Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Biochemical and Biophysical Research Communications 335 (2005) 175–180
BBRC
www.elsevier.com/locate/ybbrc

Calmodulin binds to the Rab GTPase activating protein required for insulin-stimulated GLUT4 translocation

Susan Kane, Gustav E. Lienhard *

Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755, USA

Received 24 June 2005
Available online 25 July 2005

Abstract

Recently, we described a 160 kDa protein with a Rab GTPase activating protein domain that is phosphorylated on multiple sites by the protein kinase Akt (designated AS160). Phosphorylation of AS160 in adipocytes is required for insulin-stimulated translocation of the glucose transporter GLUT4 to the plasma membrane. In the present study, we searched for proteins that interact with the GTPase activating protein (GAP) domain region of AS160 by the yeast two-hybrid screen. This search indicated that calmodulin bound to a small domain just amino terminal to the GAP domain of AS160, and this association has been confirmed by three other methods, including co-immunoprecipitation from lysates of adipocytes. The association was Ca ion dependent. The role of calmodulin binding to AS160 in insulin-stimulated GLUT4 translocation was examined through the generation of a point mutant of AS160 that did not bind calmodulin. This mutation did not interfere with the capacity of AS160 lacking Akt phosphorylation sites to inhibit GLUT4 translocation. Consequently, calmodulin binding is probably not required for the participation of AS160 in insulin-stimulated GLUT4 translocation.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Akt; Calmodulin; GLUT4; GTPase activating protein; Insulin; Rab

Insulin treatment of fat and muscle cells rapidly stimulates glucose transport. The basis for this stimulation is an increase in the amount of the glucose transporter GLUT4 in the plasma membrane. This increase is the result of movement of intracellular vesicles containing GLUT4 to the plasma membrane, followed by fusion therewith, a process referred to as GLUT4 translocation (reviewed in [1]). The complete signaling pathway(s) from the insulin receptor that leads to this trafficking of GLUT4 is not known and has been a subject of extensive investigation. A signaling pathway that has been established to participate is the one that proceeds from the insulin receptor through tyrosine phosphorylation of insulin receptor substrates, activation of phosphatidylinositol 3-kinase, generation of phosphatidylinositol 3,4,5-trisphosphate, and the activation of the serine kinase Akt. Several types of evidence indicate that activation of Akt is critical for GLUT4 translocation. Adipocytes partially depleted of Akt show reduced insulin stimulation of GLUT4 translocation [2–4], and expression of constitutively active Akt in adipocytes causes as much GLUT4 translocation as does insulin [4,5].

The connection between Akt activation and GLUT4 translocation has been unknown. Recently, we described a protein substrate of Akt in adipocytes that has properties expected for such a connection [6]. This substrate is a 160 kDa protein with a predicted GTPase activating protein (GAP) domain for the Rab family of small G proteins. It has been designated at AS160 (Akt substrate of 160 kDa). The GAP domain of AS160 is expected to act on the GTP form of Rab to convert it to the GDP form. In many systems, Rabs in the GTP form are

* Corresponding author. Fax: +1 603 650 1128.
E-mail address: gustav.e.lienhard@dartmouth.edu (G.E. Lienhard).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.07.056

known to be part of the machinery for vesicle movement and fusion. Thus, a plausible hypothesis is that phosphorylation of AS160 by activated Akt inhibits its GAP activity, thereby leading to the elevation of the GTP form of a certain Rab(s), which in turn triggers the movement of GLUT4 vesicles to the plasma membrane and/or the fusion therewith. In support of this hypothesis, when a mutant of AS160 lacking four of the Akt phosphorylation sites was expressed in 3T3-L1 adipocytes, insulin-stimulated GLUT4 translocation was markedly inhibited [6]. Moreover, the inhibitory effect of this mutant required that its GAP domain be catalytically active. Presumably, because the mutant cannot be phosphorylated on the Akt sites, it remains active in the presence of insulin, and so insulin is unable to trigger the rise in the GTP form of the Rab(s).

The present study characterizes AS160 further. We have found that AS160 is a calmodulin binding protein and examined whether calmodulin binding to AS160 is involved in insulin-stimulated GLUT4 translocation.

