View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Dartmouth Digital Commons (Dartmouth College)

Dartmouth College
Dartmouth Digital Commons

Open Dartmouth: Faculty Open Access Articles

8-1-2009

Insulin Stimulates the Phosphorylation of the Exocyst Protein Sec8 in Adipocytes

Patrick D. Lyons
Dartmouth College

Grantley R. Peck
Dartmouth College

Arminja N. Kettenbach
Dartmouth College

Scott A. Gerber
Dartmouth College

Liya Roudaia
Dartmouth College

See next page for additional authors

Follow this and additional works at: https://digitalcommons.dartmouth.edu/facoa

Part of the Biochemistry Commons

Recommended Citation

Lyons, Patrick D.; Peck, Grantley R.; Kettenbach, Arminja N.; Gerber, Scott A.; Roudaia, Liya; and Lienhard, Gustav E., "Insulin Stimulates the Phosphorylation of the Exocyst Protein Sec8 in Adipocytes" (2009). *Open Dartmouth: Faculty Open Access Articles*. 3657.
https://digitalcommons.dartmouth.edu/facoa/3657

This Article is brought to you for free and open access by Dartmouth Digital Commons. It has been accepted for inclusion in Open Dartmouth: Faculty Open Access Articles by an authorized administrator of Dartmouth Digital Commons. For more information, please contact dartmouthdigitalcommons@groups.dartmouth.edu.

Authors
Patrick D. Lyons, Grantley R. Peck, Arminja N. Kettenbach, Scott A. Gerber, Liya Roudaia, and Gustav E. Lienhard

This article is available at Dartmouth Digital Commons: https://digitalcommons.dartmouth.edu/facoa/3657

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Biosci Rep. Author manuscript; available in PMC 2015 September 16.

Published in final edited form as:

Biosci Rep. 2009 August ; 29(4): 229–235. doi:10.1042/BSR20080162.

Insulin Stimulates the Phosphorylation of the Exocyst Protein Sec8 in Adipocytes

Patrick D. Lyons[*], **Grantley R. Peck**[*], **Arminja N. Kettenbach**[†], **Scott A. Gerber**[†], **Liya Roudaia**[*], and **Gustav E. Lienhard**[*]

[*]Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755 USA

[†]Department of Genetics and Norris Cotton Cancer Center, Dartmouth Medical School, Lebanon, New Hampshire 03756, USA

Synopsis

The signal transduction pathway leading from the insulin receptor to stimulate the fusion of vesicles containing the glucose transporter GLUT4 with the plasma membrane in adipocytes and muscle cells is not completely understood. Current evidence suggests that in addition to the Rab GTPase activating protein AS160, at least one other substrate of the protein kinase Akt, which is as yet unidentified, is required. Sec8 is a component of the exocyst complex that has been previously implicated in GLUT4 trafficking. In this study we report that insulin stimulates the phosphorylation of Sec8 on Ser32 in 3T3-L1 adipocytes. Based upon the sequence around Ser32 and the finding that phosphorylation is inhibited by the phosphatidylinositol 3-kinase inhibitor wortmannin, it is likely that Akt is the kinase for Ser32. We examined the possible role of Ser32 phosphorylation in the insulin-stimulated trafficking of GLUT4, as well as the transferrin receptor, to the plasma membrane by determining the effects of overexpression of the nonphosphorylatable S32A mutant of Sec8 and the phosphomimetic S32E mutant of Sec8. Substantial overexpression of both mutants had no effect on the amount of GLUT4 or transferrin receptor at the cell surface in either the untreated or insulin-treated states. These results indicate that insulin-stimulated phosphorylation of Sec8 is not part of the mechanism by which insulin enhances the fusion of vesicles with the plasma membrane.

Keywords

Akt; exocyst; GLUT4; insulin; Sec8; transferrin receptor

Introduction

Insulin rapidly stimulates the uptake of glucose from the blood into fat and muscle cells. The basis of this effect is a rapid increase in the amount of the glucose transporter GLUT4 at the cell surface as a result of its trafficking from intracellular sites. This process is referred to as insulin-stimulated GLUT4 translocation. Insulin achieves this effect primarily by stimulating the movement of intracellular vesicles containing GLUT4 to the plasma

Address correspondence to: Dr. Lienhard, tel 603-650-1627; fax 603-650-1128; gustav.e.lienhard@dartmouth.edu.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

membrane and the fusion of these vesicles therewith [1,2]. A major signaling pathway for GLUT4 translocation proceeds from the insulin receptor to the activation of phosphatidylinositol 3-kinase and subsequent activation of the protein kinase Akt [1,2]. In fact, a recent study by James and associates showed that the rapid, direct activation of Akt in 3T3-L1 adipocytes caused as large an increase in the amount of GLUT4 at the cell surface as does insulin [3]. This finding thus suggests that the only insulin signaling pathway needed to be activated to cause GLUT4 translocation is the one resulting in activation of Akt.

