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Glucosamine-induced insulin resistance is coupled to O-linked glycosylation of Munc18c

Guoli Chen, Ping Liu, Debbie C. Thurmond, Jeffrey S. Elmendorf*

Departments of Cellular and Integrative Physiology, and Biochemistry and Molecular Biology, Indiana University School of Medicine, Center for Diabetes Research, Indianapolis, IN 46202-5120, USA

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Abstract Evidence suggests that glucosamine inhibits distal components regulating insulin-stimulated GLUT4 translocation to the plasma membrane. Here we assessed whether key membrane docking and fusion events were targeted. Consistent with a plasma membrane-localized effect, 3T3-L1 adipocytes exposed to glucosamine displayed an increase in cell-surface O-linked glycosylation and a simultaneously impaired mobilization of GLUT4 by insulin. Analysis of syntaxin 4 and SNAP23, plasma membrane-localized target receptor proteins (t-SNAREs) for the GLUT4 vesicle, showed that they were not cell-surface targets of O-linked glycosylation. However, the syntaxin 4 binding protein, Munc18c, was targeted by O-linked glycosylation. This occurred concomitantly with a block in insulin-stimulated association of syntaxin 4 with its cognate GLUT4 vesicle receptor protein (v-SNARE), VAMP2. In conclusion, our data suggest that the mechanism by which glucosamine inhibits insulin-stimulated GLUT4 translocation involves modification of Munc18c.

Key words: GLUT4; Glucosamine; Insulin resistance; Munc18c; SNARE protein

1. Introduction

Insulin normalizes elevated plasma glucose levels mainly by increasing glucose uptake into fat and muscle cells, via stimulating the translocation of the insulin-responsive glucose transporter GLUT4 from intracellular sites to the plasma membrane. GLUT4 resides in vesicles that continuously cycle from intracellular stores to the plasma membrane. Insulin increases glucose transport by greatly increasing the rate of GLUT4-containing vesicle exocytosis and slightly decreasing the rate of internalization [1]. This insulin-stimulated process requires phosphatidylinositol 3-kinase (PI3K) as well as a proposed PI3K-independent pathway leading to activation of the small GTPase TC10 [2]. Disconnect in the PI3K signal linking the insulin receptor (IR) to the GLUT4 protein has been described, and is speculated to be a molecular and physiological basis of insulin resistance in obesity and type 2 diabetes.

Insulin resistance, the inability of cells or tissues to respond to physiological levels of insulin, results in profound dysregulation of glucose transport. Evidence indicates that the glucosamine biosynthetic pathway, a quantitatively small pathway of glucose utilization, may contribute to the insulin-resistant state [3–6]. The first and rate-limiting enzyme of the hexosamine biosynthesis pathway is glutamine:fructose-6-phosphate amidotransferase (GFAT), which catalyzes the conversion of fructose-6-phosphate to glucosamine-6-phosphate. This pathway ultimately produces UDP–*N*-acetylglucosamine (UDP–GlcNAc) and UDP–*N*-acetylgalatosamine, which serve as obligatory precursors for the synthesis of glycosyl side chains of proteins and lipids. Experimentally, work from this and other laboratories suggest that defects in the PI3K and/or the TC10 signaling routes engaged by insulin to mobilize GLUT4 do not readily account for glucosamine-induced insulin resistance [7–9]. In agreement with a distally targeted glucosamine-induced defect, the insulin mimetic action of hyperosmolarity, which is independent of early components of insulin action, is also blocked in cells exposed to glucosamine [7]. Although mobilization of GLUT4 by hyperosmolarity does not entirely utilize the same signal transduction pathways of insulin action [10,11], stimulation by hyperosmolarity appears to require some of the same distally targeted proteins of insulin action that regulate GLUT4 vesicle and plasma membrane fusion [11,12].

