Glucose transporters (GLUTs)\(^2\) mediate ATP-independent facilitative diffusion of glucose across cell membranes (1, 2). All vertebrate GLUTs contain 12 transmembrane (TM) domains and a conserved N-glycosylation consensus site (Asn-Xaa-Thr/ Ser) that is typically positioned in the first or fifth extracellular loop. Responsiveness to insulin is a functional feature of GLUT4, and it is therefore believed to play an essential role in glucose homeostasis. GLUT4 is selectively expressed in insulin-responsive tissues such as adipocytes and skeletal muscle cells (3). Newly synthesized and glycosylated GLUT4 enters a continuous recycling pathway that concentrates most GLUT4 in intracellular storage vesicles under basal conditions (4–6). Stimulation by insulin induces rapid translocation of GLUT4 from these intracellular storage vesicles to the plasma membrane, resulting in an increase in glucose uptake. The molecular mechanism responsible for the insulin-regulated translocation of GLUT4 has been studied extensively in insulin-responsive cells (7–10).

It is widely accepted that type 2 diabetes is caused by the impaired ability of insulin to regulate glucose homeostasis adequately, as a result of insulin resistance in multiple tissues. GLUT4 has drawn intense attention in the context of type 2 diabetes development, and several studies have found an association between impaired translocation/recycling of GLUT4 and insulin resistance (4, 11, 12). Clarification of the molecular events involved in insulin-stimulated GLUT4 translocation is therefore of medical importance.

The glycans on glycoproteins are known to play important roles in the physicochemical properties of proteins, including their solubility, proper folding, and thermal stability, as well as in their physiological properties, including their bioactivities or intracellular/intercellular trafficking (13, 14). A GLUT4 glycosylation mutant was previously found to be nonfunctional primarily because of its very low level of expression in rat adipose cells (15). However, the precise role of the N-glycan chain on GLUT4 has not yet been clarified.

In this study, we used a GLUT4 mutant lacking the sole N-glycosylation site (N57Q) to assess the importance of the N-glycan chain on GLUT4. To this end, stable transfectants expressing C-terminally enhanced GFP (EGFP)-tagged wild-type (WT) GLUT4 or the GLUT4 glycosylation mutant were isolated using HeLa cells. The overall stability of the protein was compromised in the mutant, suggesting that the N-glycan chain contributes to quality control of newly synthesized GLUT4. Moreover, our results clearly indicated that WT and mutant GLUT4 exhibited different intracellular distributions, i.e. insulin-regulated aminopeptidase (IRAP), a marker for GLUT4 storage vesicles, markedly co-localized with WT GLUT4, whereas its co-localization with the mutant was much less apparent. Consistent with this observation, WT GLUT4 retained the ability for enhanced cell surface expression upon insulin treatment in HeLa cells, whereas the mutant failed to respond to insulin. Interestingly, WT GLUT4 also lost sensitivity to insulin when cells were treated with kifunensine (KIF), an inhibitor of endoplasmic reticulum (ER)/Golgi α-mannosidase I, strongly indicating an important role for N-glycan structure in GLUT4 trafficking. In contrast, the glucose transport activity of the cell surface transporter was not altered between

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\(^2\)The abbreviations used are: GLUT, glucose transporter; EGFP, enhanced GFP; ER, endoplasmic reticulum; IRAP, insulin-regulated aminopeptidase; KIF, kifunensine; KRB, Krebs-Ringer bicarbonate buffer; 2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; PNGase, peptide-N-glycanase; TTR, transferrin receptor; TM, transmembrane.

