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The amino acids upstream of NH(2)-terminal dileucine motif play a role in regulating the intracellular sorting of the Class III transporters GLUT8 and GLUT12

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Abstract

The transport of glucose across cell membranes is mediated by a family of facilitative glucose transporters (GLUTs). The class III glucose transporters GLUT8 and GLUT12 both contain a similar [DE]XXXL[LI] dileucine sorting signal in their amino terminus. This type of dileucine motif facilitates protein trafficking to various organelles or to the plasma membrane via interactions with adaptor protein (AP) complexes. The [DE]XXXL[LI] motif in GLUT8 is thought to direct it to late endosomal/lysosomal compartments via its interactions with AP1 and AP2. Unlike GLUT8, the [DE]XXXL[LI] motif does not direct GLUT12 to a lysosomal compartment. Rather, GLUT12 resides in the Golgi network and at the plasma membrane. In a previous study we found that exchanging the XXX (TQP) residues in GLUT8 with the corresponding residues in GLUT12 (GPN) resulted in a dramatic missorting of GLUT8 to the cell surface. We postulated that the XXX amino acids upstream of the dileucine motif in GLUT8 influence the degree of interaction between the [DE]XXXL[LI] motif and adaptor proteins. To further explore its trafficking mechanisms, we created mutant constructs to identify the role that each of the individual XXX amino acids has for regulating the intracellular sorting of GLUT8. Here we find that the XXX amino acids, specifically the position of a proline -2 from the dileucine residues, influence the affinity of APs for GLUT8 and GLUT12.

Introduction

Members of the facilitative glucose transporter protein (GLUT) family mediate the transport of hexoses across membranes of mammalian cells. Fourteen isoforms of the GLUT family have been identified based on sequence homology (Joost and Thorens, 2001, Scheepers et al., 2004). These isoforms differ in tissue distribution, subcellular localization, substrate specificity, and kinetics of hexose transport. Based on sequence homology, three classes of GLUTs have been identified: Class I (GLUT-1,-2,-3,-4), Class II (GLUT-5,-7,-9,-11) and Class III (GLUT-6,-8,-10,-12, and HMIT). Class I and Class III glucose transporters mediate the transport of glucose across membranes, while Class II GLUTs facilitate the transport of glucose and/or fructose across the cell surface (Wood and Trayhurn, 2003). The Class III GLUTs differ from other GLUTs in that they contain an N-linked glycosylation site in a large exofacial loop between transmembrane helices 9 and 10 (Joost and Thorens, 2001, Wood and Trayhurn, 2003). Class III GLUTs also contain a dileucine signal at their amino termini (Schmidt et al., 2009).

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Class III glucose transporters are expressed in various tissue types and appear to predominantly localize within intracellular compartments (Schmidt et al., 2009). Therefore, further characterizing the intracellular localization and trafficking of these transporters is key to elucidating a potential role for GLUTs in regulating hexose transport across intracellular membranes. GLUT8 is predominantly expressed in the testis and brain and its localization has been shown in the endoplasmic reticulum, the late endosome, and the lysosome (Gomez et al., 2006, Gomez et al., 2009, Kim and Moley, 2007, Schurmann et al., 2002, Ibberson et al., 2002, Ibberson et al., 2000, Augustin et al., 2005). Several studies have shown that an NH2-terminal dileucine motif directs its intracellular localization to the lysosome (Diril et al., 2009, Augustin et al., 2005). Mutation of these dileucine residues to dialanine redirects the transporter to the plasma membrane, demonstrating that these residues regulate the intracellular localization of GLUT8 (Ibberson et al., 2000, Lisinski et al., 2001). The class III transporter GLUT12 is predominantly expressed in insulin-sensitive tissue such as heart, skeletal muscle, and fat (Wood et al., 2003, Zhou et al., 2004, Stuart et al., 2006, Rogers et al., 2002). It is found both intracellularly at the Golgi network and at the cell surface, and several recent studies suggest that is translocation to the cell surface is a stimulant regulated event (Stuart et al., 2009, Wilson-O'Brien et al., 2008, Flessner and Moley, 2009). As a class III GLUT, GLUT12 also contains a dileucine motif at its amino-termini, and it is important to better characterize the role that this motif plays in the cell surface trafficking of this transporter.

The Class III glucose transporters GLUT8 and GLUT12 both contain a [DE]XXXL[LI] sorting signal in their intracellular amino-termini. Dileucine-based [DE]XXXL[LI] consensus sequences, in which X represents any amino acid, are a type of dileucine signal that interact with adaptor protein (AP) complexes (Robinson and Bonifacino, 2001, Bonifacino and Traub, 2003). AP complexes mediate the sorting of membrane proteins through endocytic and secretory pathways by interacting with dileucine- or tyrosine-based consensus sequences on the cargo protein (Bonifacino and Traub, 2003, Robinson and Bonifacino, 2001). Previous studies using glutathione *S*-transferase (GST) pulldown assays have shown that the dileucine motif in GLUT8 interacts with subunits of AP1 and AP2 complexes (Diril et al., 2009, Schmidt et al., 2006, Doray et al., 2007). The interactions between the dileucine motif in GLUT12 and AP complexes remain uncharacterized.

In our previous studies we have showed that GLUT8 and GLUT12 localize to different cellular compartments despite sharing a si milar [DE]XXXL[LI] consensus sequence in their amino termini (Flessner and Moley, 2009). In addition, GLUT8 and GLUT12 displayed differences in trafficking from the cell surface. While cell surface GLUT8 was internalized and redirected to the late endosome/lysosome (Augustin et al., 2005, Flessner and Moley, 2009), GLUT12 largely associated with the cell surface. To identify whether the differences between the transporters localization and trafficking are affected by the XXX amino acids upstream of the dileucine residues, we created mutant constructs exchanging the XXX residues in GLUT8 and GLUT12. We found that when GLUT8 is mutated to contain the GPN residues found in GLUT12, there is a dramatic resorting of GLUT8-GPN to the cell surface rather than the lysosome. When the XXX residues in GLUT12 were mutated to the TQP residues in GLUT8, there was a decrease in GLUT12-TQP at the cell surface and an increase in a punctate intracellular compartment. This data showed that the XXX amino acids within the [DE]XXXL[LI] consensus sequence play an important role in the steady state localization of GLUT8 and GLUT12. We hypothesized that this was due to differences in the affinity of interaction between the [DE]XXXL[LI] motifs and various adaptin subunits.

In the present study, we aim to further characterize the role that the XXX amino acids play in regulating the steady state localization of GLUT8 and GLUT12. We individually swapped

the amino acids -1, -2, and -3 from the dileucine motif in GLUT8 with the corresponding amino acids in GLUT12. To determine whether specific amino acid residues upstream of the dileucine motif affect the *in vivo* interaction of GLUT8 and AP1, AP2, or AP3, we knocked down AP subunits and examined its affect on the steady state localization of GLUT8 and GLUT8 mutants. We conclude that the position of a proline residue upstream of the dileucine motif impacts the affinity of AP1 and AP2 complexes for the [DE]XXXL[LI] consensus sequence in GLUT8 and GLUT12.