These considerations indicate that there are one or more Akt substrates that link insulin signaling to GLUT4 translocation. One such Akt substrate is the Rab GTPase activating protein (GAP) AS160 (also known as Tbc1d4). There is considerable evidence for the following scheme: in unstimulated adipocytes and muscle cells AS160 maintains one or more Rabs in their inactive GDP form; in response to insulin Akt phosphorylates AS160 and thereby suppresses its GAP activity; this reduction in GAP activity leads to the elevation of the active GTP form of the Rab(s), which in turn triggers GLUT4 translocation, since activated Rabs function in vesicle movement and in tethering prior to fusion [1,2,4]. However, several findings indicate that AS160 is not the only Akt substrate that participates in GLUT4 translocation. First, knockdown of AS160 in 3T3-L1 adipocytes caused only 30 % of the increase in cell surface GLUT4 as that caused by insulin [5]. If activation of Rab(s) were the only trigger for GLUT4 translocation, then the deletion of the Rab GAP would be expected to cause full GLUT4 translocation. Second, a study of the kinetics of the docking and fusion of GLUT4 vesicles in 3T3-L1 adipocytes by TIRF microscopy found that a nonphosphorylatable mutant of AS160 inhibited the tethering of GLUT4 vesicles to the plasma membrane, but did not inhibit the increased rate of fusion of tethered vesicles caused by insulin [6]. This study thus suggests that while phosphorylation of AS160 enhances the rate at which GLUT4 vesicles tether to the plasma membrane, phosphorylation of at least one other Akt substrate, as yet identified, is required to stimulate the fusion of the tethered vesicles.

The present study describes one such potential Akt substrate, the exocyst component Sec8 (also known as EXOC4). The exocyst is an octameric complex that participates in vesicle tethering that leads on to vesicle fusion [7]. Considerable evidence has implicated the exocyst, including Sec8, as part of the machinery for GLUT4 translocation (see the Discussion). We show that insulin stimulates the phosphorylation of Sec8 on Ser32, and we examine the role of this phosphorylation in GLUT4 translocation.

Experimental

Plasmids

Mouse Sec8 with a carboxy terminal Flag tag in the pShuttle-CMV vector (MP Biomedicals, Solon, OH) was a gift from Dr. Gwyn Gould (University of Glasgow, Glasgow, Scotland). Rat Sec8 in the pcDNA3.1 vector (Invitrogen, Carlsberg, CA) was a gift of Dr. Richard Scheller (Genentech, San Francisco, CA). Point mutations in Sec8 were introduced through the use of the QuickChangeIIXL site-directed mutagenesis kit from Stratagene (La Jolla, CA) and verified by DNA sequencing.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Cell culture, immunoprecipitation, and immunoblotting

3T3-L1 fibroblasts were maintained in culture and differentiated into adipocytes as previously described [8]. In some experiments 10-cm plates on day 4 of differentiation were transfected by electroporation with 100 μg of the Flag-tagged Sec8 plasmid or the S32A mutant thereof, or were cotransfected by electroporation with 100 μg of the wild type, S32A, or S32E Sec8 plasmid and 75 μg of HA-GLUT4-GFP plasmid, as described in [8], and were used 24 h later. Before use cells were serum-starved for 2 h. Cells used for immunoblotting were washed with PBS and solubilized in SDS sample buffer, and the concentration of protein was determined by the precipitating Lowry assay [9]. For the immunoprecipitation of Sec8, cells from a 10-cm plate were scraped into 0.6 ml of 40 mg/ml SDS, 10 mM dithiothreitol, 300 mM NaCl, 100 mM Hepes, pH 7.5 with protease inhibitors (10 μM leupeptin, 10 μM EP475, 1 μM pepstatin, 10 μg/ml aprotinin). The lysate was held at 100° for 5 min and cooled, and the sulfhydryl groups alkylated by the addition of 25 mM N-ethylmaleimide from a 300 mM stock. The SDS lysate was mixed with 4 ml 25 mg/ml C12E9, 150 mM NaCl, 50 mM HEPES, pH 7.5, to give an SDS/C12E9 lysate in which the C12E9 nonionic detergent was in 4-fold excess by weight over SDS. The lysate was centrifuged at 18,000 rpm for 15 min. The supernatant was either immunoadsorbed on anti-Flag conjugated to agarose (A2220, Sigma, St. Louis, MO) or with mouse monoclonal antibody against recombinant rat Sec8 (VAM-SV016, Assay Designs Inc., Ann Arbor, MI) followed by protein G-Sepharose for 16 h at 4°. The immunoadsorbates were washed three times with 5 mg/ml C12E9, 150 mM NaCl, 50 mM Hepes, pH 7.5, and solubilized in SDS sample buffer. For immunoblotting, SDS samples of cells or of the immunoprecipitates were separated by SDS PAGE and immunoblotted with either a 1:1 mixture of two phospho-Akt-substrate antibodies (9611 and 9614, Cell Signaling Technology, Beverly, MA) (referred to as the PAS antibody), anti-Flag conjugated to horseradish peroxidase (A8592, Sigma, St. Louis, MO), or the mouse monoclonal antibody against Sec8, as described previously [8]. For mass spectrometry, the SDS samples of the Sec8 immunoprecipitates were separated by SDS PAGE and stained with coomassie blue, and the Sec8 band was excised from the gel.

Mass spectrometry

Sec8 was isolated from basal and insulin-treated (160 nM for 10 min) 3T3-L1 adipocytes by immunoadsorption, as described above. The gel slices containing Sec8 were subjected to in-gel digestion with trypsin, and the peptides were analyzed by microcapillary LC-MS/MS on an LTQ Orbitrap mass spectrometer. For one estimate of the insulin effect on the stoichiometry of phosphorylation, the tryptic peptides from the gel digests were divided in half. The samples were dried by vacuum centrifugation, and then treated either with calf intestinal phosphatase (New England Biolabs, Ipswitch, MA) or with buffer alone. The samples were then desalted on OASIS HLB μElution plates (Waters, Milford, MA) and subjected to LTQ Orbitrap mass spectrometry. The extracted ion chromatographic peak areas of the phospho and cognate nonphospho Ser32 peptides in each sample were calculated using instrument software. The absence of the tryptic peptides containing phospho Ser32 from the phosphatase-treated sample established that the phosphatase treatment was complete.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Assay for cell surface GLUT4