The facilitative glucose transporter GLUT4 plays a key role in regulating whole body glucose homeostasis. GLUT4 dramatically changes its distribution upon insulin stimulation, and insulin-resistant diabetes is often linked with compromised translocation of GLUT4 under insulin stimulation. To elucidate the functional significance of the sole N-glycan chain on GLUT4, wild-type GLUT4 and a GLUT4 glycosylation mutant conjugated with enhanced GFP were stably expressed in HeLa cells. The N-glycan contributed to the overall stability of newly synthesized GLUT4. Moreover, cell surface expression of wild-type GLUT4 in HeLa cells was elevated upon insulin treatment, whereas the glycosylation mutant lost the ability to respond to insulin. Subcellular distribution of the mutant was distinct from that of wild-type GLUT4, implying that the subcellular localization required for insulin-mediated translocation was impaired in the mutant protein. Interestingly, kifunensine-treated cells also lost sensitivity to insulin, suggesting the functional importance of the N-glycan structure for GLUT4 trafficking. The Km or turnover rates of wild-type and mutant GLUT4, however, were similar, suggesting that the N-glycan had little effect on transporter activity. These findings underscore the critical roles of the N-glycan chain in quality control as well as intracellular trafficking of GLUT4.
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WT and mutant GLUT4, suggesting that the N-glycan was not important for transporter activity. Taken together, these findings indicate critical roles for the N-glycan chain in quality control and intracellular trafficking of GLUT4.

EXPERIMENTAL PROCEDURES

Plasmid Construction, Cell Culture, and Transfection—A human GLUT4 cDNA was purchased from Open Biosystems (Huntsville, AL) and subcloned into pEGFP-N1 (Clontech, Mountain View, CA). The GLUT4-EGFP N57Q mutant was constructed by substituting Asn57 (AAT) with Gln (CAG) using a QuikChange II Site-directed Mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer’s protocol. HA-GLUT4-EGFP was constructed by inserting an HA epitope into GLUT4-EGFP constructs between Glu67 and Gly68 in the first extracellular loop (16) using a QuikChange II Site-directed Mutagenesis kit, with primers 5′′-gaggcaggggcctgagtacccatacg-acctcagactacgacagcagtacagtacg-3′ and 5′′-ggtatggagctgggt-gctctggtctctggtctctggtctg-3′. See also supplemental Experimental Procedures.

HeLa cells were cultured in DMEM containing 10% FBS and antibiotics (100 units/ml penicillin and 0.1 mg/ml streptomycin). The cells were transfected with the plasmids using the FuGENE HD transfection reagent (Roche Applied Sciences), according to the manufacturer’s protocol. The transfected cells were maintained in medium supplemented with 0.8 mg/ml G418 (Nacalai Tesque, Kyoto, Japan). Cells stably expressing GLUT4-EGFP (WT or N57Q) were cultured in medium supplemented with 0.8 mg/ml G418 (Nacalai Tesque, Kyoto, Japan). Cells stably expressing GLUT4-EGFP (WT or N57Q) were cultured in medium supplemented with 0.8 mg/ml G418 (Nacalai Tesque, Kyoto, Japan). Cells stably expressing GLUT4-EGFP (WT or N57Q) were cultured in medium supplemented with 0.8 mg/ml G418 (Nacalai Tesque, Kyoto, Japan).

Antibodies and Reagents—Anti-GFP polyclonal antibody was purchased from Molecular Probes (Eugene, OR), anti-GAPDH monoclonal antibody was obtained from Millipore (Temecula, CA), and anti-β (F-7) was from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies for Western blotting analyses were purchased from GE Healthcare and for immunofluorescence from Molecular Probes. Human recombinant insulin (4 mg/ml solution) was obtained from Invitrogen, and peptide-N-glycanase (PNGase) F was from Roche Applied Sciences. A BCA protein assay kit, Sulfo-NHS-Biotin, and NHS-SS-Biotin were from Thermo Fisher Scientific (Waltham, MA).

PNGase F Digestion and Western Blot Analysis—HeLa cells stably expressing GLUT4-EGFP (WT or N57Q) were cultured in 24-well plates. The cells were washed twice with PBS and lysed in 50 μl of lysis buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 5 mM EDTA) containing Complete™ protease inhibitor EDTA-free (Roche Applied Sciences) at 4 °C. The lysates were centrifuged at 15,000 rpm for 15 min at 4 °C, and the protein concentrations were determined by the BCA protein assay. Aliquots of the lysates (3 μg of protein) were incubated with 0.5 unit of PNGase F overnight at 37 °C. Aliquots of the lysates incubated under the same conditions without PNGase F were used as controls. The incubated mixtures were subjected to 7.5% SDS-PAGE and transferred onto a PVDF membrane (Millipore). The membrane was subjected to Western blotting procedures and visualized using a LAS3000mini (Fujifilm Co., Tokyo, Japan) and Immobilon Western Reagents (Millipore).