Materials and Methods

DNA constructs and antibodies

A murine GLUT8 coding sequence insert was cloned into a pcDNA3.1+ vector (Invitrogen, Grand Island, NY, USA). An HA-epitope tag (YPYDVPDYA) was inserted in the large exofacial loop between transmembrane helices 9 and 10 as described previously (Augustin et al., 2005). Site directed mutagenesis was performed to change the amino acids T (10) to G, Q (11) to P, P (12) to N, Q (11) to A, TQP (10,11,12) to APA, and TQP (10,11,12) to GPN by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). All constructs were confirmed by sequencing.

To detect the GLUT8-HA constructs by immunofluorescence or western blotting, monoclonal mouse (Covance Research Products Inc, Berkeley, CA, USA) and rat-HA antibodies (Roche Applied Science, Indianapolis, IN, USA) were used. For colocalization studies, we used Syntaxin 8 (BD Biosciences) and a polyclonal GLUT8 antibody targeting the COOH-terminus of mouse GLUT8 (Augustin et al., 2005). Fluorescent- labeled and HRP- conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR, USA) and Pierce (Rockford, IL, USA). FITC-Dextran was purchased from Molecular Probes.

Cell culture and transfections

HEK293 and HeLa cells were maintained in Dulbecco's Modified Eagle Medium containing 10% fetal calf serum, 1% penicillin-streptomycin, 1% L-glutamine, 1% sodium pyruvate and 1% non-essential amino acids. Cells were transfected using Fugene HD (Roche, Nutley, NJ, USA). Immunofluorescent staining was performed 48 hours following transfection. For siRNA experiments, cells were reverse transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen, Grand Island, NY, USA). Twenty-four hours after transfection, the cell culture media was changed and cells were forward transfected with the GLUT constructs using Fugene HD (Roche, Nutley, NJ, USA).

Confocal microscopy

HEK293 and HeLa cells were grown on cover slips and rinsed with PBS before being fixed with 4% paraformaldehyde in PBS for 10 minutes. The cells were rinsed three times with PBS and permeabilized in 0.1% Tween for 5 minutes. Cells were rinsed three times in PBS and nonspecific antibody binding was blocked by incubating cells in 20% normal goat serum in 2% BSA/PBS for 30 minutes. Primary antibodies diluted in 2% BSA/PBS were then applied for one hour at room temperature or overnight at 4 °C. Cells were washed three times with PBS. Alexa Fluor 488 was used as a secondary antibody at a dilution of 1:500 in 2% BSA/PBS, and was applied to cells at room temperature for 20 minutes. Cells were washed three times in PBS and then the nuclei were counterstained with TOPRO-iodide (Molecular Probes, Eugene, OR, USA) diluted 1:500 in PBS for 10 minutes. Cells were mounted on slides using Vectashield (Vector Labs, Burlingame, CA, USA) and examined by confocal microscopy using a Nikon C1 microscope. Two dimensional images were obtained at a magnification of 60× with a pixel resolution of 1024 × 1024.

FITC-Dextran was used to look for colocalization of GLUTs and the lysosome. Cells were cultured in 1 mg/mL of FITC-Dextran for 6 hours, followed by an additional 2 hours in culture after washing the FITC-Dextran out of the cell culture media. The cells were then processed for immunofluorescence staining and confocal microscopy as described above.

Western blotting

The expression of GLUTs in transiently transfected HEK293 and HeLa cells was confirmed using western blot analysis of whole cell lysates. Cells were washed in PBS and lysed in cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 0.5% NP-40, and 0.2% Glycerol) containing proteinase inhibitors. Whole cell lysates were separated using SDS-PAGE electrophoresis and transferred to nitrocellulose paper. Non-specific antibody binding was blocked in 5% milk in TBST (10 mM Tris-HCl, 100 mM NaCl, and 0.1% Tween). The HA- and actin-antibodies were applied overnight at 4°C in 1% milk in TBST (1:2000). Membranes were then probed with HRP-conjugated secondary antibodies and detected using SuperSignal Dura chemiluminescence substrate (Pierce, Rockford, IL, USA).

Cell surface expression levels of GLUT8-HA and GLUT8-HA mutants

HEK293 cells were plated into 6 well plates containing small 8mm coverslips. When the cells were 50-60% confluent, they were transiently transfected with the GLUT8-HA constructs. Forty-eight hours following transfection, the cells on the coverslips were stained without permeabilization in order to detect cell surface associated GLUTs. Cells were then imaged using confocal microscopy with the gain set below the levels of saturation for antibody staining. Ten separate fields of view were imaged for cells expressing each GLUT, and the fields were selected by cross checking with two independent observers to minimize experimental bias. The levels of cell surface staining was quantified using ImageJ (NIH). Cell surface staining was normalized to the number of nuclei in each field of view. To normalize for differences in the transfection efficiency of each GLUT, cell lysates were created from the cells in each well after the removal of the coverslip for staining. The relative expression levels of GLUT8-HA and GLUT8-HA mutants were determined by western blotting, and the levels of cell surface staining were normalized to the expression levels of each GLUT.

Internalization assay for GLUT8-HA and GLUT8-HA mutants

Endocytosis of GLUTs was compared using HEK293 cells expressing GLUT8-HA, GLUT8-TAP-HA, GLUT8-APA-HA, or GLUT8-GPN-HA constructs. These GLUT constructs contain an HA-tag in the large exofacial loop between transmembrane helices 9 and 10. Cells were grown on coverslips and transiently transfected with GLUT8-HA, GLUT8-TAP-HA, GLUT8-*APA*-HA, or GLUT8*GPN*-HA as described above. Twenty-four hours after transfection the cells were incubated for one hour with 8 uL/mL monoclonal HA antibody (Covance, Princeton, NJ, USA). The cells were rinsed three times in PBS to remove any unbound HA antibody and were cultured for an additional hour without antibody. Cells were then fixed for ten minutes in 3% paraformaldehyde. Following fixation, cells were permeabilized to examine the degree of GLUT endocytosis. Nonspecific antibody binding was blocked in 20% normal goat serum in 2% BSA/PBS for 30 minutes and cells were probed with goat anti-mouse Alexa-Fluor 488 in 2% BSA/PBS for 30 minutes. The cells were then mounted in Vectashield and analyzed by confocal microscopy.

Statistical analysis

The relative cell surface expression levels of GLUTs were analyzed using ANOVA with Bonferroni post hoc test. The relative expression levels of adaptin subunit mRNA transcripts in cells treated with siRNA targeting APs compared to control cells was analyzed using a

Student T test. Significance was defined as a p value of less than 0.05. All error bars represent the standard error of the mean (SEM).