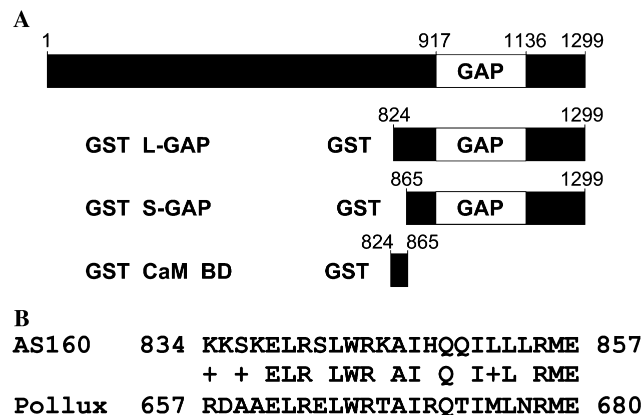

Fig. 1. GST constructs of human AS160 and comparison of AS160 and Pollux calmodulin binding domains. (A) Schematic diagrams of human AS160 (top) and of the three GST fusion proteins used in this study. The fusion proteins have been designated the long GAP (L-GAP), short GAP (S-GAP), and calmodulin binding domain (CaM BD). (B) Compares the amino acid sequence of AS160 to that of the known calmodulin binding domain of the related drosophila protein Pollux.

Materials and methods

Plasmids. The CMV-10 plasmid encoding human AS160 with a triple Flag tag at its amino terminus was as described in [7], and that encoding the mutated form with four of the Akt phosphorylation sites converted to Ala (designated 4P-AS160) was as described in [6]. Mutations of calmodulin binding domain in wild-type and 4P-AS160 were made with the the QuikChange XL site-directed mutagenesis kit from Stratagene, and the mutations were verified by DNA sequencing. For production of the glutathione *S*-transferase (GST) fusion proteins of portions of human AS160, PCR fragments of human AS160 cDNA were generated and cloned into the *Not*I/*Bam*HI sites of pGEX-5X-3 (Amersham Biosciences). The GST fusion proteins were prepared as described in the Amersham Biosciences handbook.

Antibodies. Antibodies were purchased from the following sources (catalog number in parentheses): Phospho Akt substrate antibody (9611), Cell Signaling Technology; HA tag monoclonal (MMS-101P), Berkeley Antibody Company; Cy3-conjugated goat anti-mouse immunoglobulin (115-165-146); calmodulin monoclonal antibody (05-173), Upstate; and anti-Flag antibody immobilized on agarose beads (A2220), Sigma. Affinity-purified rabbit antibody against the carboxy terminus of mouse AS160 was as described in [7].

Yeast two-hybrid screen. The yeast two-hybrid screens were performed by the Molecular Interaction Facility at the University of Wisconsin, Madison, according to their protocols, which can be found at http://www.biotech.wisc.edu/mif/.

Cell culture. 3T3-L1 fibroblasts from the American Type Culture Collection were carried as fibroblasts and differentiated, as described previously [6]. For transfections, cells on day 4 of differentiation were electroporated as described previously [6], with 75 μg HA-GLUT4-GFP and 100 μg wild-type or mutated Flag-tagged AS160 plasmid for the cells from a 10-cm plate.

Recombinant AS160. The recombinant Flag-tagged AS160 used in Fig. 1 was generated by transfecting HEK293 cells with the AS160 plasmid. Each 10-cm plate was transfected with 10 μg plasmid using Lipofectamine 2000 (Invitrogen). After 24 h, the cells were lysed in 2 ml of 40 mM Hepes, pH 7.4, 150 mM NaCl, and 1.5% nonaethyleneglycol dodecyl ether (NDE) with protease inhibitors (10 μM each of pepstatin, leupeptin, aprotinin, and EP475), the lysate was cleared by centrifugation at 10,000 rpm for 15 min, and the AS160 was adsorbed on 10 μl anti-Flag beads, which were then washed thoroughly with 40 mM Hepes, pH 7.5, 150 mM NaCl. This procedure yielded about 15 μg AS160 per 10-cm plate that was largely pure, as assessed by PAGE and Coomassie blue staining of SDS samples prepared from the beads. The recombinant Flag-tagged AS160 and mutants thereof used in Fig. 5A were generated by a slightly different procedure. In this case, SDS/NDE lysates of transfected 3T3-L1 adipocytes were prepared as described in [7], and the denatured Flag-tagged AS160 was adsorbed onto anti-Flag beads.