Cycloheximide Chase Analysis—Cells were incubated with 100 μg/ml cycloheximide (Sigma) and 10 μM MG-132 (Peptide Institute Inc., Osaka, Japan) for various periods. Cell lysates were prepared and subjected to Western blot analysis as described above. For KIF treatment, cells were incubated with 2 μg/ml KIF (Cayman Chemical, Ann Arbor, MI) for 2 days prior to cycloheximide chase assay.

Cell Surface Biotinylation and Internalization Assay for GLUT4—HeLa cells stably expressing GLUT4-EGFP (WT or N57Q) were cultured in 6-well plates. The cells were serum-starved in Krebs-Ringer bicarbonate buffer (KRB) (129 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 5 mM NaHCO3, 10 mM HEPES, 3 mM d-glucose, 2.5 mM CaCl2, 1.2 mM MgCl2, and 0.2% BSA; pH adjusted to 7.4 with NaOH) for 3 h, and then incubated with or without 100 nM insulin for 30 min at 37 °C. The cells were washed three times with ice-cold PBS and incubated with ice-cold PBS containing 1 mM Sulfo-NHS-Biotin for 1 h at 4 °C. After three washes with 100 mM glycine in PBS, the cells were lysed as described above. The biotinylated proteins were pulled down with streptavidin-Sepharose (GE Healthcare) and subjected to Western blot analysis.

Internalization assays were performed as described previously (17, 18) with minor modifications. Briefly, HeLa cells stably expressing GLUT4-EGFP (WT or N57Q) were serum-starved in KRB and then incubated with 100 nM insulin for 30 min at 37 °C. The cells were washed with ice-cold KRB and incubated on ice with ice-cold KRB containing 0.5 mg/ml NHS-SS-Biotin twice for 15 min at 4 °C. After two washes with ice-cold KRB, the cells were cultured in prewarmed KRB for 0, 30, or 60 min at 37 °C. The cells were then washed twice with ice-cold KRB containing 10% FBS, and the remaining cell surface biotin was removed by incubating the cells in a reducing solution (50 mM glutathione (reduced form), 75 mM NaCl, 0.3% NaOH, and 10% FBS) twice for 20 min at 4 °C. The reaction was quenched by 5 mg/ml iodoacetamide in KRB, and cell lysates were prepared. The biotinylated cell surface proteins were pulled down with streptavidin-Sepharose for further analyses.

Time Lapse Imaging of Living Cells—Experiments were carried out using an FV1000-D Laser scanning confocal microscope (Olympus, Tokyo, Japan) equipped with an incubator, and the cells were maintained at 37 °C throughout the experiments. HeLa cells expressing GLUT4-EGFP (WT or N57Q) were plated on glass-bottomed dishes (35-mm diameter) and serum-starved in KRB. The cells were then stimulated with insulin (100 nM), and time lapse images were acquired at 5-min intervals at 37 °C under a 5% CO2 atmosphere.
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For internalization assays, the cells were serum-starved in KRB and then stimulated with insulin (100 nM) for 30 min just prior to imaging. After washing to remove insulin, images of the cells in KRB were acquired under the same conditions described above.

Immunofluorescence Microscopy—HeLa cells expressing GLUT4-EGFP (WT or N57Q) were grown on cover glasses (12-mm diameter) placed in 24-well plates in medium and serum-starved in KRB. Cells were washed with PBS and fixed with 3% paraformaldehyde-PBS for 20 min, washed twice with PBS, permeabilized with 50 μg/ml digitonin for 15 min, and incubated with 1% BSA-PBS for 30 min. Subsequently, cells were stained with anti-IRAP (Cell Signalling Technology) or anti-transferrin receptor (TfR) (BD Transduction Laboratories) at 1:50 dilution for 1 h at room temperature followed by Alexa Fluor 546-labeled secondary antibody for 1 h at room temperature, washed, mounted with a drop of Vectashield with DAPI (Vector Laboratories, Burlingame, CA), and observed by laser scanning confocal microscopy (FV500). See also supplemental Experimental Procedures.

