Insulin-stimulated Phosphorylation of a Rab GTPase-activating Protein Regulates GLUT4 Translocation*  

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Insulin stimulates the rapid translocation of intracellular glucose transporters of the GLUT4 isotype to the plasma membrane in fat and muscle cells. The connections between known insulin signaling pathways and the protein machinery of this membrane-trafficking process have not been fully defined. Recently, we identified a 160-kDa protein in adipocytes, designated AS160, that is phosphorylated by the insulin-activated kinase Akt. This protein contains a GTPase-activating domain (GAP) for Rabs, which are small G proteins required for membrane trafficking. In the present study we have identified six sites of in vivo phosphorylation on AS160. These sites lie in the motif characteristic of Akt phosphorylation, and insulin treatment increased phosphorylation at five of the sites. Expression of AS160 with two or more of these sites mutated to alanine markedly inhibited insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes. Moreover, this inhibition did not occur when the GAP function in the phosphorylation site mutant was inactivated by a point mutation. These findings strongly indicate that insulin-stimulated phosphorylation of AS160 is required for GLUT4 translocation and that this phosphorylation signals translocation through inactivation of the Rab GAP function.  

Insulin rapidly stimulates glucose transport into fat and muscle cells by causing the insertion of additional glucose transporters of the GLUT4 isotype into the plasma membrane, in a process referred to as GLUT4 translocation. The overall process consists of generation of the specialized vesicles containing GLUT4 from the endosomal system, the movement of these vesicles from the perinuclear region to the plasma membrane, and the fusion of the vesicles with the plasma membrane (1). The steps in this process that insulin accelerates, and the complete signaling pathways from the insulin receptor that lead to their acceleration, have not yet been fully defined. One partial insulin signaling pathway that has been established to be required for GLUT4 translocation is the pathway that proceeds from the receptor through tyrosine phosphorylation of the insulin receptor substrates to activation of phosphatidylinositol 3-kinase and generation of phosphatidylinositol 3,4,5-trisphosphate. The latter leads to the activation of the protein kinase Akt and also protein kinase C \(\alpha\), and there is evidence that GLUT4 translocation requires the activation of one or both of these enzymes (reviewed in Refs. 2 and 3). However, although a substrate linking either kinase to GLUT4 translocation has been sought for several years, hitherto none has been clearly identified.  

Recently, we reported the discovery of a new substrate for insulin-activated Akt in 3T3-L1 adipocytes, which was designated AS160 for Akt substrate of 160 kDa (4). The most prominent feature of AS160 is the presence of a GAP-active domain for a Rab. Since Rabs are small G proteins that play critical roles in vesicle formation, movement, and fusion (5), we investigated the role of AS160 in GLUT4 translocation. Our present results strongly indicate that insulin-stimulated phosphorylation of AS160 is required for GLUT4 translocation and also that an active GAP domain in AS160 is required for AS160 control of GLUT4 translocation. This study thus identifies AS160 as a new and likely key connection between the phosphatidylinositol 3-kinase insulin signaling pathway and the vesicle trafficking machinery in GLUT4 translocation.  