Biotinylated calmodulin binding assay. AS160, its point mutants, or GST fusion proteins with portions of AS160 were separated by SDS–PAGE and transferred to PVDF membranes (Immobilon-P, Millipore). Membranes were blocked in buffer A (50 mM Tris–Cl, pH 7.5, 200 mM NaCl, and 50 mM $MgCl_2$) containing 1% BSA and either 0.5 mM $CaCl_2$ or 5 mM EGTA for 1 h, and then treated with 100 ng/ml biotinylated calmodulin (Calbiochem, catalog number 208697) in buffer A with 1% BSA and 0.5 mM $CaCl_2$ or 5 mM EGTA for 2 h. The membranes were then washed four times for 10 min in buffer A with $CaCl_2$ or EGTA plus 0.05 % Tween 20. Calmodulin association was detected by incubating the membranes with Streptavidin-HRP (Invitrogen, catalog number 19534), diluted at 1:50,000 in buffer A with 1% BSA and $CaCl_2$ or EGTA, followed by 4 washes in buffer A with $CaCl_2$ or EGTA plus 0.05% Tween 20 and then 2 washes without Tween 20. The membranes were incubated in chemiluminescent substrate (SuperSignal West, Pierce) and exposed to film.

GST pulldowns and immunoprecipitations. For the GST pulldowns, recombinant calmodulin (7 μg, Upstate) was incubated with glutathione beads (30 μl) with 10 μg of bound GST L-GAP and S-GAP, or 5 μg GST CaM BD or GST alone (see Fig. 1A), in 1 ml buffer B (20 mM Tris–Cl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.1% NDE, 1 mM DTT, and 100 μg/ml BSA), containing either 0.5 mM $CaCl_2$ or 5 mM EGTA, for 1 h at 4 °C on a rotating wheel. The beads were washed three times in buffer B with either 0.5 mM $CaCl_2$ or 5 mM EGTA, and the bound proteins were solubilized in SDS sample buffer at 100 °C for 5 min. Calmodulin was detected by immunoblotting a portion of the SDS samples.

To examine co-immunoprecipitation of calmodulin with AS160 from cell lysates, 10-cm plates of 3T3-L1 adipocytes were treated with 160 nM insulin for 10 min or not, washed twice with PBS, and lysed in 1 ml of 30 mM Hepes, pH 7.5, 100 mM NaCl, 3% NDE with a phosphatase inhibitor (200 nM calyculin A), and protease inhibitors

(see above) containing either 0.5 mM $CaCl_2$ or 5 mM EGTA. The lysate was centrifuged at 12,000 rpm for 15 min and the infranatant was passed through a 0.45-μm filter to remove residual triglyceride droplets. Antibody against the carboxy terminus of AS160 (5 μg per 0.5 plate) was added, and after a 2 h incubation at 4 °C the immune complexes were collected on protein A–Sepharose (20 μl) for 2 h. The beads were transferred to a new microfuge tube, washed five times with 30 mM Hepes, pH 7.5, 100 mM NaCl, and 0.3% NDE containing either 0.5 mM $CaCl_2$ or 5 mM EGTA, and the immunoprecipitates were solubilized in SDS sample buffer containing 1 mM EGTA at 100 °C for 5 min. Samples of the immunoprecipitates were then immunoblotted for calmodulin and AS160.

Immunoblotting. Proteins were separated by SDS–PAGE and transferred to PVDF membranes (Immobilon-P, Millipore). The membranes were blocked with 3% BSA in TBST (0.3% Tween 20, 150 mM NaCl, and 20 mM Tris–Cl, pH 7.5), treated with primary antibody in TBST with 1% BSA, washed with TBST and then TBS, treated with horseradish peroxidase-conjugated secondary antibody, and developed with the chemiluminescence reagent (SuperSignal West, Pierce). For detection of calmodulin in the AS160 immunoprecipitation experiments, proteins were transferred to membranes, cross-linked with glutaraldehyde, and immunoblotted according to the methods described in [8]. This method increases the sensitivity of immunoblotting for calmodulin by about 30-fold.