Measurement of Cell Surface GLUT4 Trafficking by Flow Cytometry—Flow cytometry analysis was performed as described previously (19), with minor modifications. Briefly, L6 myoblasts transiently expressing HA-GLUT4-EGFP (WT or N57Q) were cultured and differentiated in 6-well plates. Myotubes were serum-starved in KRB and then incubated in the presence or absence of 100 nM insulin for 30 min at 37 °C. The cells were transferred to 4 °C, washed with ice-cold KRB, and incubated with a 1:200 dilution of anti-HA in 2% BSA-KRB for 1 h at 4 °C. After three washes with ice-cold KRB, the cells were incubated with a 1:400 dilution of Alexa Fluor-633-labeled antiserum in 2% BSA-KRB for 1 h at 4 °C. The cells were then washed three times with KRB, detached by incubation with 1 mM EDTA-PBS for 10 min at 37 °C, and fixed with 1% paraformaldehyde-PBS for 10 min at room temperature. After three washes with 0.5% BSA-PBS, the cells were resuspended in 0.5% BSA-PBS. The fluorescence of stained cells was measured using a BD LSR flow cytometer and CellQuest Pro software (BD Biosystems). In each case, 5,000 GFP-positive cells were counted.

Glucose Transport Assay—The glucose uptake activity was measured using 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]-2-deoxy-D-glucose (2-NBDG) (Peptide Institute Inc.), as described previously (20). Briefly, cells in 24-well plates were serum-starved in KRB and then incubated in glucose-free KRB containing 100 nM insulin for 20 min at 37 °C. To measure the time course of 2-NBDG transport, the cells were incubated with 500 μM 2-NBDG in glucose-free KRB containing 100 nM insulin for various times, and the reactions were stopped by the addition of ice-cold KRB containing 0.5 mM phloretin. The cells were then washed three times with ice-cold PBS and solubilized in lysis buffer. The fluorescence intensities were measured using an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) with λ<sub>ex</sub> = 465 nm and λ<sub>em</sub> = 540 nm. The kinetic parameters were determined under similar conditions with various concentrations of 2-NBDG (100 μM, 200 μM, 500 μM, 1 mM, or 2 mM), and the reactions were carried out for 2 min to determine the initial velocity of the transport.

RESULTS

N-Glycosylation of GLUT4 Contributes to Its Stability—EGFP tagging does not affect the insulin-responsive trafficking of GLUT4 (21, 22). C-terminally EGFP-tagged WT GLUT4 or its glycosylation mutant (N57Q) was therefore transfected into HeLa cells to provide insights into the roles of the N-glycan of GLUT4.

Consistent with previous observations using rat adipose cells (15), it was found that N57Q was expressed at a lower level than WT after transient transfection into HeLa cells. Cells stably expressing similar amounts of WT or N57Q were therefore selected using a FACS, and the isolated cells were used for further analyses. The N-glycosylation of GLUT4-EGFP was confirmed by SDS-PAGE in cells with or without PNGase F treatment (Fig. 1A). Cell proliferation did not differ significantly between HeLa cells expressing WT and N57Q (supplemental Fig. S1), but transfected cells expressing N57Q were smaller (data not shown).

To examine the stability of N57Q and WT GLUT4, cycloheximide chase experiments were carried out. N57Q was degraded more rapidly than WT in HeLa cells (Fig. 1B). This finding indicates that the N-glycan chain is critical for the stability of GLUT4 and may at least partly explain the low level of expression of the mutant protein in various cells. Consistent with this idea, when the proteasomal activity was inhibited by MG-132, N57Q degradation was delayed to the level of WT, whereas that of WT was barely affected (Fig. 1B). Collectively, these findings suggest that the compromised protein stability of N57Q was caused mainly by the quality control system for newly synthesized proteins and that the N-glycan on GLUT4 is critical for this protein to escape proteasomal degradation.

