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Characterization of a Mutant GLUT4 Lacking the N-Glycosylation Site: Studies in Transfected Rat Adipose Cells

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GLUT4, the insulin-responsive glucose transporter expressed primarily in muscle and adipose tissue, contains a single N-glycosylation site. We characterized a mutant GLUT4 lacking the N-glycosylation site ($Asn^{57} \rightarrow Gln$) in primary cultures of rat adipose cells. We transiently transfected cells with expression vectors for epitope-tagged GLUT4 containing either wild-type (GLUT4-HA) or mutant (GLN57-HA) cDNA sequences. Expression of GLN57-HA in adipose cells was ~10-fold lower than for GLUT4-HA even though mRNA levels for both recombinant transporters were comparable. Biosynthetic labeling studies showed markedly decreased incorporation of [^{35}S]-methionine/cysteine into GLN57-HA relative to GLUT4-HA consistent with either a decreased synthetic rate or accelerated degradation of GLN57-HA. Interestingly, transient transfection of GLUT4-HA and GLN57-HA in COS-7 cells (which do not express endogenous GLUT4) resulted in comparable levels of protein expression for both transporters. Thus, in the physiologically relevant adipose cell, glycosylation of GLUT4 appears to play an important functional role.

GLUT4 belongs to a family of facilitative glucose transporters containing twelve membrane spanning domains with a single N-glycosylation site in the first extracellular loop (1). It is expressed in a tissue-specific manner and is the major insulin-responsive glucose transporter in muscle and adipose tissue. The translocation of GLUT4 from an intracellular pool to the cell surface accounts for most of the insulin-stimulated increase in glucose uptake observed in insulin sensitive tissues (2). However, the molecular mechanisms underlying GLUT4 translocation are unknown. In general, modifications such as N-glycosylation contribute importantly to the proper folding, stability, sorting, and function of proteins (3). Previous studies using agents such as tunicamycin or various glycosidases and glycanases to inhibit or modify glycosylation (4–10), as well as studies using glycosylation deficient mutants (11,12) have suggested that proper glycosylation is functionally important for the ubiquitously expressed GLUT1 glucose transporter. We used transient transfection of rat adipose cells in primary culture (13–16) to characterize a mutant GLUT4 lacking the N-glycosylation site ($Asn^{57} \rightarrow Gln$) in a physiologically relevant cell.

METHODS

pCIS2. pCIS2 is an expression vector containing a CMV promoter and enhancer with a generic intron located upstream from the multiple cloning site (obtained from Dr. Cornelia Gorman, Genentech) (17). This vector gives high expression of recombinant genes in transfected rat adipose cells (13) and was used as the parent vector for subsequent constructions.

GLUT4-HA. pCIS2 containing the cDNA coding for human GLUT4 with the influenza hemagglutinin antigen epitope (HA1) located in the first exofacial loop of GLUT4 was constructed as described (14).

GLN57-HA. A mutation was introduced into GLUT4-HA at codon 57 so that glutamine is substituted for asparagine in the recombinant protein. pCIS-GLUT4 (the pCIS2 vector containing the cDNA for human GLUT4 (14)) was linearized with Eco RI and amplified by PCR using an upstream primer corresponding to pCIS2 (5′-GTC CAA CTG CAG CTC G-3′) and a downstream mutagenic primer corresponding to nucleotides 307-355 in GLUT4 (5′-GCT GGG TCC CTC AGG CCC

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CTG CCT CCC CAG CCA CGT CTC *CTG* GTA GCT C-3′, point mutations introduced by the primer are underlined). The resulting 389 bp PCR product was restriction digested with ClaI and SauI to generate a 356 bp cassette that was exchanged for the normal sequence in pCIS-GLUT4. The presence of the mutation (AAT → CAG) was confirmed by direct sequencing. Finally, the nucleotide sequence encoding the HA1 epitope was inserted into the SauI site of GLUT4 as previously described (14).

Isolated rat adipose cell preparation and transfection. Isolated adipose cells were prepared from epididymal fat pads of male rats by collagenase digestion as described (13,18). Cells were transfected by electroporation and cultured overnight as described (13–16).