GLUT4 distribution. GLUT4 at the cell surface was measured by a single-cell fluorescence assay. In this assay, GLUT4 with an HA epitope tag in an extracellular loop and GFP at its carboxy terminus was expressed through electroporation of 3T3-L1 adipocytes with the HA-GLUT4-GFP plasmid. Cells were fixed with formaldehyde, and GLUT4 in the plasma membrane was then reacted with anti-HA and Cy3-conjugated goat anti-mouse immunoglobulin. For each condition, the fluorescence intensities of Cy3 and GFP were quantitated for individual cells. For each cell, the intensity of Cy3 was normalized to that of GFP, to correct for different levels of expression of HA-GLUT4-GFP. The normalized Cy3 intensity is a relative measure of GLUT4 at the cell surface. The method is described in detail in [6].

Results

Binding of calmodulin to AS160

The region of human AS160 from amino acids 824 to 1299 encompasses its predicted Rab GAP domain (Fig. 1A, L-GAP). In an effort to find a Rab that is associated with AS160, this portion of the protein, designated L-GAP, was used as the bait in the yeast two-hybrid screen with a combined human heart/liver library and also with a combined human brain/testes library. No interacting Rab was found in this screen. However, the Ca binding protein calmodulin was found repeatedly with both libraries.

A possible basis for the interaction between L-GAP and calmodulin became apparent upon examination of proteins related to AS160. One of these is the *Drosophila* protein known as Pollux (gi24644376). Pollux is a protein of 1379 amino acids that is 33% identical/48% similar to AS160 in the large region from its amino acids 117 to 1093. The function of Pollux is not known, but it has been found to bind calmodulin. Moreover, this binding has been localized to a short segment of 14 amino acids (amino acid 657–680) [9]. The corresponding region of AS160, which consists of amino acids 834–857, is present at the amino terminus of L-GAP. It is 58% identical to the peptide in Pollux (Fig. 1B). Consequently, it seemed likely that this region of the L-GAP accounted for the interaction with calmodulin. In agreement with this supposition, when a prey lacking this region (Fig. 1A, S-GAP, amino acids 865–1299) was used in the yeast two-hybrid screen with both libraries, calmodulin was not found as an interactor. The screen with S-GAP also did not yield a Rab as an interactor.

In order to determine more directly that calmodulin bound to AS160, we employed a binding assay in which biotinylated calmodulin was incubated with a membrane to which recombinant, SDS-denatured human AS160 had been transferred. The biotinylated calmodulin bound to AS160 in the presence of Ca ion, but not in its absence (Fig. 2). Subsequently, this assay was used to assess the binding of calmodulin to GST fusion proteins with L-GAP, S-GAP, and also a short region encompassing only the putative calmodulin binding sequence (Fig. 1A, designated CaM BD). As shown in Fig. 3A, the biotinylated calmodulin bound to L-GAP and CaM BD, but not to S-GAP or to GST alone. The binding assay in Fig. 3A was carried out in the presence of Ca ion. When the same assay was done in the absence of Ca ion, no binding was detected (data not shown).

The assay with biotinylated calmodulin detects binding to the proteins on a membrane in at least partially denatured form. To test whether the nondenatured GST fusion proteins would bind to calmodulin in solution, we carried out a GST pulldown assay in which the GST fusion proteins, immobilized on glutathione beads, were incubated with recombinant calmodulin, and then the washed beads were assayed for bound calmodulin by immunoblotting (Fig. 3B). By this assay as well, the L-GAP and the CaM BD bound calmodulin. Strong

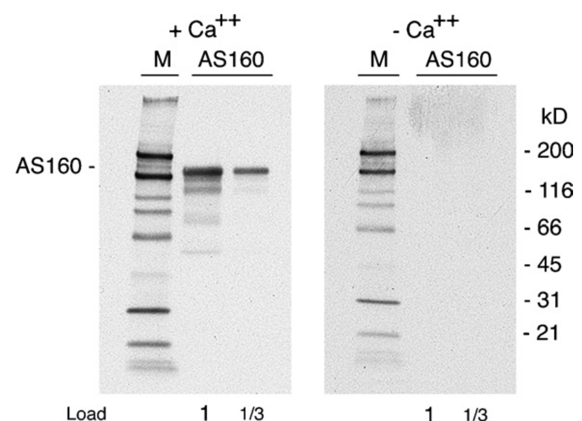

Fig. 2. Binding of calmodulin to AS160. Recombinant AS160 was separated on SDS–PAGE, transferred to a membrane, and probed with biotinylated calmodulin in the presence or absence of Ca ion, as described in the Materials and methods. The 1× load of AS160 was 400 ng. The lanes designated M show biotinylated standard marker proteins.