The stability of the GLUT4 proteins on the cell surface was also examined. Cell surface proteins were labeled with biotin and chased for specified times. The remaining biotinylated GLUT4 proteins were then detected by Western blot analysis. In sharp contrast to the overall stability, N57Q on the cell surface was found to be as stable as WT (Fig. 1C). These findings suggest that the rapid degradation of N57Q occurred in its intracellular pool but that once it reached the cell surface, its half-life was not significantly affected by the absence of the N-glycan.

N-Glycan Is Important for Insulin-mediated Cell Surface Expression of GLUT4—GLUT4 is known to change its main subcellular localization from intracellular vesicles to the cell surface in response to insulin treatment (23). We therefore examined the responses of GLUT4 proteins expressed in HeLa cells to insulin treatment. HeLa cells are known to express insulin receptors (24) as well as AS160 (25), a key molecule for mediating insulin-stimulated GLUT4 translocation, and are therefore expected to respond to insulin treatment. Insulin stimulation of HeLa cells is also known to induce PI3K signaling (26). These previous observations led us to speculate that most, if not all, of the insulin-mediated signal transductions are intact in HeLa cells.

The subcellular distributions of WT and N57Q were examined using confocal microscopy. A dominant localization in dot-like structures inside the cells was evident for WT (Fig. 2A,
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FIGURE 1. Effect of glycosylation on the stability of GLUT4-EGFP in HeLa cells. A, Western blotting of WT GLUT4-EGFP and its N57Q glycosylation mutant. Cell lysates from HeLa cells expressing GLUT4(WT)-EGFP or its mutant GLUT4(N57Q)-EGFP were analyzed by Western blotting with an anti-GFP antibody. B, cycloheximide (CHX) chase experiments for GLUT4-EGFP. Cells were incubated with 100 μg/ml CHX, with or without 10 μM MG-132 (MG) for the indicated times. The amounts of detected GLUT4-EGFP were normalized by cell numbers. The data represent the means ± S.D. (error bars) of three independent experiments. C, cell surface biotinylation chase analysis of GLUT4-EGFP. Cell surface proteins on HeLa cells expressing GLUT4(WT)-EGFP or the nonglycosylated mutant (N57Q) were labeled with biotin and incubated for the indicated times. Remaining biotinylated GLUT4-EGFP was detected by Western blotting. The data represent the means ± S.D. of three replicate samples.

left panels for WT). A similar major intracellular localization was observed for N57Q, although the dot-like structures were less clear (Fig. 2A, left panels for N57Q). The dot-like structures for WT co-localized well with IRAP, one of the known components of GLUT4-specific vesicles referred to as "GLUT4 storage vesicles" (27, 28) (Fig. 2B, panels for WT). In contrast, co-localization of N57Q with IRAP was much less apparent (Fig. 2B, panels for N57Q). Both WT and N57Q were partially distributed in TfR-containing recycling vesicles (Fig. 2C). The response to insulin in HeLa cells was examined using time lapse analysis. Time-dependent translocation of WT to the cell surface was clearly observed (Fig. 2A, WT clones 1 and 2), indicating that GLUT4-EGFP was capable of responding to insulin treatment in HeLa cells. In sharp contrast, no notable change in distribution of N57Q was observed (Fig. 2A, N57Q clones 1 and 2). Similar results were obtained for 3T3-L1 adipocytes and L6 myotubes, where GLUT4 is known to be expressed and to respond to insulin treatment (supplemental Fig. S2). Collectively, these findings suggest that the N-glycan on GLUT4 is critical for intracellular trafficking, which in turn affects its insulin-mediated translocation to the cell surface.