The relative amount of GLUT4 at the cell surface in basal and insulin-stimulated adipocytes was measured through the use of the reporter construct of GLUT4 with an HA epitope tag in the first extracellular loop and GFP fused to its carboxy terminus (HA-GLUT4-GFP). The use of this construct has been described in detail previously [8]. In this method 10-cm plates of adipocytes on day 4 of differentiation were co-transfected by electroporation with the plasmids for HA-GLUT4-GFP and Sec8, as described above, and distributed into four 35-mm wells. Twenty-four hours later the cells were serum-starved for 2 h and then treated with 160 nM insulin for 30 min or left unstimulated. Previously, in this method the cells were fixed with formaldehyde and labeled with antibody against HA and Cy3-conjugated secondary antibody, and then fluorescence images of individual cells were obtained, and the Cy3 and GFP fluorescence intensities of each cell were quantitated. In the current study, we changed the procedure for analysis. We used a slight adaptation of the flow cytometry procedure that was recently developed by Muretta et al. to quantitate the relative amount of HA-GLUT4 at the cell surface in 3T3-L1 adipocytes [10]. After insulin treatment, the 35-mm wells of cells were washed with cold PBS containing 10 mg per ml BSA (BSA/PBS), and then incubated with 0.6 ml of 5 μg/ml monoclonal mouse antibody against HA (16B12, Covance, Princeton, NJ) in BSA/PBS at 4° for 1 h. The wells were washed twice with cold BSA/PBS for 5 min and then incubated with 0.6 ml of 3 μg per ml APC-conjugated donkey anti-mouse antibody (17-4012-82, eBioscience, San Diego, CA) for 1 h at 4°. The labeled cells were washed twice with cold BSA/PBS and released from the plate by incubation with 1 mg/ml collagenase (C6885-1G, Sigma, St. Louis, MO) in PBS at 4° for 30 min. The cells were then analyzed on a Becton Dickinson FACSCalibur flow cytometer using the CellQuest™ Pro software (version 5.2). Data were subsequently analyzed using the FlowJo software (version 8.6.3). Cells were first gated to separate adipocytes from undifferentiated fibroblasts and debris, and then the GFP-positive adipocytes were gated to separate them from untransfected adipocytes. The ratio of APC signal to GFP for each cell was obtained. This ratio is a measure of the relative amount of HA-GLUT4-GFP at the cell surface normalized to the total level of its expression. For each condition approximately 4000 cells were analyzed, and the average ratio value was taken. Each value was corrected for nonspecific binding of the secondary antibody by subtracting the value obtained from a 35-mm well in which incubation with the primary antibody was omitted. In order to compare the values from separate experiments, the corrected values within each experiment were normalized to 1.0 for the vector control in the insulin-stimulated state.

In order to determine the efficiency of the electroporation transfection procedure and the extent to which co-transfection occurred, we transfected adipocytes on day 4 of differentiation with a mixture of 100 μg Flag-tagged Sec8 plasmid and 75 μg HA-GLUT4-GFP plasmid and fixed the cells after 24 h with 4 % formaldehyde. The fixed cells were permeabilized with 0.2 % saponin, and then labeled with monoclonal mouse antibody against Flag and then with a secondary Cy3-conjugated goat anti-mouse antibody, as described in [8]. The cells were examined by immunofluorescence microscopy. Among 500 cells examined, 19 % were positive for Flag and 18 % were positive for GFP. Among the GFP-positive cells, 74 % also were positive for Flag.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Assay for cell surface transferrin receptor

Adipocytes on day 4 of differentiation were co-transfected with the plasmids for HA-GLUT4-GFP and Sec8, as described above. In this case, the HA-GLUT4-GFP served as a marker for cells that were co-transfected. Twenty-four hours later the cells were serum-starved for 2 h and then treated with 160 nM insulin or not for 15 min, which is the time period required for full insulin-stimulated increase in cell surface TfR [11]. Subsequently, the cells were labeled with a monoclonal rat antibody against the mouse TfR antibody (14-0711-82, eBioscience, San Diego, CA), followed by an APC-conjugated donkey anti-rat antibody (17-4822-82, eBioscience, San Diego, CA), and analyzed by flow cytometry, as described above for the assay of cell surface HA-GLUT4-GFP. The average value of the APC signal for the GFP-positive cells provided a measure of the relative amount of endogenous TfR at the cell surface in the cells expressing ectopic Sec8. Each value was corrected for nonspecific binding of the secondary antibody as described above, and the corrected values were normalized to 1.0 for the vector control in the insulin-stimulated state.

Results

Insulin-stimulated Phosphorylation of Sec8

As part of a phosphoproteomic analysis of insulin-treated 3T3-L1 adipocytes, a phosphopeptide from Sec8 was identified by mass spectrometry (private communication from Dr. Ailan Guo, Cell Signaling Technology). The site of phosphorylation was Ser32, which is located within the sequence VIRTLpST.

In order to determine whether Sec8 was phosphorylated in response to insulin, we immunoprecipitated Sec8 from lysates of basal and insulin-treated 3T3-L1 adipocytes and then immunoblotted the isolated Sec8 with antibody against the phosphomotif generated by Akt phosphorylation (PAS antibody). Insulin treatment led to an approximately 4-fold increase in Sec8 phosphorylation as detected with the PAS antibody (Fig. 1, upper panel, compare lanes 1 and 3). Since the activation of Akt is downstream of PI3K, we determined the effect of inhibition of PI3K with the compound wortmannin on the phosphorylation of Sec8. Phosphorylation was markedly inhibited (Fig. 1, upper panel, compare lanes 3 and 6). Immunoblotting for Sec8 showed that the amount in the immunoprecipitates was the same (Fig. 1, lower panel).