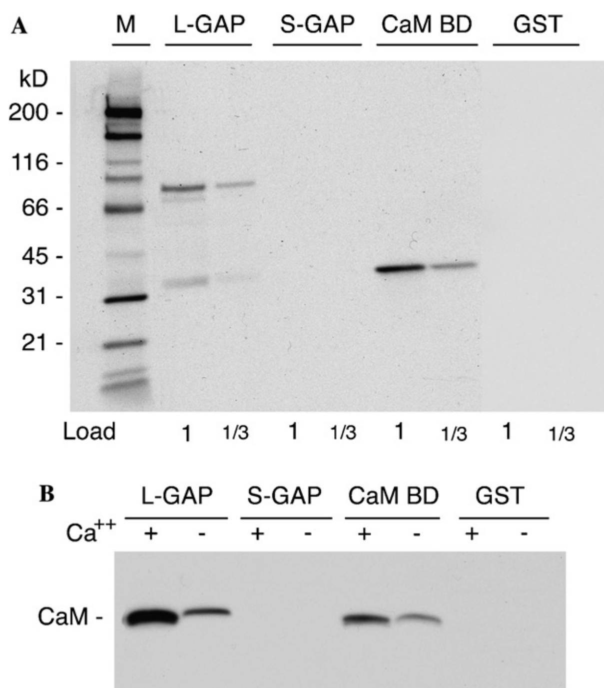

Fig. 3. Binding of calmodulin to GST fusion proteins of AS160. (A) The GST fusion proteins shown in Fig. 1A were separated by SDS–PAGE, transferred to a membrane, and probed with biotinylated calmodulin in the presence of Ca ion, as described in Materials and methods. The 1× load for L- and S-GAP was 200 ng and that for CaM BD and GST was 100 ng. (B) The GST fusion proteins on glutathione beads were incubated with calmodulin in the presence or absence of Ca ion, and then SDS samples prepared from the washed beads were immunoblotted for calmodulin, as described in Materials and methods.

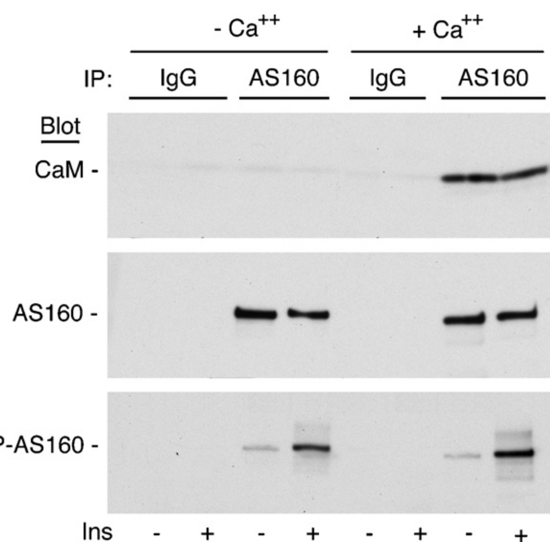

Fig. 4. Co-immunoprecipitation of calmodulin with AS160 from lysates of 3T3-L1 adipocytes. Immunoprecipitates were prepared from nonionic detergent lysates of basal and insulin-treated adipocytes in the absence or presence of Ca ion, with either an irrelevant rabbit antibody (IgG) or antibody against the carboxy terminus of AS160 (AS160). The immunoprecipitates were immunoblotted for calmodulin, AS160, and phosphorylated AS160. The load in each case was the immunoprecipitate derived from 10% of a 10-cm plate.

binding required Ca ion, but, in contrast to the assay with biotinylated calmodulin, there was some association in the absence of Ca ion.

Association of calmodulin with AS160 in 3T3-L1 adipocytes

To assess whether calmodulin and AS160 have the potential to be associated in vivo, we immunoprecipitated AS160 from nonionic detergent lysates of 3T3-L1 adipocytes prepared either with or without Ca ion and then immunoblotted the AS160 for associated calmodulin (Fig. 4). Calmodulin co-immunoprecipitated with the AS160 and the association required the presence of Ca ion. The amount of associated calmodulin was independent of whether or not cells had been treated with insulin. Upon insulin treatment AS160 is phosphorylated by the protein kinase Akt on six sites, none of which is in or near the calmodulin binding domain [6]. Immunoblotting with an antibody specific for phosphorylated Akt sites confirmed that the phosphorylation had occurred. Thus, it is likely that the association of calmodulin with AS160 is independent of the phosphorylation state of AS160.