Results

Substitutions at the -3 and -1 positions do not change GLUT8 intracellular localization

In our previous studies, we showed that GLUT8 and GLUT12 localize to different cellular compartments. While GLUT8 resides in the late endosome and the lysosome, GLUT12 localizes to the Golgi and to the plasma membrane. Several studies have indicated that the [DE]XXXL[LI] motif in the amino terminus of GLUT8 is involved in trafficking the GLUT to the lysosome. GLUT12 contains a similar [DE]XXXL[LI] motif in its amino terminus, however it plays a different role in the sorting of GLUT12. In our previous study, we found that replacing the TQP residues upstream of the dileucine motif in GLUT8 with the GPN residues upstream of the dileucine motif in GLUT12 resulted in a dramatic redirecting of GLUT8 to the cell surface. Conversely, by exchanging the TQP residues of GLUT8 with the GPN residues of GLUT12, we saw a redirecting of GLUT12 away from the cell surface to an intracellular compartment. These results demonstrated that the three amino acids immediately upstream of the dileucine motif are important in mediating the trafficking of GLUT8 and GLUT12. To better understand the role that these amino acids play in the localization of GLUTs, we individually mutated the amino acids at XXX positions in GLUT8 to the corresponding amino acid from GLUT12 (Figure 1). HEK293 and HeLa cells were transiently transfected with GLUT8-TQP-HA, GLUT8-*G*QP-HA, GLUT8-T*P*P-HA, GLUT8-TQ*N*-HA and GLUT8-*GPN*-HA. After 48 hours of expression, cells were fixed and stained with an antibody directed against the HA epitope. As expected, wild-type GLUT8 localized to an intracellular compartment, while GLUT8-*GPN* localized primarily at the cell surface (Figure 2). Two of the constructs with a single amino acid mutation, GLUT8-*G*QP-HA and GLUT8-TQ*N*-HA, localized to an intracellular compartment similar to wild type GLUT8. The glutamine to proline mutation, however, caused the GLUT8-T*P*P-HA construct to sort to the cell surface.

Proline substitution at the -2 position redirects GLUT8 to the cell surface

To determine if the redirecting of GLUT8-T*P*P-HA to the cell surface is due to loss of the glutamine at the XXX amino acid position, or the addition of a proline to the XXX position, we created two additional mutant constructs. In the first mutant construct, we substituted an alanine for the glutamine. In the second mutant construct, we positioned a proline at position -2 surrounded by alanine residues. Both constructs were transfected into HEK293 and HeLa cells and the subcellular localization of each construct was assessed by immunofluorescent staining. The GLUT8-T*A*P-HA construct localized to an intracellular compartment, similar to wild type GLUT8 (Figure 3). GLUT8-*APA*-HA, however, localized to the cell surface similar to GLUT8-*GPN*-HA. We therefore determined that the position of the proline residue upstream of the dileucine in the [DE]XXXL[LI] motif is a key player in directing the localization of GLUT8 and GLUT12.

Several of the GLUT8 mutations, including those in GLUT8-*G*QP-HA, GLUT8-TQ*N*-HA, and GLUT8-T*A*P-HA do not appear to alter the intracellular distribution of each GLUT relative to wild type GLUT8. To determine if these GLUT8 mutants also localize to the late endosomes and lysosomes like wild-type GLUT8, we assayed for colocalization between the GLUTs and the late endosomal marker Syntaxin 8. As expected, we observed colocalization between wild-type GLUT8 and Syntaxin 8 (Figure 4Aa-c). We also observed colocalization between the mutant GLUT8T*A*P and Syntaxin 8, demonstrating that the loss of the glutamine at position (XXX) does not alter the localization of the GLUT8 transporter (Figure 4Ad-f). However, no colocalization was observed between GLUT8-*APA*-HA or

GLUT8-*GPN*-HA and Syntaxin 8, showing that the addition of a proline at position XXX in GLUT8 redirects the transporter from the late endosome and the lysosome to the cell surface (Figure4Ag-i, j-l). To further characterize the compartment to which GLUT8-T*A*P-HA localizes, we exposed GLUT8-HA or GLUT8-TAP-HA –expressing HEK293 cells with FITC-dextran for six hours. The cells were stained with an antibody against the HA epitope and examined by confocal microscopy (Figure 4B). Both GLUT8-HA and GLUT8-T*A*P were found in the lysosomal membrane surrounding the endocytosed FITC-dextran (Figure 4B). This further confirms that mutations that do not result in the redistribution of GLUT8 to the cell surface do not alter the intracellular localization of GLUT8 relative to the wild type. Finally, to determine whether the threonine (-3) and the proline (-1) in wild type GLUT8 and in the GLUT8-T*A*P mutant impact the intracellular localization of the GLUTs, we compared the localization of GLUT8-HA, GLUT8-T*A*P-HA, and GLUT8-*APA*-HA to a GLUT8-*AAA*-HA mutant containing only alanine residues at the XXX position. A small percentage of GLUT8-*AAA*-HA traffics to the cell surface, however a majority of GLUT8-*AAA*-HA localizes to a punctate intracellular compartment similarly to GLUT8-HA and GLUT8-TAP-HA (Figure 5A). To determine whether GLUT8-*AAA*-HA also localizes to lysosomal membranes, HEK293 and HeLa cells were transiently transfected in GLUT8-HA and GLUT8-*AAA*-HA and stained to assay for colocalization between the GLUTs and Syntaxin 8. We observed colocalization between GLUT8-*AAA*-HA and Syntaxin 8 (Figure 5B), demonstrating that the threonine and proline residues at the -3 and -1 positions of GLUT8 do not impact the intracellular localization of the GLUTs.

Proline substitution at -2 of affects internalization of GLUT8 mutant

Next we performed an assay to determine whether the mutations impact the internalization of GLUT8 from the cell surface. Previous work in our lab has shown that GLUT8 associated with the cell surface is internalized and trafficked through the endosomal system to the late endosomes and the lysosomes. In contrast, cell surface associated GLUT12 is not internalized but largely remains associated with the plasma membrane. In these studies, cells were transfected with empty vector, GLUT8, GLUT8-TAP-HA, GLUT8-APA-HA, or GLUT8-GPN-HA constructs and incubated with monoclonal HA antibody for one hour, or incubated with monoclonal HA antibody for one hour and subsequently washed to rinse away unbound HA antibody, and incubated in the absence of HA antibody for an additional hour. During a one hour incubation, GLUT8 and GLUT8TAP were internalized from the cell surface and redirected to an intracellular compartment (Figure 6). GLUT8-APA and GLUT8-GPN, however, remained associated with the cell surface after both a one and two hour incubation, similar to GLUT12. These internalization assays demonstrate that the position of the proline in the [DE]XXXL[LI] motif impacts the trafficking of each transporter from the cell surface.

Proline substitution at -2 affects GLUT8 interaction with adaptor proteins AP1 and AP2

To determine whether the alterations of the XXX amino acids in the [DE]XXXL[LI] motif alter the affinity between GLUT8 and adaptor proteins, we used an siRNA approach to knock down the expression of AP1, AP2, and AP3 subunits. HEK293 cells were treated with siRNA for forty-eight hours before assaying for knockdown. The HEK293 cells had a greater than 90% transfection efficiency for the siRNA as determined by flow cytometry and confocal microscopy (Supplemental Figure A). Knockdown was assessed using real time PCR for each adaptor protein subunit forty-eight hours following transfection. The mu subunit of AP2 had a 91% knockdown (Figure 7A), the gamma subunit of AP1 had a 65% knockdown (Figure 8A), and the delta subunit of AP3 had an 81% knockdown (Supplemental Figure B). Twenty-four hours after the HEK293 cells were transfected with RNAi targeting the adaptor protein subunits, cells were transfected with empty vector, GLUT8-HA, GLUT8-TAP-HA, GLUT8-APA-HA, or GLUT8-GPN-HA. Cells were

