 vesicle distribution. We found that low glucose in the media promoted AS160 phosphorylation, followed by the increase of GLUT4myc on the cell surface of CHO cells (data not shown). Therefore, the AMPK/AS160 signaling pathway seems to have the following physiological role: AMPK senses intracellular glucose levels as an energy gauge, and when intracellular glucose levels fall, AMPK is activated and phosphorylates AS160, followed by an increase in cell-surface GLUT4 to supply energy by taking glucose into cells.

It has been reported that AMPKα2 has a more important role than AMPKα1 in the phosphorylation of AS160 after exercise or by AICAR in skeletal muscles [24, 26]. However, we did not detect AMPKα2 protein expression by Western blot analysis in L6 myotubes (data not shown). Although CHO cells lack AMPKα2 [28], we found that the activated Gaq signals apparently phosphorylated AS160 via AMPK in CHO cells (Fig. 2A, 2B, 3A). Therefore, we investigated the role of AMPKα1 and showed that the knockdown of AMPKα1 in L6 myotubes resulted in a decrease in phosphorylation of AS160 by the Gaq signal and this promoted the retention of GLUT4 vesicles in the basal state (Fig. 3D, 4B).

In 3Y1 cells stably expressing AS160, the absolute amount of cell-surface GLUT4myc in the basal state decreased in parallel with the amount of exogenous AS160 in cells expressing wild type and the 4P mutant of AS160 (Fig. 5B), indicating that AS160 has a function in GLUT4 vesicle retention at the intracellular membrane compartment [15, 20]. However, as shown in Fig. 5B, 3Y1 cells stably expressing AS160 indicated the decrement of GLUT4myc amount, indicating that the variation of its intracellular membrane compartment by AS160 might shorten its protein life-span although a transient suppression of AS160 expression seems not to affect it in 3T3L1 adipocytes as shown in Fig. 4A. Interestingly, as shown in Fig. 5D, wild type AS160 in 3Y1 cells promoted GLUT4myc translocation by PMA or NaF. In contrast, the 4P mutant of AS160 did not promote GLUT4myc translocation to the same degree as wild type AS160, indicating that GLUT4 translocation by PMA or NaF is regulated by AS160. Moreover, in 3Y1 cells stably expressing the 4P mutant of AS160, the degree of GLUT4myc translocation by PMA was identical, but GLUT4myc translocation by NaF was significantly higher compared to parental cells. This suggests that there are other predicted phosphorylation sites, other than the four Ser/Thr sites of the 4P mutant of AS160, likely to be implicated in the GLUT4 translocation by NaF [14]. On the other hand, although insulin phosphorylated exogenous AS160 at the same rate as PMA or NaF (Fig. 5C), insulin never seemed to promote GLUT4myc translocation in 3Y1 cells (Fig. 5D). These data seem to indicate that there are other signaling pathways necessary to promote GLUT4 translocation by insu-
lin in 3Y1 cells, whereas PMA and NAF can activate GLUT4 translocation.

We also found that the expression of exogenous AS160 distributes GLUT4 throughout the cytoplasm of 3Y1 cells (Fig. 5E). Although the precise localization of GLUT4 has not been elucidated, this cytoplasmic redistribution might lead to a decrease in cell-surface GLUT4 and could indicate the function of basal GLUT4 retention at the intracellular membrane compartment. As expected, this redistribution of GLUT4 was identical in cells expressing wild type or 4P mutant of AS160. In addition, the AS160 was shown not to be co-localized with GLUT4 in 3Y1 cells (data not shown). The precise meaning of this GLUT4 redistribution by AS160 remains to be identified.

We here provide evidence that AS160 is implicated in GLUT4 vesicle distribution regulated by Gαq signaling pathway that is mediated at least in part by AMPKα1 and the insulin/Akt signaling pathway. Our data also suggest that phosphorylation of AS160 is essential for the release of GLUT4 vesicle from the intracellular membrane compartment. Therefore, phosphorylation of AS160 might not be sufficient for triggering the GLUT4 translocation, but necessary for the promotion of GLUT4 translocation by various stimuli and other signaling pathways are likely required to promote GLUT4 translocation by either insulin or the Gαq signaling pathways.

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