Assay for cell surface epitope-tagged GLUT4. 20 h after electroporation, adipose cells were processed as described (13–16) and treated with insulin at final concentrations of 0, 0.024, 0.072, 0.3, or 60 nM at 37°C for 30 min. Cell surface epitope-tagged GLUT4 was determined by using the anti-HA antibody 12CA5 in conjunction with [^{125}I]-sheep anti-mouse IgG as described (14). Cells transfected with the parent vector (pCIS2) were used to determine non-specific binding of the antibodies. Typically, non-specific binding was ~30% of total binding to cells transfected with GLUT4-HA and maximally stimulated with insulin (14). The actual specific counts were comparable from experiment to experiment (see figure legends). The lipid weight from a 200 μl aliquot of cells was determined as described (19) and used to normalize the data for each sample.

Immunoprecipitation and immunoblotting of epitope-tagged GLUT4 in transfected adipose cells. Total membrane fractions prepared from adipose cells transfected with either pCIS2, GLUT4-HA, or GLN57-HA (3 μg DNA/cuvette) were immunoprecipitated with anti-HA antibody 12CA5 followed by SDS-PAGE and immunoblotting with anti-GLUT4 antibody as described (16).

Determination of relative mRNA levels of epitope-tagged glucose transporters in transfected adipose cells. Adipose cells were transfected with either GLUT4-HA or GLN57-HA (10 cuvettes per group, 3 μg of DNA per cuvette). 20 h after electroporation, cells from the two groups were mixed together and total RNA isolated. cDNA corresponding to mRNA for both wild-type and mutant epitope-tagged glucose transporters was obtained using reverse transcriptase PCR methods (20). An antisense oligonucleotide including sequence corresponding to the HA1 epitope was used to prime first strand cDNA synthesis as well as for the downstream primer in the PCR (5′-TCA GCA TAA TCA GGA ACA TCA TAA GGA TAA TCG ATC-3′). A sense oligonucleotide corresponding to pCIS2 sequence was used as the upstream primer in the PCR for amplification of cDNA (5′-GTC CAA CTG CAG CTC G-3′). Relative amounts of mRNA for GLUT4-HA and GLN57-HA were estimated by primer extension analysis. A ^{32}P-labeled oligonucleotide corresponding to nucleotides 291-311 of the sense strand of GLUT4-HA was created using polynucleotide kinase in the presence of γ-[^{32}P]-ATP (^{32}P-5′-AGA AGG TGA CTT GAA CAG AGC T-3′). The ^{32}P-labeled oligonucleotide (100,000 cpm in 1 μl) was annealed to amplified cDNA (30 ng) by heating to 95°C for 5 min, letting cool slowly to 42°C, and then placing on ice. A nucleotide solution was made by mixing 2 μl water, 5 μl ddTTP (5 mM), and 1 μl each dATP, dCTP, and dGTP (all at 10 mM). To extend the primer, we added 1 μl each of nucleotide solution, Sequenase 2.0, and dithiothreitol (0.1 M) to the reaction tube containing labeled primer and cDNA. This mixture was incubated at 42°C for 10 min before adding 10 μl stop solution (provided in Sequenase kit). In the case of the wild-type sequence, the primer was extended by 5 nucleotides prior to termination as a result of incorporation of ddTTP. For the mutant sequence, the primer was extended 12 nucleotides prior to termination. The primer extension reaction was subjected to electrophoresis at 60 W for 2.5 h through 17.5% Sequagel. Primer extension products were visualized by autoradiography of dried gels, and relative intensities were determined by quantitation on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Biosynthetic labeling of epitope-tagged GLUT4 in transfected adipose cells. Adipose cells were transfected with GLUT4-HA or GLN57-HA (45 cuvettes/group, 3 μg DNA/cuvette). Cells pooled from 15 cuvettes were placed in 6 cm petri dishes for overnight incubation. 20 h after electroporation, cells from each dish were washed two times with DMEM (containing 5% albumin without cysteine or methionine), resuspended in 4 ml media, and placed into new 6 cm dishes for incubation at 37°C, 5% CO_2. After 2 hr, 400 μCi of [^{35}S]-methionine/cysteine (1261 Ci/mmol, ICN Pharmaceuticals; Costa Mesa, CA) was added to each dish for 30 min, 1 h, or 2 h. After incubation with labeled methionine/cysteine for the appropriate time, cells were washed twice with TES (16) and total membrane fractions were prepared and subjected to immunoprecipitation with the anti-HA antibody 12CA5 followed by SDS-PAGE (16). Labeled epitope-tagged GLUT4 was visualized by autoradiography and quantitation was done by PhosphorImager analysis.