In order to establish that Ser32 was the phosphosite reacting with the PAS antibody, we expressed wild-type Sec8 with a carboxy terminal Flag tag and the corresponding S32A mutant in 3T3-L1 adipocytes, isolated the Sec8-Flag from lysates of basal and insulin-treated cells, and immunoblotted the Sec8 with the PAS antibody. As expected, the wild-type Sec8 reacted with the PAS antibody and insulin treatment increased its reactivity (Fig. 2, upper panel, lanes 3 and 4). On the other hand, no reaction with the PAS antibody was found with the S32A mutant Sec8-Flag. The recovery of wild-type and S32A Sec8-Flag was approximately the same, as assessed by an anti-Flag immunoblot (Fig. 2, lower panel). The Flag blot showed a doublet, with the upper band of the expected size for Sec8 and a lower band approximately 10 kDa smaller than the 110 kDa Sec8. Most likely the lower band is a proteolytic cleavage product of Sec8 that is missing the amino terminus. It retained the

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

carboxy terminal Flag tag, and probably lacked the amino terminus, since it showed no phosphorylation of amino terminal Ser32 in the PAS immunoblot (Fig. 2, upper panel).

We have also estimated the extent of insulin-stimulated phosphorylation of Ser32 and the stoichiometry of phosphorylation by mass spectrometry. Sec8 was isolated from SDS/C12E9 lysates of basal and insulin-treated 3T3-L1 adipocytes by immunoprecipitation with antibody against Sec8 followed by SDS PAGE. For both conditions the preparation was carried out with five 10-cm plates and yielded approximately 500 ng of purified Sec8, as assessed by the relative intensity of the Sec8 band compared to that of known amounts of standard proteins upon staining of the gel with coomassie blue (data not shown). The gel slices containing Sec8 were digested with trypsin, and the tryptic peptides that contained Ser32 and phospho Ser32 were detected by mass spectrometry. Because of partial cleavage, the tryptic peptides found were TLSTSSDDVEDR, TLSTSSDDVEDRENEK, and TLSTSSDDVEDRENEKGR and the corresponding three peptides with phosphorylation at Ser32 (TLpS..). The sum of the ion intensities for three Ser32 phosphopeptides derived from insulin-treated cells was 3.6 times larger than that for these phosphopeptides derived from the basal cells. The ratio of the ion intensities for the three individual phosphopeptides in the two states ranged from 3.4 to 4.4. This method confirmed the insulin-stimulated increase in Ser32 phosphorylation of approximately 4-fold that was seen by immunoblotting with the PAS antibody (Fig. 1).

The stoichiometry of Ser32 phosphorylation on Sec8 in the insulin state was estimated in two ways. In the first method, we calculated the ratio of the total ion intensities of the three phospho Ser32 peptides to the total ion intensities for all six peptides (phosphorylated peptides and their nonphosphorylated cognates). This method gave a value of 7.6%. A caveat of this method is that it assumes the ionization behavior and sample handling precision of the phospho and nonphospho cognate peptides in analysis by LC-MS/MS is approximately the same. In the second method, we split the tryptic digest in half, dephosphorylated the peptides in one half by treatment with calf intestinal phosphatase, and then determined the sum of the ion intensities of the nonphosphorylated Ser32 peptides in both samples. In this method, the total amount of Sec8 Ser32 peptides is given by the sum of the ion intensities of these nonphosphorylated peptides in the phosphatase-treated sample, the amount of nonphosphorylated Sec32 peptides in the original sample is given by the sum of the ion intensities of the nonphosphorylated Sec32 peptides in sample without phosphatase, and thus the amount of phosphorylated Ser32 peptides is given by the difference between the two sums. Consequently, this method allows estimation of the percent phosphorylation solely from the ion intensities of the nonphosphorylated peptides, and so does not depend upon the assumption that the phosphorylated and nonphosphorylated peptides behave the same in the MS analysis. By this method the percentage of Sec8 with phospho Ser32 in the insulin state was 8.8 %. Thus, both methods gave similar values, and the stoichiometry of Sec8 phosphorylation in response to insulin treatment was relatively low.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Effect of Sec8 Phosphorylation on GLUT4 Translocation

In order to determine whether Sec8 phosphorylation participates in insulin-stimulated GLUT4 translocation, we examined the effects of overexpression of the nonphosphorylatable S32A mutant and the phosphomimetic S32E mutant of Sec8, as well as that of wild-type Sec8. Our rationale was that substitution of the S32A Sec8 for wild-type Sec8 in the exocyst would inhibit insulin-stimulated translocation if phosphorylation of Sec8 were required for this process, whereas substitution of the S32E Sec8 mutant might elicit GLUT4 translocation in the absence of insulin. For this analysis we used untagged forms of Sec8, since an epitope tag at the amino terminus could potentially interfere with the effect of phosphorylation, which occurs near the amino terminus, and an epitope tag at the carboxy terminus would disrupt the PDZ domain binding motif present at the carboxy terminus of Sec8 [12]. Sec8 binds to the PDZ domain of SAP97 via this motif in 3T3-L1 adipocytes [12]. Figure 3A shows the effects of overexpressing these mutant forms of Sec8, as well as wild-type Sec8, on cell surface GLUT4 in 3T3-L1 adipocytes. In comparison to the vector control, none of the forms of Sec8 had any significant effect on amount of GLUT4 at the cell surface in either the basal or insulin state. As expected from previous studies [8], insulin caused a 7-fold increase in the amount of GLUT4 at the cell surface.