To confirm the effects of the N-glycan on GLUT4 translocation, cell surface biotinylation experiments were carried out with or without insulin treatment. The cell surface expression
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Figure 3. Biochemical characterization of translocation of GLUT4-EGFP WT and N57Q in HeLa cells. A, cell surface biotinylation of GLUT4-EGFP. HeLa cells expressing GLUT4-EGFP (WT) or the nonglycosylated mutant (N57Q) were serum-starved and then stimulated with insulin. The cell surface proteins were labeled with biotin and pulled down by streptavidin (SA) conjugated beads. Biotinylated GLUT4-EGFP was detected by Western blotting. B, quantification of cell surface GLUT4-EGFP. The GLUT4 surface-to-input ratios were normalized as the average value of WT insulin-untreated samples (WT insulin −) set to 1. The data represent the means ± S.D. (error bars) of triplicate samples.

of WT was markedly increased upon insulin treatment (Fig. 3A, lanes 2 and 3), whereas no notable difference was observed for N57Q (Fig. 3A, lanes 5 and 6). Quantification of the data in Fig. 3A showed that the cell surface expression level of N57Q under basal conditions was higher than that of WT, possibly as a result of correct targeting of N57Q to the intracellular storage vesicles (Fig. 3B). It also demonstrated the insulin induced up-regulation of cell surface expression of WT, but not N57Q. These results suggest that the N-glycan on GLUT4 is critical for its insulin-mediated translocation.

The N57Q mutation is associated with a distinct cellular distribution as well as insulin insensitivity of the GLUT4 protein; however, it is possible that the change in the protein structure induced by the N57Q mutation, rather than the N-glycan, is responsible for the altered behavior of the GLUT4 protein. We therefore examined the effects of KIF, an inhibitor of ER/Golgi α-mannosidase I, on the insulin sensitivity of WT GLUT4, to determine whether its insulin sensitivity depended on the N-glycan structure. HeLa cells expressing GLUT4-EGFP (WT)-EGFP were treated with 2 μg/ml KIF for 2 days. As shown in Fig. 4A, GLUT4 in KIF-treated cells produced a faster migrating band on SDS-PAGE than KIF-untreated GLUT4 (compare lane 1 with lane 4). KIF-treated GLUT4 also became sensitive to treatment with endo-β-N-acetylgalactosaminidase H digestion (compare lane 4 with lane 5), clearly suggesting a change in the overall N-glycan structure on GLUT4 in the presence of KIF. After confirming the change in N-glycan structure, we examined the effect of KIF on GLUT4 stability. As shown in Fig. 4B, KIF had little effect on the stability of GLUT4 protein, indicating that the N-glycan structure was not critical for GLUT4 protein stability. The effects of KIF on GLUT4 insulin responsiveness were examined by cell surface biotinylation assay (Fig. 4C). Surprisingly, WT GLUT4 in KIF-treated cells lost its insulin responsiveness and, as with N57Q (Fig. 3B), showed increased cell surface expression levels compared with KIF-untreated cells under basal conditions. These results collectively indicate that the insulin responsiveness, but not the protein stability, of GLUT4 is N-glycan structure-dependent. Flow cytometry revealed similar results in L6 myotubes expressing HA-GLUT4-GFP, which possesses an HA epitope in the first extracellular loop. Cell surface GLUT4 was labeled with Alexa Fluor 633 via the HA epitope. As shown in Fig. 5, enhanced cell surface expression of HA-GLUT4(WT)-EGFP in L6 myotubes was apparent upon insulin treatment (left panel), whereas no notable change was observed for N57Q (middle panel). Moreover, WT HA-GLUT4-GFP lost its insulin responsiveness in KIF-treated L6 cells (right panel), implying that the effect of N-glycan structure on insulin-mediated translocation is a general phenomenon, rather than a cell type-specific event.

N-Glycan Is Also Important for Internalization of GLUT4 upon Removal of Insulin—To evaluate further the effects of the N-glycan on GLUT4 translocation, GLUT4 internalization following insulin removal from the medium was also investigated. The insulin response is known to be reversible, and the distribution of GLUT4 returns to basal levels upon removal of insulin (5). Time lapse analyses clearly showed the apparent internalization of WT to form dot-like structures upon the removal of insulin, whereas the localization of N57Q did not appear to change (Fig. 6A). These findings further indicate that the N-glycan on GLUT4 is important for its translocation in response to insulin.