Transfection of COS-7 cells. COS-7 cells were grown to 70% confluence in 100 mm plates in DME/F12 medium containing 10% FCS and transfected with 10 μg plasmid DNA (pCIS2, GLUT4-HA, or GLN57-HA) using the calcium phosphate method (21). 42 hr after transfection, cells were scraped from the plates (two plates were combined for each sample), homogenized in 500 μl buffer A (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, 10 μg/ml leupeptin, 1 mM PMSF) and centrifuged at 10,000 g for 5 min. The supernatant was centrifuged for 90 min at 200,000 g, the resulting pellets were resuspended in 25 μl buffer A containing 1% Triton X-100 and processed as described above for immunoprecipitation and immunoblotting of adipose cells.

Statistical analysis. Paired t-tests were used where appropriate. p values less than 0.05 were considered significant. Insulin dose-response curves were fit to data as described (14–16).

RESULTS

Insulin-stimulated translocation of epitope-tagged GLUT4. We compared insulin dose-response curves for cell surface epitope-tagged GLUT4 in rat adipose cells transfected with either GLUT4-HA or GLN57-HA and (Fig. 1). In the absence of insulin, levels of cell surface GLUT4-HA and GLN57-HA were not significantly different. In cells expressing GLUT4-HA, insulin caused an ~2.5 fold increase in cell surface epitope-tagged GLUT4 with an ED_{50} of approximately 0.03 nM. In contrast, we were unable to detect a significant increase in cell surface epitope-tagged GLUT4 in response to insulin in cells transfected with GLN57-HA.

Expression of GLUT4-HA and GLN57-HA. To compare levels of expression of GLUT4-HA and GLN57-HA in transfected adipose cells, we immunoprecipitated epitope-tagged glucose transporters from total membrane fractions using an antibody directed against the HA epitope followed by immunoblotting with an anti-GLUT4 antibody (Fig. 2). Expression of GLN57-HA was markedly decreased relative to GLUT4-HA (~10-fold difference).

mRNA levels for GLUT4-HA and GLN57-HA. To determine if differences in expression of GLUT4-HA and GLN57-HA in transfected adipose cells were due to differences in transcription, we compared relative levels of mRNA for GLUT4-HA and GLN57-HA. After isolating total RNA, we performed RT-PCR using a primer that included sequence corresponding to the HA epitope. Thus, we amplified only mRNA for GLUT4-HA and GLN57-HA. The amplified RT-PCR products were used as templates to generate primer extension products reflecting the relative levels of recombinant wild-type and mutant mRNA. We estimate that the ratio of mRNA levels for GLUT4-HA and GLN57-HA was approximately 1:1.4 (Fig. 3).

Biosynthetic labeling. To determine if differences in protein synthesis or degradation account for differences in steady-state levels of GLUT4-HA and GLN57-HA we labeled transfected adipose cells with [^{35}S]-methionine-cysteine for 30, 60, or 120 min. The amount of label incorporated into GLN57-HA was ~4-fold lower than for GLUT4-HA at 30 min. Although the amount of labeled transporter increased with each successive time point (Fig. 4), label incorporation into GLN57-HA remained ~4-fold less than that for GLUT4-HA.