continued in culture for an additional twenty-four hours before staining the cells with a hemagglutinin antibody. To facilitate the detection of cell surface-associated glucose transporters, the cells were not permeabilized. Decreasing expression of the mu2 subunit of adaptor protein 2 resulted in a dramatic increase in GLUT8-HA and GLUT8-TAP-HA at the cell surface (Figure 7B). The mu2 knockdown did not impact the levels of GLUT8-APA-HA or GLUT8-GPN-HA associated with the cell surface. This data indicates that GLUT8 as well as the GLUT8-TAP-HA mutant both interact with AP2 *in vivo*. Mutating the [DE]XXXL[LI] motif in GLUT8 such that the proline is in the -2 position, and not -1 upstream of the dileucine motif, affects the ability of AP2 to interact with and direct the trafficking of GLUT8. Similarly, knocking down the gamma subunit of AP1 caused a redistribution of GLUT8 and GLUT8-*TAP* to the cell surface (Figure 8B). It did not alter the distribution of GLUT8-*APA* or GLUT8-*GPN*. This data suggests that GLUT8 and GLUT8-T*A*P interact with AP1 for trafficking to the late endosome and lysosome but that GLUT8-*APA* and GLUT8-*GPN* do not interact with AP1. Knockdown of the delta subunit of AP3 does not have any affect on the cell surface localization of GLUT8, GLUT8-T*A*P, GLUT8-*APA*, or GLUT8-*GPN*, suggesting that GLUT8 does not interact with AP3 *in vivo* (Supplemental Figure B).

Discussion

The physiological role of the class III family of glucose transporters is not yet understood. Several class III glucose transporters have been shown to localize predominantly to intracellular membranes rather than to the cell surface, raising the possibility that these GLUTs function by regulating the transport of hexoses across intracellular membranes. GLUT8 has been shown to localize in late endosomal and lysosomal compartments. Several studies have indicated that a [DE]XXXL[LI] sorting motif in the amino terminus of GLUT8 is necessary for its steady state localization to a late endosomal compartment (Diril et al., 2009, Augustin et al., 2005). Previous studies have shown that interactions between the [DE]XXXL[LI] sorting motif and AP1 and AP2 sort GLUT8 to a late endosomal compartment (Schmidt et al., 2006, Diril et al., 2009). In the present study, we confirmed that GLUT8 interacts with AP1 and AP2, but not AP3, in HEK293 cells. We further showed that the XXX amino acids within the [DE]XXXL[LI] motif direct the steady state or cell surface localization of the GLUT8 cargo protein.

Our previous studies had demonstrated that the GPN residues within the [DE]XXXL[LI] motif of GLUT12 traffic the protein to the cell surface, whereas the analogous TQP residue in GLUT8 direct the protein to the late endosome and the lysosome (Flessner and Moley, 2009). Replacing the TQP residues in GLUT8 with the GPN residues in GLUT12 redirected GLUT8 away from the lysosome and to the cell surface. Replacing the GPN residues in GLUT12 with the TQP residues in GLUT8 redirect GLUT12 to a punctate intracellular compartment rather than the cell surface. In the present study, we set out to further characterize the role that specific amino acids within the [DE]XXXL[LI] motifs of GLUT8 and GLUT12 play in directing the localization of GLUTs.

Individual mutation of the XXX amino acids in GLUT8 to the corresponding amino acids in GLUT12 showed that while the position of some amino acids acutely affected the localization of GLUT8, others did not impact the localization of the cargo protein. The steady state localization of the GLUT8*G*QP and GLUT8TQ*N* mutants remained in the late endosome/lysosome similar to wild-type GLUT8. This data demonstrates both that the threonine and the proline residues at positions -3 and -1 to the dileucine motif in GLUT8 are not responsible for the protein's localization to the lysosomal compartment. Previous studies have demonstrated that a proline residue -1 from a dileucine motif may preferentially bind to AP3 (Rodionov et al., 2002). Proteins that strongly interact with AP3, such as LIMPII, are

directed to the late endosome/lysosome(Honing et al., 1998). Our data indicates that for GLUT8, a proline residue -1 from the dileucine motif is not critical for its steady state localization in the lysosome. This data also demonstrates that neither the addition of a glycine residue at -3 to the dileucines nor the addition of the asparagine residue at -1 to the dileucines is sufficient to redirect to localization of GLUT8 to the cell surface. Furthermore, we find that GLUT8-*AAA* primarily localizes to a lysosomal compartment, demonstrating that the threonine and proline residues at -3 and -1 to the dileucines are not necessary to direct GLUT8 to its lysosomal compartment. The GLUT8T*P*P mutation, however, was sufficient to redirect the transporter from the lysosome to the cell surface. This data suggested that either the loss of a glutamine or the addition of a proline residue at -2 position is sufficient to dramatically alter the steady state localization of GLUT8 to the cell surface. To distinguish between these two possibilities, we created two more mutations to GLUT8's [DE]XXXL[LI] motif: GLUT8-T*A*P and GLUT8-*APA*. Here we find that GLUT8-T*A*P localizes to the lysosome whereas GLUT8-*APA* traffics to the plasma membrane. This confirms that the presence of a proline residue -2 to the dileucine motif is sufficient to redirect GLUT8 cargo protein to the cell surface rather than the lysosome.

In addition to influencing the steady state localization of GLUT8, the amino acids upstream of the dileucine residues also influence the cell surface internalization of the membrane protein. In our previous study we showed that cell surface-associated GLUT8 is internalized and subsequently trafficked to the late endosome/lysosome within a five hour time period in CHO and HEK293 cells (Flessner and Moley, 2009, Augustin et al., 2005). During this same time period, cell surface GLUT12 remains largely associated with the plasma membrane. In this study we showed that GLUT8 and GLUT8T*A*P, which at steady state localize to the lysosome, are both internalized from the cell surface and redirected to an intracellular compartment within one and two hour time periods. GLUT8-*APA* and GLUT8-*GPN*, however, remain associated with the plasma membrane similarly to GLUT12. This data shows that both the steady state localization of GLUT8 and its trafficking from the cell surface are influenced by the XXX amino acids in its NH_2-terminal [DE]XXXL[LI] motif.

Our data suggest that the residues adjoining the dileucines in the [DE]XXXL[LI] motif may regulate the trafficking of GLUT8 and GLUT12 by regulating the affinity of the interactions between the GLUTs and AP complexes. Here, we used small interfering RNA targeting the adaptin subunits delta1, mu2, and gamma1 to examine the *in vivo* interactions between AP complexes and our GLUT mutants. By knocking down these subunits, we confirmed that AP1 and AP2, but not AP3, interact with GLUT8 *in vivo*. Previous studies have shown an interaction between GLUT8 and AP1 and AP2 complexes using GST pulldowns and siRNA targeting adaptin subunits (Diril et al., 2009, Doray et al., 2007, Schmidt et al., 2006). A recent study by Diril et al. showed that while GLUT8 interacts with AP1 and AP2, it does not rely on interactions with the lysosome associated AP3 to be targeted to the lysosomal membrane. Our results showed that knocking down the delta subunit of AP3 does not alter the cell surface levels of GLUT8, further confirming that the GLUT8 is not a cargo protein for AP3. While the lack of interaction between AP3 and GLUT8 was surprising, previous studies have shown that the localization of lysosome-associated proteins is not always altered by depletion of AP3 subunits (Janvier and Bonifacino, 2005). By contrast, we showed that knocking down either the mu2 subunit of AP2 and the gamma1 subunit of AP1 results in an increase in GLUT expression at the cell surface. This further confirms that AP1 and AP2 interact with GLUT8 *in vivo*. In this study, we also showed that the interactions between GLUT8 and AP complexes are altered by mutations in the amino acids within the [DE]XXXL[LI] motif. Both wild type GLUT8 and GLUT8T*A*P are redirected to the cell surface in cells treated with siRNA targeting gamma1 and mu2 subunits. By contrast, treating GLUT8APA- and GLUT8GPN- expressing cells with siRNA to knock down AP1 and AP2 subunits does not alter the overall cell surface expression level of either GLUT

mutants. This suggests that in contrast to GLUT8 and GLUT8T*A*P, that GLUT8*APA* and GLUT8*GPN* do not interact with AP1 or AP2 *in vivo*. Our data suggests that the differences in internalization from the cell surface between GLUT8 and GLUT12 lies in the position of a proline residue in the [DE]XXXL[LI] motif of each GLUT. The fact that GLUT12, unlike GLUT8, is not rapidly internalized from the cell surface and redirected to an intracellular compartment suggests a lack of interaction between GLUT12 and AP2 or AP1 due to the position of its proline residue.