The mechanism of membrane fusion entails the pairing of protein complexes in the vesicle compartment (v-SNAREs, for vesicle SNAP receptors) with cognate receptor complexes at the target membrane (t-SNAREs, for target membrane SNAP receptors). Several v- and t-SNARE proteins have been identified that specifically participate in the insulin-regulated docking and fusion of GLUT4 vesicles with the plasma membrane [1,13–15]. Syntaxin 4 appears to function as the required plasma membrane t-SNARE, whereas VAMP2 is the predominant v-SNARE for insulin-stimulated GLUT4 vesicle docking and fusion [16–20]. Several accessory proteins such as Munc18c have been shown to play important functional roles in regulation of insulin-stimulated t- and v-SNARE interactions [12,21–24].

Clear functional consequences of abnormal glycosylation events are only beginning to be elucidated but most modifications affect a protein–protein association or affect protein activity directly [25]. Recently, glycosylation has been impli-

*Corresponding author. Fax: (1)-317-274 3318.
E-mail address: jelmendo@iupui.edu (J.S. Elmendorf).

Abbreviations: GLUT4, insulin-responsive glucose transporter; SNARE, *s*oluble *N*-ethylmaleimide-sensitive fusion protein *a*ttachment protein *re*ceptor; VAMP, vesicle-associated membrane protein; PI3K, phosphatidylinositol 3-kinase; GFAT, glutamine:fructose-6-phosphate amidotransferase; GlcNAc, *N*-acetylglucosamine; EGFP, enhanced green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SNAP23, synaptosome-associated protein of 23 kDa; IR, insulin receptor

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cated to have a functional role in SNARE protein interactions [26]. Given that both insulin- and hyperosmolarity-stimulated GLUT4 translocations are negatively affected by glucosamine and that they apparently share proteins involved in membrane docking and fusion events, the purpose of the present study was to determine whether increases in the hexosamine biosynthesis pathway impair SNARE protein interaction involved in GLUT4 translocation. Here we demonstrate that Munc18c isolated from cells exposed to glucosamine is O-linked glycosylated. Furthermore, the insulin-stimulated association of VAMP2 and syntaxin 4 is prevented by glucosamine treatment. The following sections describe these findings in detail.

2. Materials and methods

2.1. Cell culture

Murine 3T3-L1 preadipocytes were obtained from the American Type Tissue Culture repository and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and 10% calf serum at 37°C in an 8% CO_2 atmosphere. Confluent cultures were induced to differentiate into adipocytes as previously described [27]. All studies were performed on adipocytes, which were between 8 and 12 days old following differentiation. Prior to all experimental treatments, the differentiated adipocytes were serum starved in DMEM containing 5.5 mM glucose for 2 h at 37°C.

2.2. Glucosamine induction of insulin resistance

As we have previously reported [7], we utilized a glucosamine-induced model described by Ross et al. [8] that induces insulin resistance without affecting neither the cellular ATP levels nor the early insulin-regulated phosphorylation of substrates. Briefly, prior to induction of insulin resistance adipocytes were treated with DMEM/5.5 mM glucose media containing 10% fetal bovine serum (FBS) for two days. Cells were then treated with DMEM containing 10% FBS and 2 mM glucosamine and lacking glucose, glutamine, and insulin for 12–14 h. The culture media contained 1 mM pyruvate as an additional energy source. Also, 2 mM L-glucose was added to adjust the osmolarity of sugars in control groups.

2.3. Transient transfection

Differentiated adipocytes were electroporated (0.16 kV and 960 μF) as previously described [21]. As indicated in the figure legends, transfection experiments were performed with 50 μg of enhanced green fluorescent protein (EGFP)-tagged plasmid DNA for analysis of EGFP fluorescence. Following electroporation, the adipocytes were re-plated on glass coverslips and allowed to recover for 16–18 h before use.

2.4. Plasma membrane sheet assay

Preparation of plasma membrane sheets from the adipocytes was performed essentially by the method of Robinson et al. [28] with minor modifications as previously described [27]. Following the isolation of plasma membrane sheets, these purified membranes were used for indirect immunofluorescence. The plasma membrane sheets were fixed for 20 min at 25°C in a solution containing 2% paraformaldehyde, 70 mM KCl, 30 mM HEPES, pH 7.5, 5 mM $MgCl_2$, and 3 mM EGTA as previously described [27]. The membrane sheets were then blocked in 5% donkey serum for 60 min at 25°C, incubated for 60 min at 25°C with a 1:100 dilution of RL-2, a monoclonal mouse antibody against O-linked *N*-acetylglucosylation (Affinity Bioreagents Inc.) in 5% donkey serum followed by incubation with a 1:50 dilution of rhodamine red-X-conjugated donkey anti-mouse immunoglobulin G (Jackson Immunoresearch Inc.) for 60 min at 25°C.