**EXPERIMENTAL PROCEDURES**  
**Plasmids**—The plasmid encoding GLUT4 with a HA tag in an extracellular domain and GFP fused to the carboxyl terminus was as described in Ref. 6. The CMV-10 plasmid encoding human AS160 with a triple FLAG tag at the amino terminus was as described in Ref. 4. Mutations in AS160 in this vector were made with the QuikChange XL site-directed mutagenesis kit from Stratagene, and the mutations were verified by DNA sequencing.  
**Antibodies**—Antibodies were purchased from the following sources (catalog number in parentheses): mouse monoclonal anti-FLAG (F-3165), Sigma; mouse monoclonal anti-HA (MMS-101P), Berkeley Antibody Company; Cy3-conjugated goat anti-mouse immunoglobulin (115-165-146), Jackson ImmunoResearch. Affinity-purified rabbit antibody against the carboxyl terminus of mouse AS160 was as described in Ref. 4.  
**Purification of AS160 and Mass Spectrometry**—AS160 was purified from five 10-cm plates of unstimulated 3T3-L1 adipocytes and five plates treated with 160 nM insulin for 10 min. As described in detail in Ref. 4, cells were lysed in SDS/dithiothreitol, excess nonionic detergent was added, and the AS160 was isolated by immunoprecipitation with 30 μg of antibody against the carboxyl terminus of mouse AS160 as described in Ref. 4.  
**Cell Culture and Electroporation**—3T3-L1 fibroblasts from the Amer...
American Type Culture Collection were carried as fibroblasts and differentiated as described previously (4). For transfections, cells at day 4 of differentiation were detached with 0.25 mg/ml trypsin, 0.5 mg/ml collagenase, washed with PBS, and electroporated in a 0.5-mL cuvette (cells from approximately one 10-cm plate) at 0.18 kV and 975 microfarads in a Bio-Rad Gene Pulser II with 75 μg HA-GLUT4-GFP and 100 μg FLAG-tagged AS160 plasmid. After electroporation the cells were plated in four wells of a six-well plate containing glass cover slips. After 24 h the cells were put into serum-free medium for 2 h, treated with 160 ng insulin or not for 30 min, washed with PBS, and fixed with 4% formaldehyde in PBS for 5 min. Approximately 20% of the cells were transfected, as assessed by the GFP fluorescence. For each AS160 plasmid co-transfected with the HA-GLUT4-GFP, the extent of co-transfection was assessed by permeablizing fixed cells with 0.2% saponin, staining with anti-HA followed by Cy3-conjugated anti-mouse immunoglobulin, and determining whether cells with GFP fluorescence also exhibited Cy3 fluorescence. Under these conditions, each cell exhibited Cy3 fluorescence. For each combination, transfected, unfixed cells were solubilized in SDS sample buffer and immunoblotted for AS160, as described in Ref. 4.

Fluorescence Microscopy and GLUT4 Translocation—The appearance of HA-GLUT4-GFP in the plasma membrane was detected by reaction of its extracellular HA tag with anti-HA and Cy3-conjugated anti-mouse immunoglobulin, according to the method described in Ref. 1. A brief description is as follows. The fixed, nonpermeabilized cells on coverslips were reacted with 5 μg/ml anti-HA in PBS, 2% fetal calf serum, washed, reacted with 1/800 dilution of the Cy3-conjugated secondary antibody, washed, and mounted on slides. Fluorescence images on the GFP and Cy3 settings were acquired on a BX 51 Olympus microscope with a Sensicam QED CCD camera and analyzed with the IPLab software (Scanalytics). Ref. 8 provides details of the microscopic equipment. Each transfected cell in a field was outlined, and its average Cy3 and GFP fluorescence intensities measured. The background intensities measured from the same area of an untransfected cell in the same field, were subtracted. To normalize for differences in the expression of HA-GLUT4-GFP between cells, the Cy3 fluorescence intensity was then divided by the GFP fluorescence intensity for the same cell. A typical experiment consisted of transfecting aliquots from one preparation of cells with HA-GLUT4-GFP plus separately the 3'-FLAG-CMV-10 vector, wild-type AS160 in this vector, and several mutants of AS160 in this vector. Cells from each combination were treated with insulin or left in the basal state, and the normalized Cy3 intensity was measured for at least 50 transfected cells from each condition on coded slides to obviate investigator bias. The fold GLUT4 translocation was then calculated as the ratio of the average value of the normalized Cy3 intensity for the insulin state to that for the basal state, less 1.0.

RESULTS

Phosphorylation Sites on AS160—We have reported previously that AS160 isolated from insulin-treated cells was phosphorylated on Ser588 and Thr642 (4). To characterize the phosphorylation of AS160 more completely, we purified AS160 from basal and insulin-treated 3T3-L1 adipocytes by immunoprecipitation with antibody against the carboxyl terminus, digested it with trypsin, and searched for predicted phosphopeptides by tandem ion MS/MS. We used the program Scansite to predict the likely sites of Akt phosphorylation (9) and searched for the seven phosphopeptides corresponding to the sites with the best scores. Six of these phosphopeptides, as well as the corresponding nonphosphorylated peptides, were identified in both the basal and insulin samples, and the sites of phosphorylation were deduced (Fig. 1). The sequences surrounding the six sites in mouse AS160 are: RSRCSS311V, RRRHAS341A, RSLTSS370L, RGRGLS588M, RRAHT642F, and RRKTSS751T. Thus, with the exception of Ser570, these sites are all in the RXXXX/S/T motif preferred by Akt (9). The phosphopeptide corresponding to a seventh possible Akt site at Ser597 was not detected, although the nonphosphorylated peptide was detected.