Identifying the protein sorting motifs that regulate the steady state localization and the trafficking of GLUT8 and GLUT12 may better clarify the physiological roles for both class III glucose transporters. Our study has shown that a [DE]XXXL[LI] motif controls the localization and sorting of GLUT8 and GLUT12. Furthermore, the XXX amino acids within the [DE]XXXL[LI] motif determine the affinity of interactions between AP1 and AP2 and GLUT8 and GLUT12.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Augustin R, Riley J, Moley KH. GLUT8 contains a [DE]XXXL[LI] sorting motif and localizes to a late endosomal/lysosomal compartment. Traffic. 2005; 6:1196–212. [PubMed: 16262729]

Bonifacino JS, Traub LM. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem. 2003; 72:395–447. [PubMed: 12651740]

Diril MK, Schmidt S, Krauss M, Gawlik V, Joost HG, Schurmann A, Haucke V, Augustin R. Lysosomal localization of GLUT8 in the testis--the EXXXLL motif of GLUT8 is sufficient for its intracellular sorting via AP1- and AP2-mediated interaction. Febs J. 2009; 276:3729–43. [PubMed: 19523115]

Doray B, Lee I, Knisely J, Bu G, Kornfeld S. The gamma/sigma1 and alpha/sigma2 hemicomplexes of clathrin adaptors AP-1 and AP-2 harbor the dileucine recognition site. Mol Biol Cell. 2007; 18:1887–96. [PubMed: 17360967]

Flessner LB, Moley KH. Similar [DE]XXXL[LI] motifs differentially target GLUT8 and GLUT12 in Chinese hamster ovary cells. Traffic. 2009; 10:324–33. [PubMed: 19076329]

Gomez O, Ballester B, Romero A, Arnal E, Almansa I, Miranda M, Mesonero JE, Terrado J. Expression and regulation of insulin and the glucose transporter GLUT8 in the testes of diabetic rats. Horm Metab Res. 2009; 41:343–9. [PubMed: 19194835]

Gomez O, Romero A, Terrado J, Mesonero JE. Differential expression of glucose transporter GLUT8 during mouse spermatogenesis. Reproduction. 2006; 131:63–70. [PubMed: 16388010]

Honing S, Sandoval IV, Von Figura K. A di-leucine-based motif in the cytoplasmic tail of LIMP-II and tyrosinase mediates selective binding of AP-3. Embo J. 1998; 17:1304–14. [PubMed: 9482728]

Ibberson M, Riederer BM, Uldry M, Guhl B, Roth J, Thorens B. Immunolocalization of GLUTX1 in the testis and to specific brain areas and vasopressin-containing neurons. Endocrinology. 2002; 143:276–84. [PubMed: 11751619]

Ibberson M, Uldry M, Thorens B. GLUTX1, a novel mammalian glucose transporter expressed in the central nervous system and insulin-sensitive tissues. J Biol Chem. 2000; 275:4607–12. [PubMed: 10671487]

Janvier K, Bonifacino JS. Role of the endocytic machinery in the sorting of lysosome-associated membrane proteins. Mol Biol Cell. 2005; 16:4231–42. [PubMed: 15987739]

Joost HG, Thorens B. The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members (review). Mol Membr Biol. 2001; 18:247–56. [PubMed: 11780753]

Kim ST, Moley KH. The expression of GLUT8, GLUT9a, and GLUT9b in the mouse testis and sperm. Reprod Sci. 2007; 14:445–55. [PubMed: 17913964]

Lisinski I, Schurmann A, Joost HG, Cushman SW, Al-Hasani H. Targeting of GLUT6 (formerly GLUT9) and GLUT8 in rat adipose cells. Biochem J. 2001; 358:517–22. [PubMed: 11513753]

Robinson MS, Bonifacino JS. Adaptor-related proteins. Curr Opin Cell Biol. 2001; 13:444–53. [PubMed: 11454451]

Rodionov DG, Honing S, Silye A, Kongsvik TL, Von Figura K, Bakke O. Structural requirements for interactions between leucine-sorting signals and clathrin-associated adaptor protein complex AP3. J Biol Chem. 2002; 277:47436–43. [PubMed: 12370188]

Rogers S, Macheda ML, Docherty SE, Carty MD, Henderson MA, Soeller WC, Gibbs EM, James DE, Best JD. Identification of a novel glucose transporter-like protein-GLUT-12. Am J Physiol Endocrinol Metab. 2002; 282:E733–8. [PubMed: 11832379]

Scheepers A, Joost HG, Schurmann A. The glucose transporter families SGLT and GLUT: molecular basis of normal and aberrant function. JPEN J Parenter Enteral Nutr. 2004; 28:364–71. [PubMed: 15449578]

Schmidt S, Joost HG, Schurmann A. GLUT8, the enigmatic intracellular hexose transporter. Am J Physiol Endocrinol Metab. 2009; 296:E614–8. [PubMed: 19176349]

Schmidt U, Briese S, Leicht K, Schurmann A, Joost HG, Al-Hasani H. Endocytosis of the glucose transporter GLUT8 is mediated by interaction of a dileucine motif with the beta2-adaptin subunit of the AP-2 adaptor complex. J Cell Sci. 2006; 119:2321–31. [PubMed: 16723738]

Schurmann A, Axer H, Scheepers A, Doege H, Joost HG. The glucose transport facilitator GLUT8 is predominantly associated with the acrosomal region of mature spermatozoa. Cell Tissue Res. 2002; 307:237–42. [PubMed: 11845330]

Stuart CA, Howell ME, Zhang Y, Yin D. Insulin-stimulated translocation of glucose transporter (GLUT) 12 parallels that of GLUT4 in normal muscle. J Clin Endocrinol Metab. 2009; 94:3535–42. [PubMed: 19549745]

Stuart CA, Yin D, Howell ME, Dykes RJ, Laffan JJ, Ferrando AA. Hexose transporter mRNAs for GLUT4, GLUT5, and GLUT12 predominate in human muscle. Am J Physiol Endocrinol Metab. 2006; 291:E1067–73. [PubMed: 16803853]

Wilson-O'brien AL, Dehaan CL, Rogers S. Mitogen-stimulated and rapamycin-sensitive glucose transporter 12 targeting and functional glucose transport in renal epithelial cells. Endocrinology. 2008; 149:917–24. [PubMed: 18039784]

Wood IS, Hunter L, Trayhurn P. Expression of Class III facilitative glucose transporter genes (GLUT-10 and GLUT-12) in mouse and human adipose tissues. Biochem Biophys Res Commun. 2003; 308:43–9. [PubMed: 12890477]