There are two possible explanations for the observed insulin insensitivity of N57Q: (i) no response to the signal or (ii) balanced exocytosis/endocytosis, i.e., active recycling between the cell surface protein and intracellular pool at a steady state. To distinguish between these two possibilities, internalization assays were carried out for WT and N57Q upon removal of insulin. Cell surface WT and N57Q were labeled with a disulfide-cleavable biotinylation reagent on ice, and their internalization was induced by raising the temperature to 37 °C. Cell surface biotin was removed at specified times by treating the cells with glutathione, and the proteins with remaining biotin, representing endocytosed GLUT4, were quantified. WT at the cell surface was found to be internalized upon the removal of insulin in a time-dependent manner (Fig. 6B, lanes 2–4), whereas no obvious increase in the incorporation of N57Q was observed (Fig. 6B, lanes 6–8). These findings imply that the insensitivity to insulin may simply be caused by a lack of the insulin response, rather than by dysregulated endocytosis/exocytosis.

Glucose Transport Activity of GLUT4 Is Not Affected by Glycosylation—GLUT1, a ubiquitously expressed GLUT involved in basal glucose transport, is known to require its N-glycan for full transporter activity (29, 30). The effects of the N-glycan on GLUT4 transporter activity were examined by analyzing the uptake of 2-NBDG, a fluorescent glucose analog, in HeLa cells expressing WT or N57Q. In the presence of insulin, the
cells expressing either protein exhibited glucose uptake that was well above the background level (Fig. 7), suggesting that WT and N57Q are both active transporters. Arbitrary kinetic parameters were determined by carrying out a transporter activity assay with various concentrations of 2-NBDG. $K_m$ and the arbitrary turnover rate (arbitrary $V_{\text{max}}/K_m$) were determined after normalizing the amount of cell surface expression of WT or N57Q based on the results of the cell surface biotinylation assay. As shown in Table 1, both kinetic parameters were similar between WT and N57Q, indicating that the $N$-glycan is dispensable for cell surface expression and the consequent transport activity of GLUT4.

**DISCUSSION**

Glycans are known to play various important roles in the properties of carrier proteins (13, 14). Although the $N$-glycans on some GLUT proteins have been shown to be involved in modulating GLUT localization/functions (29–35), the biological role of the $N$-glycan on GLUT4 remains obscure. The present study therefore investigated the roles of the $N$-glycan on
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FIGURE 6. Internalization assay of GLUT4-EGFP upon removal of insulin from the medium. A, time lapse imaging of GLUT4-EGFP internalization. HeLa cells expressing GLUT4-EGFP (WT) or the nonglycosylated mutant (N57Q) were serum-starved and stimulated with insulin. The cells were washed to remove insulin from the medium, and localization of WT or N57Q was monitored by confocal microscopy. The time (h:min:s) is indicated at the top of each image. Scale bars, 10 µm. B, after cell surface labeling of GLUT4 using NHS-SS-biotin, the cells were incubated for 0, 30, or 60 min to allow internalization of the biotinylated proteins. The remaining cell surface biotin was then stripped by treatment in a reducing solution. The internalized biotinylated proteins were precipitated by streptavidin (SA)-Sepharose and analyzed by Western blotting (WB). The values indicate percent of protein remaining when the amount at time 0 was set to 100 (average value of three independent experiments).

FIGURE 7. Effect of glycosylation on glucose uptake. The time courses of glucose analog 2-NBDG uptake in untransfected HeLa cells (control) and HeLa cells expressing GLUT4-EGFP (WT) or the nonglycosylated mutant (N57Q) are shown. The data represent the averages of duplicate samples.