We used two methods to estimate the extent of overexpression of the various forms of Sec8 under the same conditions as those employed for the assay of cell surface GLUT4. First, SDS samples of a portion of the transfected cells used for the assay of cell surface GLUT4 were immunoblotted for Sec8. The cells transfected with the Sec8 forms showed increased amounts of Sec8 relative to the vector control (Fig 3B). In addition smaller amounts of the 100 kDa proteolytic fragment of Sec8 previously seen with expression of the Flag-tagged Sec8 (Fig. 2) were also present. The total amounts of Sec8, relative to the vector control taken as 1, were 1.8, 1.6, and 3.4 for the cells transfected with wild-type, S32A, and S32E Sec8, respectively, as measured by densitometry (see Fig 3B legend). Thus, the relative amounts of the ectopic wild-type, S32A, and S32E Sec8, compared to a value of 1 for the endogenous Sec8, were 0.8, 0.6 and 2.4, respectively. Since only 20 % of the cells was actually transfected with the Sec8 plasmids (see Experimental), the relative amounts of the ectopic Sec8 in the transfected cells themselves were 5 times higher. Hence, the ratios of ectopic wild-type, S32A, and S32E Sec8 to endogenous Sec8 in the transfected cells were 4.0, 3.0, and 12 to 1.

The second method that we used to estimate the extent of overexpression of Sec8 in the transfected cells employed mass spectrometry. Adipocytes were transfected with S32A or S32E Sec8 and HA-GLUT4-GFP to mimic the conditions for measurement of cell surface GLUT4. Then the Sec8 was isolated by immunoprecipitation and SDS PAGE from SDS/C12E9 lysates of basal cells, digested with trypsin, and the amounts of the tryptic peptides containing Ser32 and Ala32 or Ser32 and Glu32 in each digest were measured by mass spectrometry (see above). By this approach, the ratio of S32A Sec8 to wild-type Sec8 was 0.5 to 1, and the ratio of S32E to wild-type Sec8 was 2.3 to 1. Upon correction of these values for the fact that the mutant Sec8 was expressed in only 20 % of the cells, the ratio of ectopic S23A and S23E Sec8 to wild-type Sec8 in the transfected cells were 2.5 and 11.5 to

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

1, respectively. Thus, the two methods give almost the same values for the extent to which the mutant forms of Sec8 were overexpressed relative to the endogenous wild-type.

Effect of Sec8 Phosphorylation on Transferrin Receptor Translocation

Insulin is known to increase the amount of the transferrin receptor (TfR) at the cell surface in 3T3-L1 adipocytes by approximately two-fold [11]. The basis of this effect is a two-fold increase in the rate constant for exocytosis of TfR-containing vesicles [11]. Moreover, the exocyst complex appears to act in the trafficking of the TfR, since deletion of the Sec15 component of the exocyst as the result of a mouse mutation markedly inhibits the rate of exocytosis of the TfR receptor in reticulocytes [13]. Consequently, we investigated whether the insulin-elicited phosphorylation of Sec8 might participate in the insulin effect on the trafficking of the TfR. In identical fashion to the assay for cell surface GLUT4, we transfected 3T3-L1 adipocytes with wild-type, S32A, and S32E Sec8 together with HA-GLUT4-GFP. In this case the HA-GLUT4-GFP served as a marker for cells that were co-transfected with Sec8 plasmids. The relative amount of endogenous TfR at the cell surface in cells expressing GFP was then measured by flow cytometry assay. Overexpression of wild-type, S32A, and S32E Sec8 had no significant effect on cell surface TfR in either the basal or insulin-stimulated state compared to the vector control (Fig. 4). As expected, insulin treatment caused a 2.3-fold increase in cell surface TfR.

Discussion

Our results clearly demonstrate that insulin stimulated the phosphorylation of Sec8 on Ser32 in 3T3-L1 adipocytes. The finding that the PI3K inhibitor wortmannin inhibited the phosphorylation shows that the kinase that accounts for this phosphorylation is downstream of PI3K. The established motif for Akt phosphorylation is RXRXXS/T [14], whereas the sequence surrounding phospho Ser32 is VIRTLpST, and hence lacks the Arg at the −5 position found in fully established Akt phosphorylation sites. Nevertheless, Akt may be the kinase that phosphorylates Ser32, especially since Akt is known to prefer Thr at the −2 position [14]. Alternatively, it is possible that another basophilic kinase downstream of PI3K, such as protein kinase C λ or ζ [14, 15], is responsible for the phosphorylation. The stoichiometry of phosphorylation was about 8 %. While this degree of phosphorylation is low, it is sufficient that a functional effect might be expected.

The exocyst and the Sec8 component thereof have been implicated in insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes. Partial knockdown of Sec8 and two other exocyst components, Sec6 and Exo70, partially inhibited insulin-stimulated glucose transport in these cells [12]. GLUT4 vesicles from 3T3-L1 adipocytes contain Rabs 8, 10, and 11 and RalA [16, 17]. Rabs 8 and 11 in the GTP form are known to interact with the Sec15 component of the exocyst [18], and Rab 10 closely resembles Rab 8. Ral A in its GTP form is also known to interact with the Sec5 and Exo84 components of the exocyst [7, 17]. Insulin treatment of 3T3-L1 adipocytes causes increased localization of Sec8 and Sec6 at the plasma membrane [19, 20]. These findings suggest that the tethering of GLUT4 vesicles to the plasma membrane occurs via the exocyst. Thus, it seemed plausible that insulin-stimulated phosphorylation of the exocyst might contribute to the insulin-increased rate of

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

GLUT4 vesicle fusion with the plasma membrane (see Introduction) and hence to the increase in cell surface GLUT4.