Wood IS, Trayhurn P. Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. Br J Nutr. 2003; 89:3–9. [PubMed: 12568659]

Zhou Y, Kaye PL, Pantaleon M. Identification of the facilitative glucose transporter 12 gene Glut12 in mouse preimplantation embryos. Gene Expr Patterns. 2004; 4:621–31. [PubMed: 15465484]

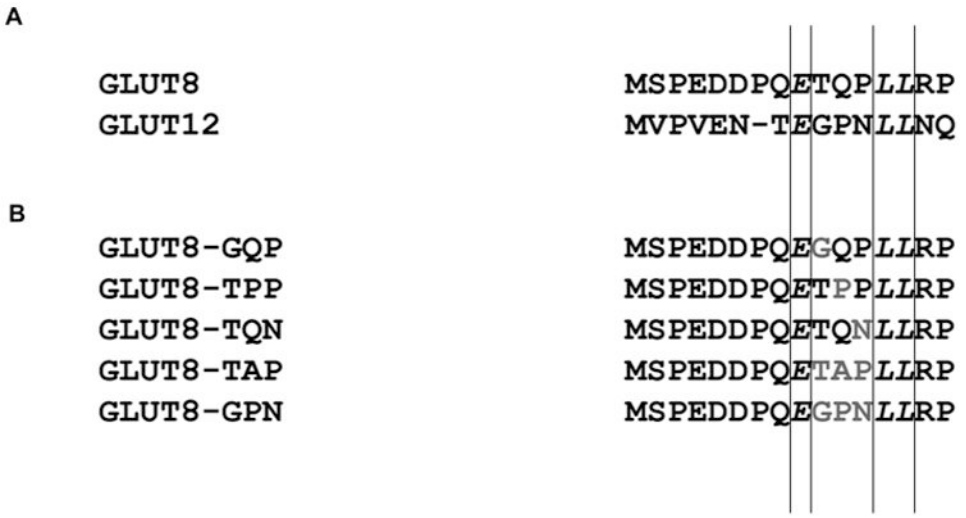

Figure 1. NH_2-termini of GLUT constructs

A. The amino terminus of both wild type murine GLUT8 and wild type murine GLUT12 contains a [DE]XXXL[LI] motif. The motif differs between GLUT8 and GLUT12 by containing different amino acids in the positions -1, -2, and -3 to the dileucine motif. *B.* The [DE]XXXL[LI] motif in the amino terminus of GLUT8 was mutated to the constructs shown in order to explore the role that these amino acids play in the distinct sorting of GLUT8 and GLUT12.

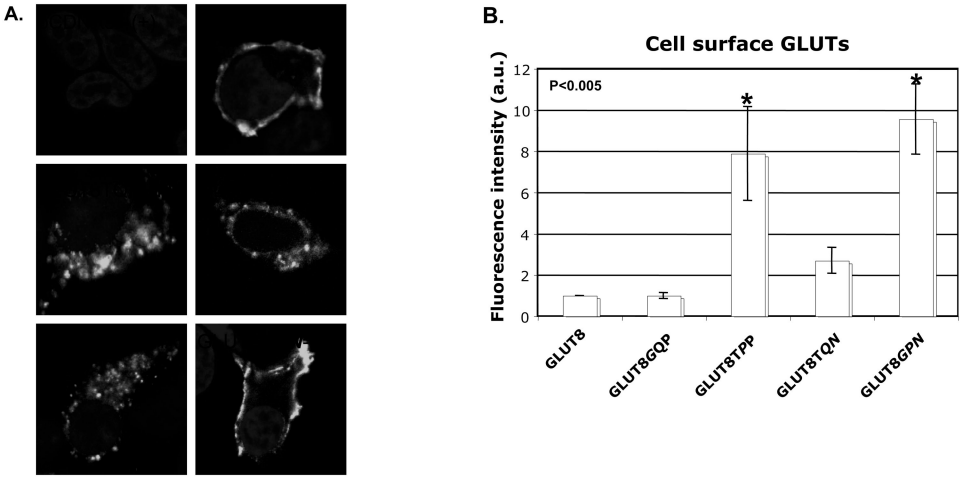

Figure 2. The amino acids -1, -2, and -3 to the dileucine motif affect the cellular localization of GLUT8

A. The amino acids -1, -2, and -3 to the dileucine motif of GLUT8 were individually mutated to the corresponding amino acids from GLUT12. HEK293 cells were transiently transfected with each GLUT and were stained and examined by immunofluorescence microscopy. Like wild type GLUT8, GLUT8-*G*QP-HA and GLUT8-TQ*N*-HA localize to an intracellular compartment. The GLUT8-*GPN*-HA construct, which contains the amino acids found in GLUT12, localizes to the plasma membrane. Similarly, the GLUT8-T*P*P-HA construct localizes to the cell surface. Images were obtained using a 60× oil objective lens. *B.* The cell surface expression of the mutant constructs GLUT8-T*P*P-HA and GLUT8-*GPN*-HA is significantly greater than that of wild type GLUT8-HA or the mutant constructs GLUT8-*G*QP-HA or GLUT8-TQ*N*-HA (n=8).

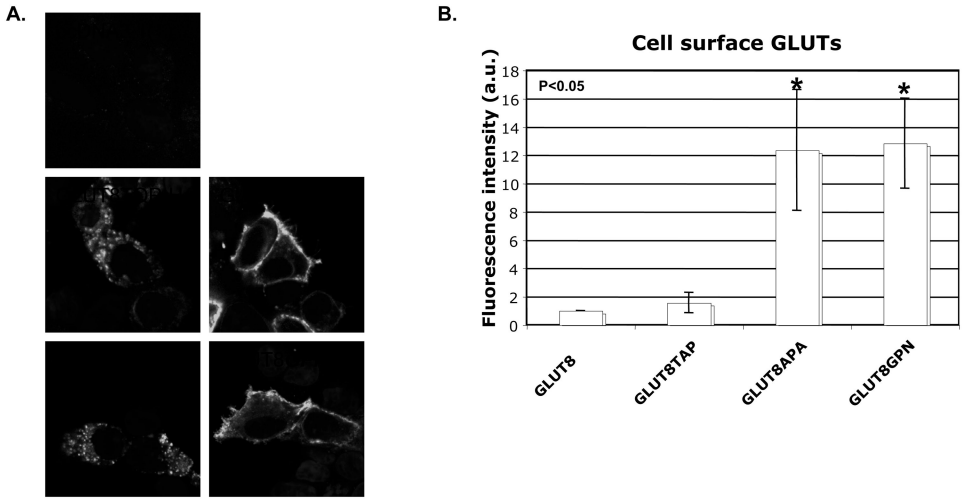

Figure 3. The position of a proline residue -2 to the dileucine motif directs the localization of GLUT8 and GLUT12

A. To determine whether glutamine or proline affects the cellular localization of GLUT8, HEK293 cells were transfected and stained with an antibody directed against the HA-tag for GLUT8-HA, GLUT8-T*A*P-HA, GLUT8-*APA*-HA, and GLUT8-*GPN*-HA. Both GLUT8-HA and GLUT8-T*A*P-HA localized to an intracellular compartment, whereas GLUT8-*APA*-HA and GLUT8-*GPN*-HA trafficked to the cell surface. Images were obtained using a 60× oil objective lens. *B.* The amount of GLUT8-*APA*-HA and GLUT8-*GPN*-HA localized at the plasma membrane was significantly greater than that of GLUT8-HA or GLUT8-T*A*P-HA (n=8).