Transient transfection of COS-7 cells. To examine the effect of N-glycosylation of GLUT4 in a heterologous cell type, we transiently transfected GLUT4-HA and GLN57-HA into COS-7 cells

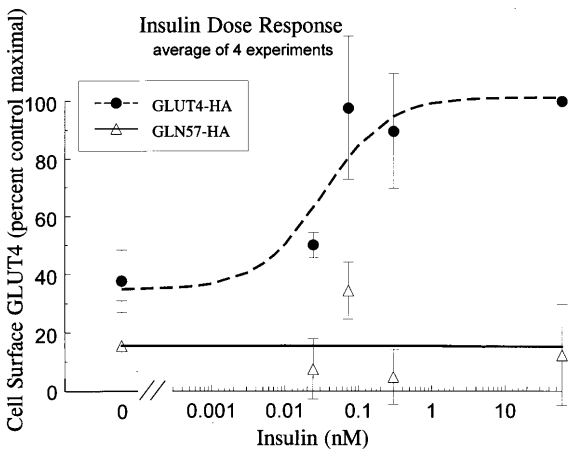


FIG. 1. Recruitment of epitope-tagged GLUT4 to the cell surface of adipose cells transfected with GLUT4-HA ((lb)) or GLN57-HA (△). 3 μg/cuvette of plasmid DNA was used in each experiment. Cells from 30 cuvettes were pooled for the GLUT4-HA and GLN57-HA groups. A group transfected with pCIS2 alone (10 cuvettes) was used to determine non-specific binding. Results are means ± SEM of four independent experiments. Data are expressed as a percentage of cell surface GLUT4 for the control group (GLUT4-HA) treated with 60 nM insulin. The actual value for cell-associated radioactivity for the control group at 60 nM insulin was 1029 ± 81 cpm.

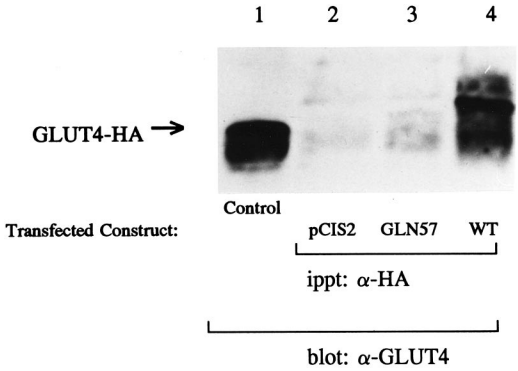


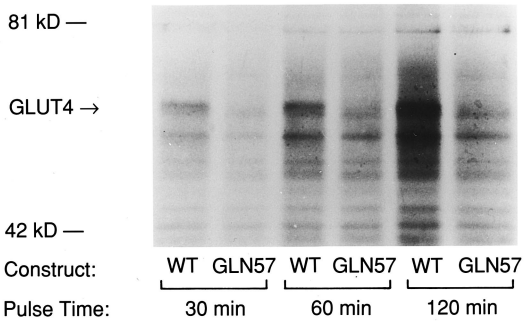
FIG. 2. Expression of epitope-tagged glucose transporters in transfected adipose cells. Total membrane fractions prepared from cells transfected with pCIS2, GLN57-HA, or GLUT4-HA (3 μg DNA/cuvette, 20 cuvettes/group) were immunoprecipitated with anti-HA antibody followed by immunoblotting with GLUT4 antibody. Cell extract from rat skeletal muscle was run as a positive control (lane 1). Intensities of bands determined by scanning densitometry for pCIS2 (lane 2), GNL-57-HA (lane 3), and GLUT4-HA (lane 4) are 180, 950, and 11840 respectively (in arbitrary density units). This is a representative blot of an experiment that was performed independently twice.

(COS-7 do not express endogenous GLUT4). In contrast to adipose cells, expression of GLN57-HA was only ~30% less than that for GLUT4-HA (Fig. 5). In addition, a broader band was seen on immunoblot for GLUT4-HA as compared with GLN57-HA. This presumably reflects glycosylation of GLUT4-HA and its absence on GLN57-HA since treatment of adipose cell extracts with N-glycanse results in a decrease in the apparent molecular mass of GLUT4-HA to a level similar to that of GLN57-HA and is without effect on GLN57-HA (data not shown).