We took two approaches to examine the question of the potential regulatory role of Sec8 phosphorylation. First, we determined the effect of overexpression of the nonphosphorylatable S32A mutant of Sec8 on cell surface GLUT4. The approximately 3-fold overexpression of this mutant, relative to the endogenous wild-type Sec8, had no effect on the amount of cell surface GLUT4 in the insulin state. If phosphorylation of Sec8 stimulated GLUT4 vesicle fusion, we would have expected to see substantial inhibition of the amount of GLUT4 at the cell surface in the insulin state. With the 3-fold overexpression of this nonphosphorylatable mutant, three fourths of the exocyst complexes participating in GLUT4 vesicle tethering would be expected to contain the nonphosphorylatable S32A mutant. Hence, in this situation any stimulatory effect of phosphorylation should be reduced by approximately 75 %. Second, we determined the effect of overexpression of the S32E mutant of Sec8, in which the negatively charged Glu side chain mimics the negatively charged phosphoryl group of Sec8 phosphorylated on Ser32. This approach assumes that the glutamate side chain is a functional mimic of the phosphoryl group; this assumption has proven to be the case for some, but not all phosphorylations. Overexpression of S32E by approximately 12-fold relative to the endogenous Sec8 had no effect on the amount of cell surface GLUT4 in the insulin state. In this case, if phosphorylation stimulated GLUT4 vesicle fusion, we might have expected to find an increase in cell surface GLUT4 in the basal state. The reason is that with 12-fold overexpression of this mutant, almost all the assembled exocyst complexes would be expected to contain the S32E form of Sec8, and hence the stimulatory effect of phosphorylation would be evident in the absence of insulin. Both these approaches thus indicate that the phosphorylation of Sec8 does not increase the rate at which GLUT4 vesicles fuse with the plasma membrane. It is interesting to note that the overexpression obtained with the S32E mutant was 3-fold greater than that obtained with the wild-type Sec8 and 4-fold greater than that with the S32A mutant. This result suggests that the presence of a negatively charged side chain at position 32 reduces the rate the degradation of Sec8.

We also examined the effect of Sec8 phosphorylation on insulin-stimulated trafficking of the TfR to the plasma membrane, since the exocyst has also been implicated in the recycling of the TfR from the endosomes to the plasma membrane. Hereto, it seemed plausible that phosphorylation of the exocyst might stimulate either tethering to, or fusion, of endosome-derived TfR vesicles with the plasma membrane and thereby account for the insulin-elicited two-fold increase in the rate constant for TfR exocytosis [11]. The stimulatory effect of insulin on TfR trafficking differs from that of insulin on GLUT4 trafficking, since the former involves the fusion of endosomal-derived vesicles with the plasma membrane and the latter involves the fusion of specialized GLUT4 vesicles with the plasma membrane [1]. Thus, the absence of an effect of the phosphorylation of Sec8 on GLUT4 trafficking was not evidence against an effect on TfR trafficking. However, the results with the Sec8 mutants indicate that phosphorylation of Sec8 also does not regulate the insulin stimulation of TfR recycling to the plasma membrane.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Acknowledgments

We are indebted to Dr. Ailan Guo at Cell Signaling Technology for the initial finding of Sec8 phosphorylation and to Drs. Joe Muretta and Cynthia Mastick at the University of Nevada for assistance in flow cytometry assays. This research was supported by National Institutes of Health grants DK25336 and DK42816 to G.E.L and RR018787 to S.A.G.

Abbreviations

C12E9	nonaethyleneglycol dodecyl ether
GAP	GTPase activating protein
PI3K	phosphatidylinositol 3-kinase
TfR	transferrin receptor

References

1. Huang S, Czech MP. The GLUT4 glucose transporter. Cell Metab. 2007; 5:237–252. [PubMed: 17403369]
2. Zaid H, Antonescu CN, Randhawa VK, Klip A. Insulin action on glucose transporters through molecular switches, tracks, and tethers. Biochem J. 2008; 413:201–215. [PubMed: 18570632]
3. Ng Y, Ramm G, Lopez JA, James DE. Rapid activation of Akt2 is sufficient to stimulate GLUT4 translocation in 3T3-L1 adipocytes. Cell Metab. 2008; 7:348–356. [PubMed: 18396141]
4. Sakamoto K, Holman GD. Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. Am J Physiol Endocrinol Metab. 2008; 295:E29–37. [PubMed: 18477703]
5. Equez L, Lee A, Chavez JA, Miinea CP, Kane S, Lienhard GE, McGraw TE. Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein. Cell Metab. 2005; 2:263–272. [PubMed: 16213228]
6. Jiang L, Fan J, Bai L, Wang Y, Chen Y, Yang L, Chen L, Xu T. Direct quantification of fusion rate reveals a distal role for AS160 in insulin-stimulated fusion of GLUT4 storage vesicles. J Biol Chem. 2007; 283:8508–8516. [PubMed: 18063571]
7. Wu H, Rossi G, Brennwald P. The ghost in the machine: small GTPases as spatial regulators of exocytosis. Trends Cell Biol. 2008; 18:397–404. [PubMed: 18706813]
8. Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, Lienhard GE. Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. J Biol Chem. 2003; 278:14599–14602. [PubMed: 12637568]
9. Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem. 1977; 83:346–356. [PubMed: 603028]
10. Muretta JM, Romenskaia I, Mastick CC. Insulin releases Glut4 from static storage compartments into cycling endosomes and increases the rate constant for Glut4 exocytosis. J Biol Chem. 2008; 283:311–323. [PubMed: 17967900]
11. Tanner LI, Lienhard GE. Insulin elicits a redistribution of the transferrin receptor in 3T3-L1 adipocytes through an increase in the rate constant for receptor externalization. J Biol Chem. 1987; 262:8975–8980. [PubMed: 3298247]
12. Inoue M, Chiang SH, Chang L, Chen XW, Saltiel AR. Compartmentalization of the exocyst complex in lipid rafts controls Glut4 vesicle tethering. Mol Biol Cell. 2006; 17:2303–2311. [PubMed: 16525015]
13. Garrick MD, Garrick LM. Loss of rapid transferrin receptor recycling due to a mutation in Sec15l1 in hbd mice. Biochim Biophys Acta. 2006; 1773:105–108. [PubMed: 17087999]
14. Obata T, Yaffe MB, Leparc GG, Piro ET, Maegawa H, Kashiwagi A, Kikkawa R, Cantley CC. Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. J Biol Chem. 2000; 275:36108–36115. [PubMed: 10945990]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