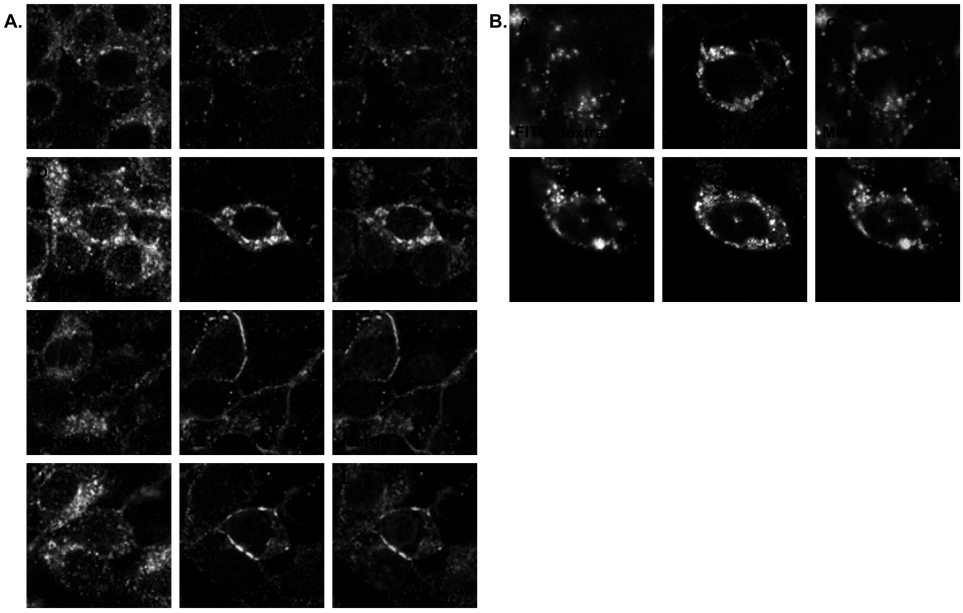

Figure 4. GLUT8 and the GLUT8TAP mutant localize to the late endosome/lysosome

HEK293 cells were transiently transfected with GLUTs and stained and examined by immunofluorescence microscopy. *A*. GLUT8 and GLUT8-TAP both colocalized with the late endosomal/lysosomal marker Syntaxin 8 (c, f). GLUT8-*APA* and GLUT8-*GPN*, which reside predominantly at the cell surface, did not colocalized with Syntaxin 8 (n=3) (i, l). Red channel: syntaxin 8 (a, d, g, j); Green channel: GLUT8 constructs (b,e,h,k); *B*. Both GLUT8 and GLUT8-TAP reside in the lysosomal membrane that surrounds the internalized FITC-dextran (n=3) (c, f); Red channel: FITC-dextran (a, d); Green channel: GLUT8 constructs (b, e). Images were obtained using a 60× oil objective lens.

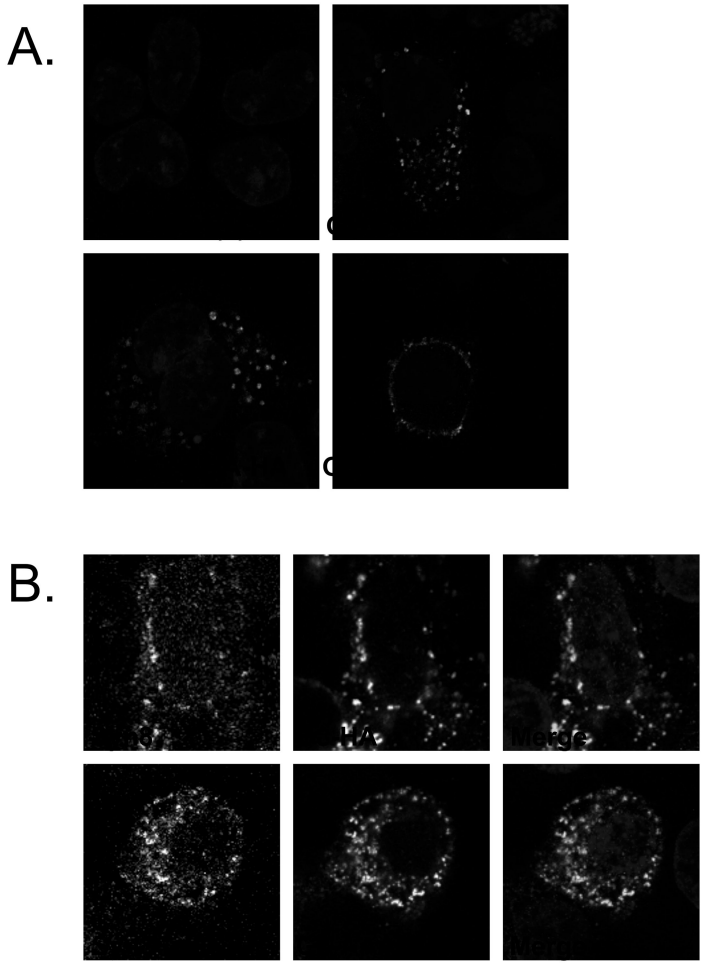

Figure 5. GLUT8 and the GLUT8AAA mutant localize to the late endosome/lysosome

A. To determine whether threonine (-3) or proline (-1) affects the cellular localization of GLUT8, HEK293 cells were transfected and stained with an antibody directed against the HA-tag for GLUT8-HA, GLUT8-*APA*-HA, and GLUT8-*AAA*-HA. GLUT8-HA, GLUT8-T*A*P-HA, and GLUT8-*AAA*-HA localized to an intracellular compartment, whereas GLUT8-*APA*-HA trafficked to the cell surface. Images were obtained using a 60× oil objective lens. *B.* Both GLUT8-HA and GLUT8-*AAA*-HA (Green channel) colocalized with the late endosomal/lysosomal marker Syntaxin 8 (Red channel). (n=3).

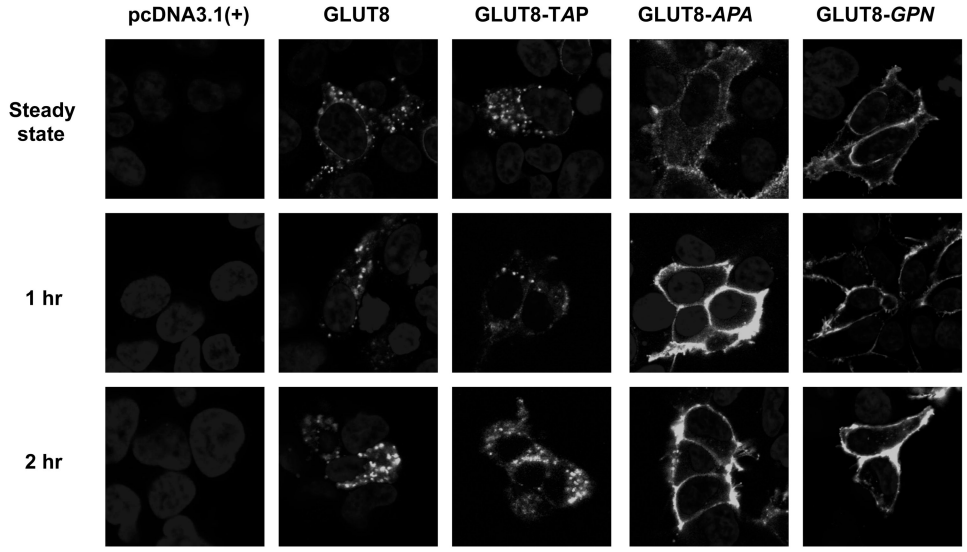

Figure 6. Cell surface associated GLUT8 and GLUT8TAP are internalized and trafficked to an intracellular compartment