To estimate the effect of insulin on the phosphorylation of each site, we measured the total ion intensities of each phosphorylated phosphate and of the corresponding nonphosphorylated peptide in the basal and insulin samples. For each sample, the total ion intensity of each phosphorylated phosphate and that of the corresponding nonphosphorylated phosphate were expressed as percentages of their combined total ion intensities. The ratio of these percentages for each phosphorylated/non-phosphorylated pair in the basal sample was then compared with the ratio in the insulin sample, to assess the effect of insulin on the extent of phosphorylation (7). These ratios were as follows (listed as phosphorylation site, ratio for phosphorylated form to that for nonphosphorylated form in the basal sample, corresponding ratio in the insulin sample): Ser318, 27/73, 76/24; Ser341, 80/20, 96/4; Ser370, 16/84, 45/54; Ser588, 14/86, 63/17; Thr642, 27/73, 74/26; and Thr751, 10/90, 75/25. Since the factor relating the total ion intensity of each peptide to its actual amount in the sample varies from peptide to peptide, these values do not give the actual molar ratios of phosphopeptide to nonphosphopeptide. Nevertheless, the large increase in this ratio in the insulin sample compared with that in the basal sample for the peptides encompassing Ser318, Ser341, Ser370, Ser588, Thr642, and Thr751 demonstrates that insulin caused a marked increase in phosphorylation at these sites.

Effect of AS160 on GLUT4 Translocation—Insulin-stimulated translocation of GLUT4 to the cell surface was followed by immunofluorescence in 3T3-L1 adipocytes expressing HA-GLUT4-GFP. The extracellularly oriented HA epitope tag on the surface of fixed nonpermeabilized cells was detected by labeling with anti-HA antibody followed by Cy3-labeled secondary antibody. The effects of wild-type AS160 and various mutants thereof on GLUT4 translocation were examined by co-transfecting the adipocytes with the AS160 plasmid and the HA-GLUT4-GFP plasmid. Fig. 2, top, shows representative images for cells transfected with vector, wild-type AS160, or AS160 in which four of the phosphorylation sites have been mutated to alanine (4P mutant). In the vector control, intracellular GLUT4, given by the GFP signal, was concentrated in the perinuclear region, and insulin treatment caused a marked increase in the GLUT4 at the cell surface, given by the Cy3 signal. The cells expressing wild-type AS160 appeared similar to the vector control. However, the cells expressing the 4P mutant exhibited a pronounced inhibition of insulin-stimulated GLUT4 appearance at the cell surface.

As described under “Experimental Procedures,” images of the type in Fig. 2 were quantitated, and the fold translocation of GLUT4 in response to insulin was calculated. Fig. 3 summarizes the data from a number of experiments. In each experiment, the vector control was included, and the fold translocation for it was determined. In 12 separate experiments the values for the control ranged from 3.1 to 13.6, with the median value at 6.7. Thus, in each experiment there was substantial translocation, but the fold effect varied. The variation may be due to differences between passages of the 3T3-L1 adipocytes. To compare all the data, we have normalized the values for insulin-stimulated GLUT4 translocation to a value of 1.0 for the vector. Fig. 3 shows that expression of wild-type AS160 had no significant effect on translocation. However, expression of
The 4P mutant markedly reduced translocation, to 21% of value for the vector control.

Rab GAP domains contain an arginine residue that is critical for activity, and mutation of this residue to lysine abolishes activity (10). Alignment of the GAP domain of AS160 with other Rab GAP domains (11) showed that this key arginine is Arg973 of AS160. Consequently, we examined the effect of the R973K mutant (R/K) on GLUT4 translocation. In addition, the effect of mutations in both the phosphorylation sites and the GAP domain largely reversed the inhibition by the phosphorylation site mutations alone (Figs. 2 and 3).

To be sure that differences in expression levels could not account for the effects of the various mutants, we immunoblotted SDS samples of the transfected cells for AS160, both with an antibody against the FLAG tag (Fig. 4, upper panel) and with an antibody against the carboxyl terminus of mouse AS160 (Fig. 4, lower panel). Approximately equal amounts of the wild-type and mutant forms of FLAG-tagged human AS160 were expressed. The blot with the antibody against the carboxyl terminus also showed the endogenous mouse AS160, which is of slightly greater mobility since it lacks the FLAG tag. From the relative signals in the lower panel of Fig. 4 and the ~20% transfection efficiency, we estimate that the transfected cells express about 15 times more FLAG-tagged human AS160 than endogeneous mouse AS160, which differs from mouse AS160 by four out of 12 amino acids in the carboxyl terminus.