15. Chou MM, Hou W, Johnson J, Graham LK, Lee MH, Chen CS, Newton AC, Schaffhausen BS, Toker A. Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. Curr Biol. 1998; 8:1069–1077. [PubMed: 9768361]
16. Miinea CP, Sano H, Kane S, Sano E, Fukuda M, Peranen J, Lane WS, Lienhard GE. AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. Biochem J. 2005; 391:87–93. [PubMed: 15971998]
17. Chen XW, Leto D, Chiang SH, Wang Q, Saltiel AR. Activation of RalA is required for insulin-stimulated GLUT4 trafficking to the plasma membrane via the exocyst and the motor protein Myo1c. Devel Cell. 2007; 13:391–404. [PubMed: 17765682]
18. Wu S, Mehta SQ, Pichaud F, Bellen HJ, Quiocho F. Sec15 interacts with Rab11 via a novel domain and affects Rab11 localization in vivo. Nat Struct Mol Biol. 2005; 12:879–885. [PubMed: 16155582]
19. Inoue M, Chang L, Hwang J, Chiang SH, Saltiel AR. The exocyst complex is required for targeting of Glut4 to the plasma membrane by insulin. Nature. 2003; 422:629–633. [PubMed: 12687004]
20. Ewart M, Clarke M, Kane S, Chamberlain LH, Gould GW. Evidence for a role of the exocyst in insulin-stimulated Glut4 trafficking in 3T3-L1 adipocytes. J Biol Chem. 2005; 280:3812–3816. [PubMed: 15550383]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

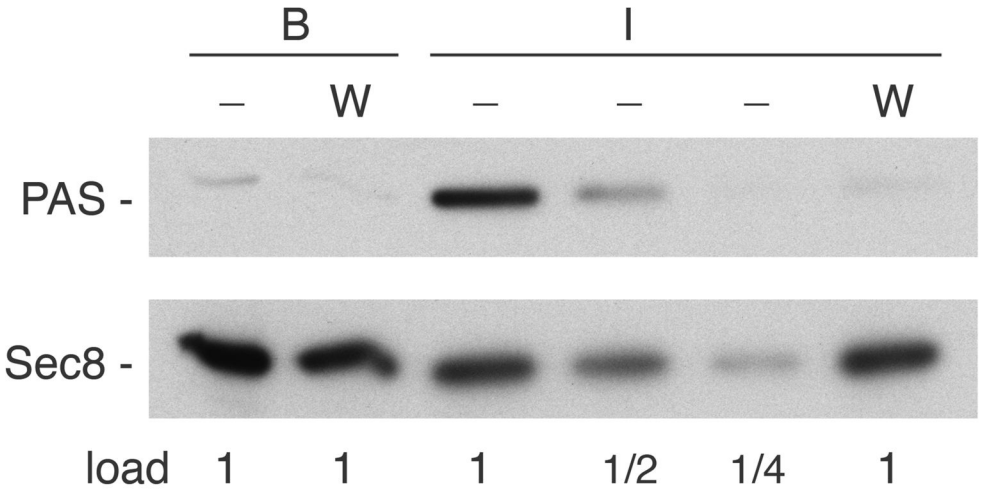

Figure 1. Insulin-stimulated phosphorylation of Sec8

3T3-L1 adipocytes were left untreated (B) or treated with either 200 nM wortmannin for 45 min (W) for or 160 nM insulin for 10 min (I), or both. SDS/C12E8 lysates were prepared, and Sec8 was immunoprecipitated. The Sec8 immune complexes were immunoblotted with antibody against the phosphomotif typical of Akt substrates (PAS) (*top panel)* and with antibody against Sec8 (*bottom panel)*. The 1X loads were derived from 20% and 0.25% of a 10-cm plate for the PAS and Sec8 blots, respectively. A replicate of this experiment gave similar results.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

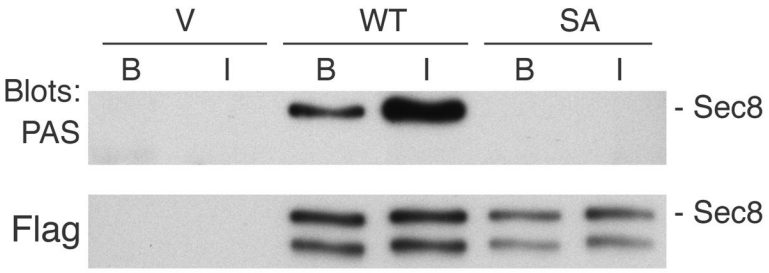

Figure 2. Identification of serine-32 in Sec8 as a site of insulin-stimulated phosphorylation