HEK293 cells were transiently transfected with GLUT8-HA, GLUT8-TAP-HA, GLUT8-*APA*-HA, or GLUT8-*GPN*-HA. At steady state, GLUT8-HA and GLUT8-TAP-HA predominantly reside in an intracellular compartment. GLUT8-*APA*-HA and GLUT8-*GPN*-HA reside in the plasma membrane. A monoclonal HA antibody was added to the cell culture media and incubated for one hour (1 hr), or washed out after one hour and cultured for an additional hour (2 hr). At both the one hour and two hour time points, GLUT8-HA and GLUT8-TAP-HA are internalized from the cell surface and redirected to an intracellular compartment. After one and two hours in culture, a majority of the labeled GLUT8-*APA*-HA and GLUT8-*GPN*-HA remains associated with the plasma membrane (n=3). Images were obtained using a 60× oil objective lens.

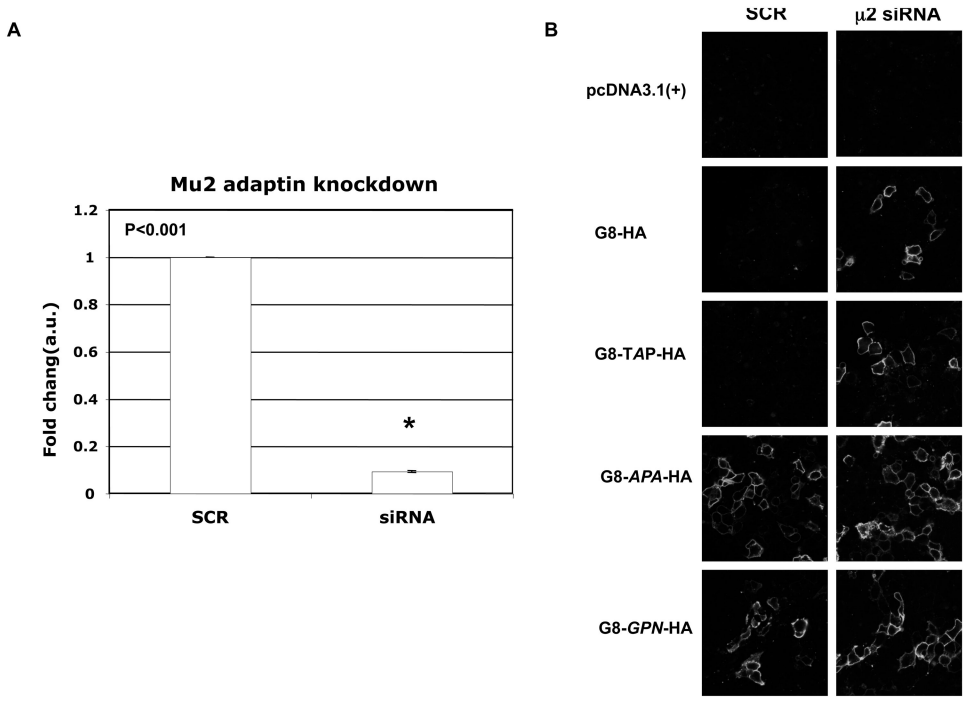

Figure 7. AP2 interacts with GLUT8 and the mutant GLUT8-TAP in HEK293 cells

A. The levels of RNA transcripts for the mu2 subunit of AP2 were knocked down by 91% in HEK293 cells by siRNA (n=3). B. RNA*i* treated HEK293 cells were transfected with GLUT8 or the GLUT8 mutants (n=3). The levels of GLUT8-HA and GLUT8-TAP-HA on the plasma membrane increased dramatically in cells transfected with RNA*i* targeting AP2. The cell surface levels of GLUT8-APA-HA and GLUT8-GPN-HA were not altered by the depletion of AP2. Images were obtained using a 60× oil objective lens.

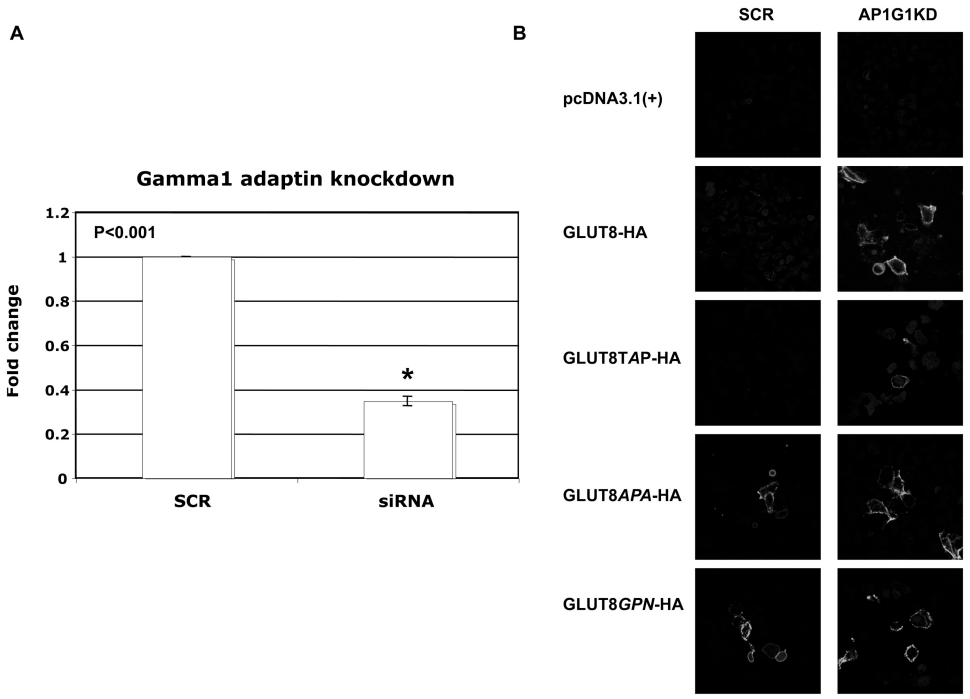

Figure 8. AP1 interacts with GLUT8 and the mutant GLUT8-T*AP* in HEK293 cells

A. The transcript levels for the gamma1 subunit of AP-1 were depleted by 65% in HEK293 cells following treatment with siRNA (n=3). *B.* RNAi treated HEK293 cells were transiently transfected with GLUT8 or the GLUT8 mutants and stained without permeabilization (n=2). The cell surface associated GLUT8-HA and GLUT8-TAP-HA were greater in cells transfected with *si*RNA targeting AP1 than in cells transfected with negative control *si*RNA. The levels of GLUT8-APA-HA and GLUT8-GPN-HA at the plasma membrane were not altered by the depletion of AP1. Images were obtained using a 60× oil objective lens.