Disruption of calmodulin binding by mutation of AS160

In order to examine the role of calmodulin binding in AS160 function, we sought to prepare a mutant of AS160 that did not bind calmodulin. Hydrophobic residues, such as Leu and Trp, have been established to be key ones in calmodulin binding sites (calmodulin target database, http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html). Consequently, we mutated the LeuTrp, which is conserved in the middle of calmodulin binding sequence in human AS160 and *Drosophila* Pollux (Fig. 1B), to GlyGly. This mutation was placed in both Flag-tagged wild-type AS160 and in a mutant of AS160 in which four of the Akt phosphorylation sites are mutated to Ala [6] (4P-AS160). These proteins were expressed in 3T3-L1 adipocytes and isolated by immunoprecipitation with anti-Flag beads. Calmodulin binding was assessed with the biotinylated calmodulin assay. As expected, biotinylated calmodulin bound to the wild-type AS160 and to the 4P-AS160. However, no binding to AS160 LW/GG or to 4P-AS160 LW/GG was detected (Fig. 5).

Effect of calmodulin binding on AS160 function in GLUT4 translocation

Overexpression of wild-type AS160 in 3T3-L1 adipocytes has no effect on insulin-stimulated translocation of GLUT4 to the plasma membrane, but expression of the 4P-AS160 blocks translocation [6]. Consequently, to determine the effect of abrogation of calmodulin binding on AS160 function, we examined the effects of both the

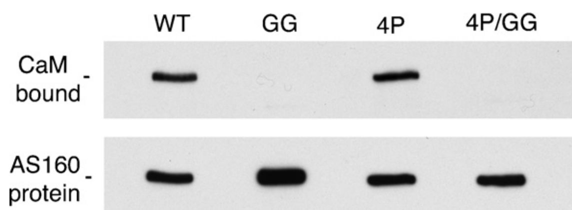

Fig. 5. Disruption of calmoduling binding by mutation of LeuTrp in the calmodulin binding domain of AS160. Flag-tagged AS160 and point mutants thereof were isolated by immunoprecipitation from SDS/nonionic detergent lysates of transfected 3T3-L1 adipocytes, separated by SDS–PAGE, transferred to a membrane, and probed with biotinylated calmodulin. Subsequently, the membrane was stripped by treatment with 5 mM EGTA and then probed with antibody against the carboxy terminus of AS160. The forms of AS160 were: WT, wild-type; GG, the L842G/W843G mutant; 4P-AS160; 4P/GG, AS160 with the combined mutations of the 4P and GG forms.

AS160 LW/GG mutant and the 4P-AS160 LW/GG mutant on the insulin-stimulated translocation of GLUT4. The relative amount of GLUT4 in the plasma membrane of 3T3-L1 adipocytes was measured by a single-cell immunofluorescence assay with HA-GLUT-GFP. Fig. 6 shows the results of this analysis. With expression of either wild-type Flag-tagged AS160 or AS160 LW/GG, insulin caused a large increase in GLUT4 at the cell surface. Thus, disruption of calmodulin binding in the context of wild-type AS160 had no effect. Moreover, expression of either 4P-AS160 or 4P-AS160 LW/GG markedly inhibited insulin-stimulated translocation of GLUT4. Consequently, the inhibitory effect of the AS160 mutant lacking the Akt phosphorylation sites was not dependent upon calmodulin binding. In each of the three experiments shown in Fig. 6, the expression of Flag-tagged AS160 and the mutants thereof relative to each other and that of endogenous AS160 was assessed by immunoblotting SDS lysates of the transfected cells with the antibody against its carboxy terminal peptide,