GLUT4 using HeLa cells. GLUT4 is known to be a long lived protein ($t_{1/2}$ of about 40 h) in mature adipocytes or muscle cells (36). Moreover, it has previously been shown that WT GLUT4 is not degraded through the ER-associated protein degradation process, but is mainly targeted to lysosomes for degradation (37). Our present results clearly indicate that the N-glycan on GLUT4 is critical for preventing GLUT4 from undergoing premature proteasomal degradation through the quality control machinery, which most likely involves ER-associated protein degradation. Consistent with this observation, previous reports have indicated the importance of N-glycans for ER-associated protein degradation of carrier proteins (38, 39). In contrast, once the N57Q glycosylation mutant escaped from the quality control system and reached the cell surface, its stability at the cell surface did not appear to be dramatically different from that of WT. Taken together, the results of this study suggest that the N-glycan on GLUT4 is critical for quality control of the protein, but is less important for its cell surface stability.

Both live imaging and biochemical approaches demonstrated that GLUT4 expressed in HeLa cells was able to respond to insulin in an N-glycan-dependent manner, suggesting that HeLa cells, despite their lack of GLUT4 expression (40), retain the basic machinery required for insulin-mediated cellular signaling. Similar findings have previously been reported for non-insulin-responsive fibroblasts (21), although the detailed molecular mechanism of translocation may not be exactly the same as that in insulin-responsive adipocytes (41). Under basal conditions, part of the WT protein is co-localized with endogenous IRAP, whereas another part is co-localized with the Tfr, a general recycling endosomal marker, suggesting that intracellular vesicles containing GLUT4 may comprise at least two distinct populations in HeLa cells. On the other hand, the nonglycosylated mutant co-localized with Tfr-positive vesicles, and less with IRAP-containing vesicles, implying that the N-glycan on GLUT4 is important for the intracellular localization required for its insulin responsiveness. Cell surface expression of the N57Q mutant under basal conditions was also noted to be higher than that of WT, further suggesting that the mutant apparently lacks proper targeting to GLUT4 storage vesicles. Strikingly, KIF treatment of WT GLUT4 clearly indicated the importance of the N-glycan structure on GLUT4 in terms of its insulin responsiveness. Similar results were obtained for GLUT4 in L6 myotubes, implying that the effect of N-glycan structure on insulin-mediated translocation may be a general phenomenon, rather than the cell type-specific event. A plausible explanation for these observations is that a specific structural element of N-glycan may be critical for the localization of GLUT4 to the appropriate intracellular pool essential for insulin-mediated translocation, and part of the N-glycan structure has the potential to act as a sorting signal. At present, however, candidate molecules responsible for recognizing the N-glycan structure remain unclear.

In sharp contrast to the case for GLUT1, in which a critical role of the N-glycan in transporter activity has been reported (29, 30), both WT and N57Q functioned as active transporters.

| TABLE 1 |
|---|
|**Kinetic parameters of wild-type GLUT4 and its N57Q glycosylation mutant** |
| | GLUT4 | $K_m$ | $V_{max}$ | Arbitrary turnover rate* |
| | m$m^3$ pmol/min/10$^6$ cells |
| WT | 1.8 | 46.6 | 14.3 |
| N57Q | 1.5 | 19.3 | 12.8 |

*The arbitrary turnover rate was determined based on the total surface expression of each protein assessed by cell surface biotinylation, and the protein concentration of N57Q was set to 1.
with similar kinetic parameters. No crystal structure data are available for any members of the facilitated GLUT family (42), but TM7, TM10, and TM11 of GLUT4 have been suggested to serve as binding sites for glucose (5), separate from the sole glycosylation site present in the extracellular loop between TM1 and TM2.

A defect in the ability of the insulin response to regulate the metabolic event is one of the key physiological dysfunctions of type 2 diabetes. Type 2 diabetes is characterized by the loss of insulin sensitivity, and reduced sensitivity or function of GLUT4 would therefore be a key factor in the development of this disease. The results of the present study provide evidence that the N-glycan on GLUT4 not only plays a general role in protein stability, but also has a function in GLUT4 sorting and insulin responsiveness. It should be noted that several studies have demonstrated changes of N-glycan profiles under diabetic conditions (43–46), and the effects of glycan profile alterations, especially in GLUT4, on the insulin resistance phenotype in type 2 diabetes should therefore be carefully investigated in future studies. Clarification of the key factors involved in N-glycan-related intracellular trafficking will be imperative for understanding the precise roles of the N-glycan functions in GLUT4.

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