3T3-L1 adipocytes were transfected with vector (V), wild-type Sec8-Flag (WT) or S32A-Sec8-Flag (SA) and either left untreated (B) or treated with 160 nM insulin for 10 min (I). SDS/C12E9 lysates were prepared and immunoprecipitated with anti-Flag. The immune complexes were immunoblotted with the PAS antibody *(top panel)* or Flag antibody *(bottom panel)*. The loads for the PAS and Flag blots were derived from 10 and 5 % of a 10-cm plate, respectively. A replicate of this experiment gave similar results.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

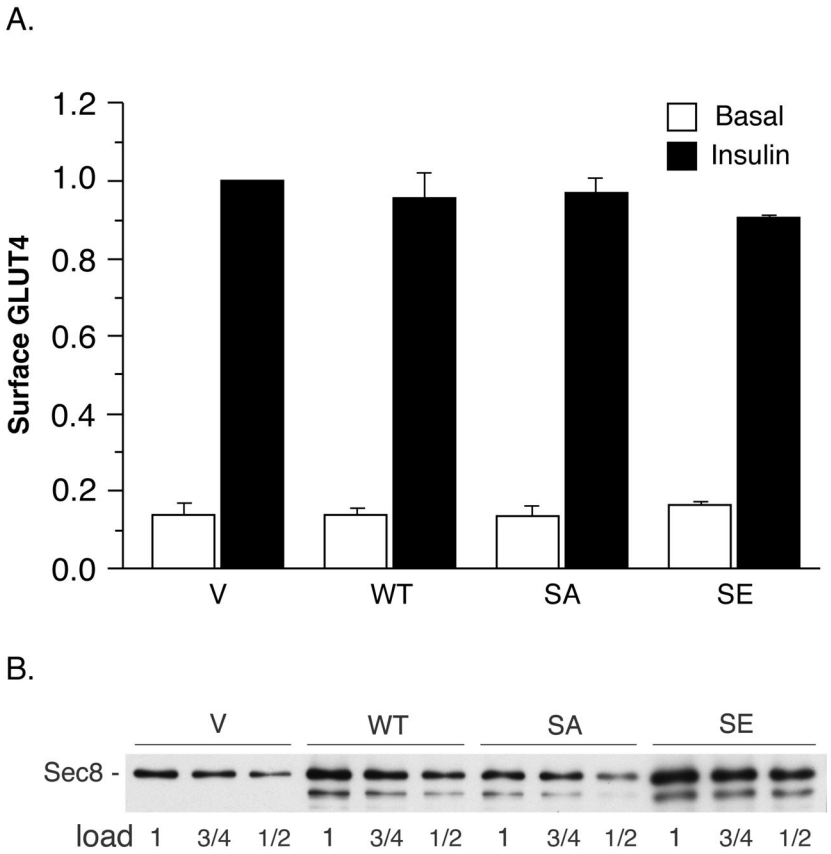

Figure 3. Effect of Sec8 phosphorylation on GLUT4 translocation

Part A. 3T3-L1 adipocytes were transfected with HA-GLUT4-GFP and either vector (V), wild-type Sec8 (WT), S32A-Sec8 (SA), or S32E-Sec8 (SE). The relative amount of HA-GLUT4-GFP at the cell surface in basal and insulin-stimulated 3T3-L1 adipocytes was measured by flow cytometry, as described in the Experimental. The values are the averages +/− S.E.M from four separate experiments with the exception of those for the SE mutant. The latter values are the averages +/− the range from two experiments. Part B. SDS samples of total cell lysates were prepared from a portion of the cells from an experiment in Part A and immunoblotted for Sec8. The 1X load corresponds to 1.0 μg of total protein. A replicate done with SDS samples from another separate experiment in Part A showed similar results. The relative amounts of Sec8 in the SDS samples from the two experiments were measured by scanning densitometry of the immunoblot film, and found to be 1.8 +/− 0.0, 1.6 +/− 0.2, and 3.4 +/− 0.2 (average value +/− the range) for the WT, SA, and SE samples, relative to the V sample.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

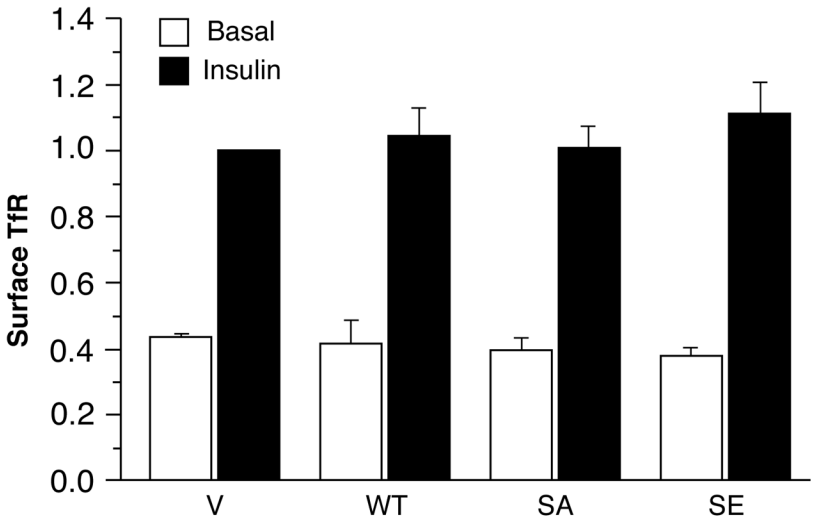

Figure 4. Effect of Sec8 phosphorylation on TfR translocation

3T3-L1 adipocytes were transfected with HA-GLUT4-GFP and either vector (V), wild-type Sec8 (WT), S32A-Sec8 (SA), or S32E-Sec8 (SE). The relative amount of TfR at the cell surface in basal and insulin-stimulated 3T3-L1 adipocytes was measured by flow cytometry, as described in Experimental. The values are the averages +/− the range from two separate experiments.