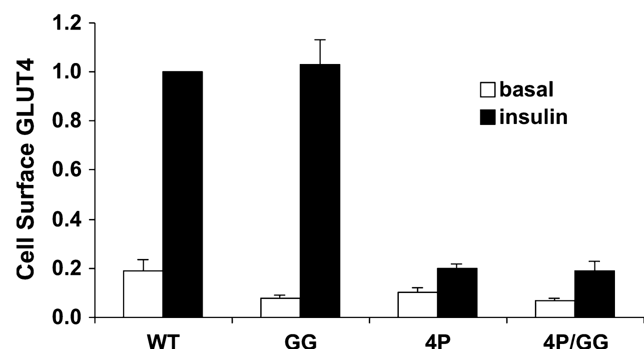

Fig. 6. Effect of the disruption of AS160 calmodulin binding on GLUT4 translocation. 3T3-L1 adipocytes were transfected with HA-GLUT4-GFP and either Flag-tagged AS160 or one of the point mutants thereof (see legend of Fig. 5). HA-GLUT4-GFP at the cell surface in the basal and insulin state, was measured as described in Materials and methods. The values for three separate experiments have been normalized to a value of 1.0 for the wild-type AS160 in the insulin state; the error bars show the SEM.

as described previously [6]. As expected from our previous results [6], in each experiment the Flag-tagged AS160, AS160 LW/GG, 4P-AS160, and 4P-AS160 LW/GG were approximately equally expressed, and the expression was approximately fivefold or more than that of the endogenous AS160 in the transfected cells (data not shown).

Discussion

The results presented here demonstrate that AS160 is a calmodulin binding protein. In three different assays, this association either required Ca ion (binding of biotinylated calmodulin, co-immunoprecipitation) or was stronger in its presence (GST pulldown). Consequently, it appears that calmodulin must be complexed with Ca ion for tight association of the two proteins. This situation is typical of many calmodulin binding proteins [10]. The results also show that the binding occurs through a small domain of AS160 that is located just amino terminal to its GAP domain.

The occurrence of this calmodulin association then led us to examine its possible role in the function of AS160. In order to do so, we generated the LW/GG mutants of wild-type AS160 and 4P-AS160 that did not bind calmodulin, and examined the effects of overexpression of these mutants on insulin-stimulated GLUT4 translocation. The AS160 LW/GG mutant had no effect on GLUT4 translocation. This result suggests that calmodulin binding is not required for the function of AS160 in insulin-stimulated GLUT4 translocation. However, it does not prove it, since the cells also contain the endogenous wild-type AS160 that can function normally. The result with the 4P-AS160 LW/GG mutant is more definitive. This mutant inhibited GLUT4 translocation to the same extent as 4P-AS160. Consequently, the inhibitory effect of the nonphosphorylatable 4P mutant does not depend upon calmodulin binding. As described in Introduction, the inhibitory effect of the 4P mutant is most likely due to its continued activity as a Rab GAP in the presence of insulin. If this is the case, the GAP activity does not require calmodulin binding. Moreover, since our previous findings indicate that insulin-stimulated phosphorylation of AS160 shuts off its GAP activity [6], it seems unlikely that calmodulin binding is also required for this shut-off. If calmodulin binding were required for the shut-off, then the AS160 LW/GG mutant should remain active as a GAP in the presence of insulin and so inhibit GLUT4 translocation. However, it did not. These considerations indicate that calmodulin binding to AS160 is not involved in insulin-stimulated GLUT4 translocation.

The absence of a role for calmodulin binding to AS160 in GLUT4 translocation is consistent with the results of earlier studies on the role of calcium in insulin action. Insulin treatment of adipocytes does not elevate

the intracellular concentration of free calcium [11,12]. Consequently, the concentration of the Ca-chelated calmodulin in either basal or insulin-treated adipocytes is unlikely to be sufficient to result in substantial binding to AS160. On the other hand, it should be noted that Ca ion appears to play at least a permissive role in GLUT4 translocation, since treatment of 3T3-L1 adipocytes with the Ca chelator BAPTA-AM markedly inhibits insulin-stimulated GLUT4 translocation [13,14].

Although the binding of calmodulin to AS160 is probably not required for AS160 to participate in signaling insulin-stimulated GLUT4 translocation, this association could be part of a signaling pathway that contributes to the stimulation of GLUT4 translocation and so glucose transport by exercise in muscle. Exercise increases the concentration of cytosolic Ca ion, and elevated Ca ion causes an increase in glucose transport [15,16]. Thus, a reasonable hypothesis is that elevated Ca/calmodulin, caused by exercise, binds to AS160 and inhibits its Rab GAP activity. As a consequence, the GTP form of the Rab(s) required for GLUT4 translocation increases, and GLUT4 translocation is triggered. This mechanism is similar to the one proposed for the role of AS160 in insulin-stimulated GLUT4 translocation (see Introduction), except that the GAP activity of AS160 is inhibited by Ca/calmodulin binding, rather than phosphorylation by Akt. In the future, it should be possible to test this proposal by introduction of the AS160 LW/GG mutant into muscle. This mutant should not be inhibitable by Ca/calmodulin, and so if the proposal is correct, it should block exercise-stimulated glucose transport.