DISCUSSION

An enormous amount of work has been done to characterize the biological roles of protein glycosylation (22). However, it remains difficult to predict precise roles of glycosylation *a priori.* We used site-directed mutagenesis to create a mutant GLUT4 where the N-glycosylation site is abolished. We characterized this mutant in the physiologically relevant rat adipose cell using transient transfection (13). To distinguish between recombinant and endogenous glucose transporters we engineered an epitope tag (HA) into both wild-type (GLUT4-HA) and mutant (GLN57-HA) recombinant GLUT4. We previously demonstrated that GLUT4-HA behaves similarly to endogenous GLUT4 with respect to insulin-stimulated translocation (14–16). In the present study,

FIG. 3. Quantitation of relative mRNA levels for GLUT4-HA and GLN57-HA in transfected adipose cells. Primer extension was performed on DNA derived from mRNA for GLUT4-HA and GLN57-HA obtained by RT-PCR of total RNA from transfected cells (see methods). For GLUT4-HA, primer extension results in a 26 bp product while for GLN57-HA, primer extension results in a 33 bp product. By PhosphorImager analysis, the ratio of intensity of the 33 bp band (GLN57-HA) to the 26 bp band (GLUT4-HA) is 1.4 to 1.



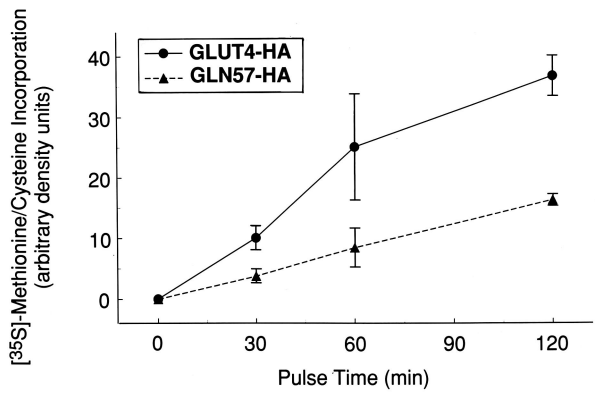


FIG. 4. Biosynthetic labeling of GLUT4-HA (WT) and GLN57-HA (GLN57) in transfected adipose cells. Cells were pulsed with [^{35}S]-methionine/cysteine for 30, 60, or 120 min. Total membrane fractions were isolated, immunoprecipitated with an anti -HA antibody and subjected to SDS-PAGE followed by autoradiography. A representative blot from an experiment that was repeated independently twice is shown (upper panel). Immunoblotting with an anti-GLUT4 antibody was done on duplicate blots to verify the location of the epitope-tagged GLUT4 bands (not shown). The average of the Phosphorimager quantitation analysis for two independent experiments is shown in the lower panel (mean ± S.D.).

adipose cells expressing GLUT4-HA showed a significant increase in cell surface GLUT4-HA after insulin stimulation as expected. In contrast, adipose cells transfected with GLN57-HA did not have a detectable increase in cell surface GLN57-HA after insulin stimulation. This is due, in part, to a 10-fold decrease in expression for GLN57-HA as compared to GLUT4-HA. That is, our assay may not be sensitive enough to detect an increase in cell surface GLN57-HA under conditions where total GLN57-HA expression is so low. Alternatively, N-glycosylation of GLUT4 may be required for insulin-stimulated translocation to the cell surface. Studies in CHO cells transfected with glycosylation deficient mutants of GLUT1 suggested that N-glycosylation of GLUT1 is important for both catalytic activity of the transporter as well as proper subcellular localization (11,12). Unfortunately, we were unable to assess these characteristics of GLN57-HA because of low expression levels.

To determine if differences in steady-state levels of GLUT4-HA and GLN57-HA were due to differences in transcription, we estimated relative mRNA levels. Each step of the assay after transfection was performed on a single pooled sample. Therefore, technical issues such as efficiency of RNA preparation, linearity of PCR, and efficiency of primer extension should not contribute to differences observed in relative mRNA levels. Since, there was slightly more mRNA for GLN57-HA than for GLUT4-HA, it is unlikely that decreased transcription accounts for the decrease in steady-state expression of GLN57-HA.