2.5. Preparation of total cell extracts and immunoprecipitation

Total cell extracts were prepared from 100-mm plates of 3T3-L1 adipocytes following the appropriate treatment. Cells from each plate were washed two times with ice-cold phosphate-buffered saline and scraped into 1 ml of lysis buffer (25 mM Tris, pH 7.4, 50 mM NaF, 10 mM $Na_3P_2O_7$, 137 mM NaCl, 10% glycerol and 1% NP40) containing 1.0 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4, 5 μg ml^{-1} aprotinin, 2.5 μg ml^{-1} leupeptin, and 1 μg ml^{-1} pepstatin A by rotation for 15 min at 4°C. Insoluble material was separated from the soluble extract by microcentrifugation for 15 min at 4°C. Protein concentration was determined, and samples were either subjected directly to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, as described in Section 2.7) or immunoprecipitated for syntaxin 4, Munc18c or VAMP2. Briefly, 3–5 mg of cellular protein were immunoprecipitated with 4.5 μg of rabbit anti-syntaxin 4 polyclonal antibody (Chemicon International Inc.) and 2.0 μg of rabbit anti-Munc18c affinity-purified polyclonal antibody (generously provided by Dr. Jeffrey Pessin, University of Iowa) for 2 h at 4°C. Immune complexes were recovered by the addition of protein A-Sepharose (Amersham Pharmacia Biotech) and subjected to SDS–PAGE (as described in Section 2.7).

2.6. Subcellular fractionation

Adipocyte subcellular membrane fractions were obtained using the differential centrifugation method previously described [21] with slight modification. Briefly, control and insulin-stimulated 3T3-L1 adipocytes were washed and resuspended in HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, and 255 mM sucrose containing 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} pepstatin, 10 μg ml^{-1} aprotinin, and 5 μg ml^{-1} leupeptin). Cell lysates were prepared by shearing the cells through a 22-gauge needle 10 times. Lysates were then centrifuged at $19\,000\times g$ for 20 min at 4°C. The low speed pellet was obtained by centrifugation of the resulting supernatant at $41\,000\times g$ for 20 min at 4°C. Supernatant was removed and centrifuged at $180\,000\times g$ for 75 min at 4°C to generate the high speed pellet and cytosol (supernatant) fractions. The crude plasma membrane pellet was obtained by resuspending the pellet from the initial $19\,000\times g$ centrifugation in HES buffer followed by layering onto a 1.12 M