We also examined the effect of cumulative mutations to alanine of the six phosphorylation sites in AS160 on GLUT4 translocation. Most of the inhibition was achieved with the combined T588A and S642A mutations (Fig. 5).

**DISCUSSION**

Our results show that AS160 undergoes a marked increase in phosphorylation at five sites in response to insulin. Since each site lies within the consensus sequence for Akt phosphorylation, and since we have shown previously that Akt phosphorylates the Thr642 site (4), it seems likely that activated Akt is responsible for phosphorylation at these sites, although it remains possible that one or more other insulin-activated kinases, such as protein kinase C α/δ, also participates.

Expression of AS160 mutated at two or more of its phosphorylation sites markedly inhibited insulin-stimulated GLUT4 translocation, whereas equivalent expression of wild type AS160 had no effect. This result strongly indicates that phosphorylation of AS160 is necessary for GLUT4 translocation to occur. Expression of AS160 with combined mutations in the phosphorylation sites and the Rab GAP domain largely reversed the inhibition given by AS160 mutated at the phospho-

**FIG. 4.** Expression of AS160 and mutants thereof in 3T3-L1 adipocytes. SDS samples of the transfected cells described in the legends to Figs. 2 and 3 containing equal amounts of protein were immunoblotted for the exogenous FLAG-tagged human AS160 (hAS160) with anti-FLAG (upper panel) and for this form plus the endogeneous mouse AS160 (mAAS160) with antibody against the carboxyl terminus of mouse AS160. The 1× loads were 5 μg (upper panel) and 35 μg (lower panel). A repetition of this experiment with a second set of transfected cells gave similar results.

**FIG. 5.** Effect of AS160 phosphorylation site mutations on GLUT4-GFP translocation. The translocation was measured and calculated as described under “Experimental Procedures” and “Results,” and in the legend to Fig. 3. The AS160 was mutated to alanine at: single sites, Ser588 and Thr642; Ser588 and Thr642 (2P); 2P sites plus Ser570 (3P); 3P sites plus Ser341 (4P); 4P sites plus Ser770 (5P); and 5P sites plus Ser341 (6P).
rlation sites alone. This result demonstrates that the inhibitory effect of AS160 mutated at its phosphorylation sites requires a functional Rab GAP domain. A hypothesis that explains these findings is the following: insulin-stimulated translocation of GLUT4 requires a Rab in its active GTP form; in the unstimulated state this Rab is maintained in its inactive GDP form by the GAP domain of AS160; phosphorylation of AS160 inhibits its GAP activity toward this Rab, through an effect either on GAP function and/or on localization of the protein; as a consequence the GTP form of the Rab increases, and the Rab-dependent step(s) in GLUT4 translocation proceed.

If the above hypothesis is correct, it might be thought that AS160 with an inactive GAP domain would trigger translocation in the absence of insulin, whereas in fact expression of the GAP mutant of AS160 did not have this effect. One possibility is that extent of expression of the GAP mutant was not sufficient for it to block the action of the endogenous AS160. A second possible explanation is that generation of the GTP form of a Rab is only one of several signals required for GLUT4 translocation. There is considerable evidence that a second signaling pathway involving activation of the Rho-type G protein TC10 and the rearrangement of cortical actin is required for GLUT4 translocation (3). This pathway is independent of Akt activation. In addition, there is suggestive evidence that Akt phosphorylation of the syntaxin 4-interacting protein Synip may be required for GLUT4 translocation (12, 13).

This study indicates that AS160 is a key component linking the phosphatidylinositol 3-kinase insulin signaling pathway to the vesicle trafficking machinery in GLUT4 translocation. In the future it will be important to identify the Rab(s) on which AS160 acts and to determine whether phosphorylation of AS160 directly inhibits its GAP activity. Rab4 and Rab11 are present in the intracellular vesicles that contain GLUT4 (reviewed in Ref. 14). Consequently these two Rabs are candidates to be substrates for the GAP function of AS160. We are currently attempting to determine whether AS160 exhibits GAP activity toward either of these Rabs or toward another one of the ~60 Rabs present in mammals (5).

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