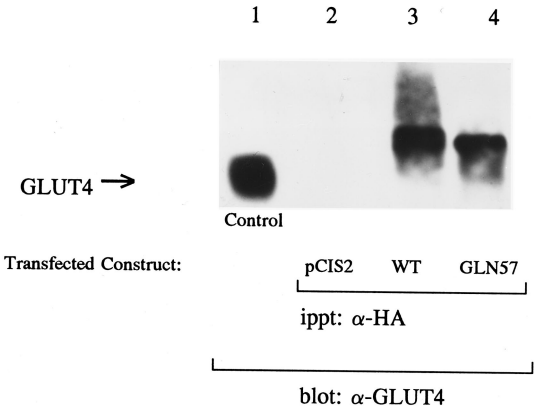


FIG. 5. Expression of GLUT4-HA and GLN57-HA in transfected COS-7 cells. Total membrane fractions prepared from cells transfected with pCIS2, GLUT4-HA, or GLN57-HA were immunoprecipitated with an anti-HA antibody followed by immunoblotting with an anti-GLUT4 antibody in conjunction with chemiluminescent detection. Cell extract from rat skeletal muscle was run as a positive control (lane 1). Intensities of bands determined by scanning densitometry for pCIS2 (lane 2), GLUT4-HA (lane 3), and GLN57-HA (lane 4) are 100, 20600, and 13600 respectively (in arbitrary density units). This is a representative blot of an experiment that was repeated independently three times.

Our results imply that GLN57-HA has either a decreased rate of synthesis, accelerated rate of degradation, or both. We metabolically labelel transfected adipose cells under conditions where we hoped to estimate initial rates of biosynthesis for GLUT4-HA and GLN57-HA. If one assumes a single compartment for distribution of glucose transporters, measurement of label incorporation at early time points (relative to the half-life of the protein) reflects synthesis with little contribution from degradation. Estimates of degradation rates can then be made since steady-state levels are known. If one applies this analysis to our labeling studies it would appear that the synthesis of GLN57-HA is markedly impaired. However, it seems unlikely that a point mutation in the coding region of the cDNA would cause a significant inhibition of the rate of translation and synthesis. A more plausible interpretation of our data involves the assumption that multiple compartments for distribution of GLUT4 exist (23,24). It is possible that the non-glycosylated mutant GLUT4 is accumulating in the ER where it undergoes rapid degradation because it is not folded properly or does not contain proper signals to undergo further processing and intracellular targeting. The small amount of mutant GLUT4 that escapes the ER accounts for the small amount of labeled GLN57-HA that we detect. Thus, accelerated degradation of a sequestered mutant is also consistent with our data. There are many examples of proteins where lack of glycosylation results in accelerated degradation due to sequestration and susceptibility to degradative enzymes (22).

To study N-glycosylation of GLUT4 in a heterologous cell type, we transiently transfected COS-7 cells (cells that do not express endogenous GLUT4). Unlike adipose cells, levels of GLUT4-HA and GLN57-HA were roughly comparable in transfected COS-7 cells. These experiments help to rule out the possibility that the decrease in detection of GLN57-HA in transfected adipose cells is due to technical issues such as impaired recognition of GLN57-HA by the anti-HA or anti-GLUT4 antibodies. In addition, the COS cell experiments support our interpretation of accelerated degradation of GLN57-HA in adipose cells since it seems improbable that the point mutation in GLN57-HA would cause a cell-specific impairment in translation. The fact that levels of expression for the wild-type and mutant GLUT4 were similar in COS-7 cells but not in adipose cells is interesting because the ability of GLUT4 to translocate appropriately in response to insulin is tissue specific. Studies by Ebina and colleagues have shown that in CHO cells co-expressing GLUT4 and either insulin receptors, EGF receptors or PDGF receptors, modest translocation of GLUT4 occurs with ligand stimulation (25–27). However, in most studies, when GLUT4 is expressed by recombinant methods in heterologous cell types such as CHO, COS, or NIH-3T3

cells, GLUT4 does not translocate appropriately in response to insulin even when the cells are also overexpressing insulin receptors (28–30). This suggests that these tissue culture cells are missing machinery present in insulin target tissues (e.g., muscle and adipose cells) that is required for regulation of GLUT4 translocation by insulin. Invoking teleology, the COS-7 cell may be indifferent to the absence of glycosylation on GLUT4 while the physiologically relevant adipose cell is able to appropriately recognize and degrade non-glycosylated forms of GLUT4.

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