Acknowledgments

We are indebted to Cristinel Miinea for the preparation of some recombinant proteins. This study was supported by a grant (DK 025336) from the National Institutes of Health.

References

[1] R.T. Watson, M. Kanzaki, J.E. Pessin, Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes, Endocr. Rev. 25 (2004) 177–204.

[2] S.S. Bae, H. Cho, J. Mu, M.J. Birnbaum, Isoform-specific regulation of insulin-dependent glucose uptake by Akt/protein kinase B, J. Biol. Chem. 278 (2003) 49530–49536.

[3] Z.Y. Jiang, Q.L. Zhou, K.A. Coleman, M. Chouinard, Q. Boese, M.P. Czech, Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing, Proc. Natl. Acad. Sci. USA 100 (2003) 7569–7574.

[4] T. Katome, T. Obata, R. Matsushima, N. Masuyama, L.C. Cantley, Y. Gotoh, K. Kishi, H. Shiota, Y. Ebina, Use of RNA interference-mediated gene silencing and adenoviral overexpression to elucidate the roles of AKT/protein kinase B isoforms in insulin actions, J. Biol. Chem. 278 (2003) 28312–28323.

[5] A. Zeigerer, M.K. McBrayer, T.E. McGraw, Insulin stimulation of GLUT4 exocytosis, but not its inhibition of endocytosis, is dependent on RabGAP AS160, Mol. Biol. Cell 15 (2004) 4406–4415.

[6] H. Sano, S. Kane, E. Sano, C.P. Miinea, J.M. Asara, W.S. Lane, C.W. Garner, G.E. Lienhard, Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation, J. Biol. Chem. 278 (2003) 14599–14602.

[7] S. Kane, H. Sano, S.C. Liu, J.M. Asara, W.S. Lane, C.W. Garner, G.E. Lienhard, A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain, J. Biol. Chem. 277 (2002) 22115–22118.

[8] D. Hulen, A. Baron, J. Salisbury, M. Clarke, Production and specificity of monoclonal antibodies against calmodulin from *Dictyostelium discoideum*, Cell Motil. Cytoskeleton 18 (1991) 113–122.

[9] X.Z. Xu, P.D. Wes, H. Chen, H.S. Li, M. Yu, S. Morgan, Y. Liu, C. Montell, Retinal targets for calmodulin include proteins implicated in synaptic transmission, J. Biol. Chem. 273 (1998) 31297–31307.

[10] P. Cohen, C.B. Klee (Eds.), Molecular Aspects of Cellular Regulation, Elsevier, Amsterdam, 1998.

[11] A. Klip, T. Ramlal, Cytoplasmic Ca2+ during differentiation of 3T3-L1 adipocytes. Effect of insulin and relation to glucose transport, J. Biol. Chem. 262 (1987) 9141–9146.

[12] K.L. Kelly, J.T. Deeney, B.E. Corkey, Cytosolic free calcium in adipocytes. Distinct mechanisms of regulation and effects on insulin action, J. Biol. Chem. 264 (1989) 12754–12757.

[13] J.P. Whitehead, J.C. Molero, S. Clark, S. Martin, G. Meneilly, D.E. James, The role of Ca2+ in insulin-stimulated glucose transport in 3T3-L1 cells, J. Biol. Chem. 276 (2001) 27816–27824.

[14] D.S. Worrall, J.M. Olefsky, The effects of intracellular calcium depletion on insulin signaling in 3T3-L1 adipocytes, Mol. Endocrinol. 16 (2002) 378–389.

[15] J.O. Holloszy, A forty-year memoir of research on the regulation of glucose transport into muscle, Am. J. Physiol. Endocrinol. Metab. 284 (2003) E453–E467.

[16] L.O. Pereira, A.H. Lancha, Effect of insulin and contraction upon glucose transport in skeletal muscle, Prog. Biophys. Mol. Biol. 84 (2004) 1–27.