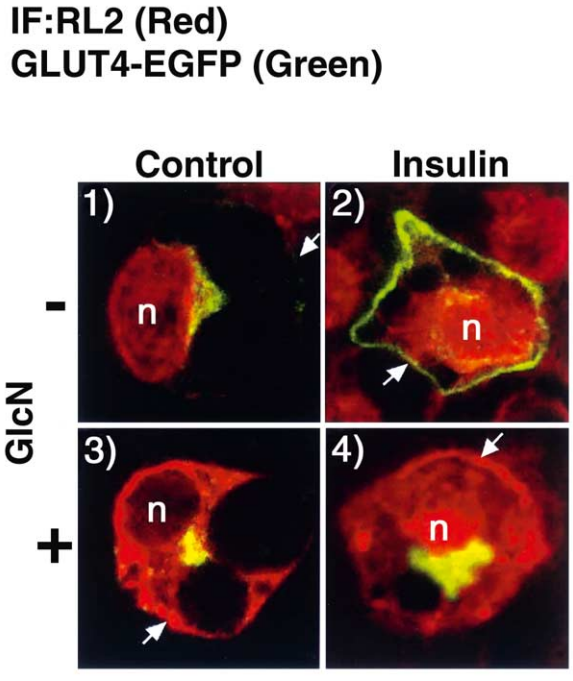

Fig. 1. Glucosamine exposure results in O-linked glycosylation of cell-surface proteins concomitant with a blockade in insulin-stimulated GLUT4–EGFP translocation. 3T3-L1 adipocytes were electroporated with 50 μg of the GLUT4–EGFP cDNA (panels 1–4). Following a 24 h recovery, the cells were incubated in the absence (−GlcN, panels 1 and 2) or presence (+GlcN, panels 3 and 4) of 2 mM glucosamine for 12 h as described in Section 2. Cells were subsequently left either untreated (control, C, panels 1 and 3) or treated with 100 nM insulin (insulin, panels 2 and 5) for 15 min. Cells were then subjected to immunofluorescence microscopy with an antibody that recognizes polypeptides containing O-linked *N*-acetylglucosamine residues (RL2). Nuclear (n) and plasma (arrow) membranes are denoted. Representative observations from three independent experiments are shown.

sucrose cushion for centrifugation at 100 000 × *g* for 60 min. The plasma membrane layer was removed from the sucrose cushion and centrifuged at 40 000 × *g* for 20 min. All pelleted fractions were resuspended in a detergent-containing lysis buffer and assayed for soluble protein content.

2.7. Electrophoresis and immunoblotting

Whole cell lysates, immunoprecipitated proteins, and subcellular fractions were subjected to electrophoresis on a 12% SDS–PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and immunoblotted with antibodies indicated in the individual figure legends. All immunoblots were subjected to enhanced chemiluminescence detection (Amersham Pharmacia Biotech).

2.8. Statistical analysis

All values are presented as means ± S.E., and significant differences between means were evaluated by paired Student's *t*-test. For testing multiple pairs, analysis of variance was used to determine differences among groups. Where a significant difference was indicated, the Newman–Keuls Test was used to determine significant differences between groups. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of glucosamine on the cellular distribution of O-linked glycosylated proteins and GLUT4 translocation

GLUT4–EGFP-expressing cells pretreated with 2 mM glucosamine for 12 h displayed a marked increase in O-linked glycosylation at the cell surface compared to cells pretreated in the absence of glucosamine (Fig. 1, compare panels 1–4). Consistent with other reports, nuclear membrane labeling was detected in all cells [29–31]. In the absence of glucosamine exposure, insulin treatment for 15 min stimulated a characteristic increase in GLUT4–EGFP translocation (Fig. 1, compare panels 1 and 2). In contrast, the ability of insulin to mobilize GLUT4–EGFP to the cell surface was blocked in glucosamine-exposed cells (Fig. 1, panel 4). Consistent with the increased cell-surface immunoreactivity, highly purified plasma membrane sheets prepared from cells pretreated with glucosamine displayed a marked increase in immunofluorescence detection of O-linked glycosylation compared to sheets from untreated cells (Fig. 2A, compare panels 1 and 2). In addition, immunoblot analyses of plasma membrane fractions show that the ability of insulin to mobilize GLUT4 to the plasma membrane is blocked following glucosamine exposure (Fig. 2B). Collectively, these data clearly show that the glucosamine-induced blockade in GLUT4 translocation occurs concomitant with O-linked glycosylation of plasma membrane-localized proteins.

3.2. Effect of glucosamine on the cellular distribution and O-linked glycosylation state of SNARE and SNARE-associated proteins

We next assessed whether key SNARE and SNARE-associated proteins involved in GLUT4 translocation were affected by glucosamine treatment. As expected, insulin stimulation for 15 min markedly increased plasma membrane-associated VAMP2 (Fig. 3A, compare lanes 1 and 2). Also the basal-state levels of syntaxin 4 and its binding protein, Munc18c, in the plasma membrane were increased following insulin stimulation (Fig. 3A, compare lanes 1 and 2). In cells treated with glucosamine, neither the syntaxin 4, Munc18c, nor VAMP2 basal levels were affected. However, the insulin-stimulated plasma membrane contents of these SNARE proteins were markedly decreased by glucosamine exposure (Fig. 3A, lanes 3 and 4). Densitometry analysis of three independent experiments clearly shows that glucosamine caused defective insulin-stimulated redistribution of these key GLUT4 effector proteins (Fig. 3B–D). Given that cells ex-

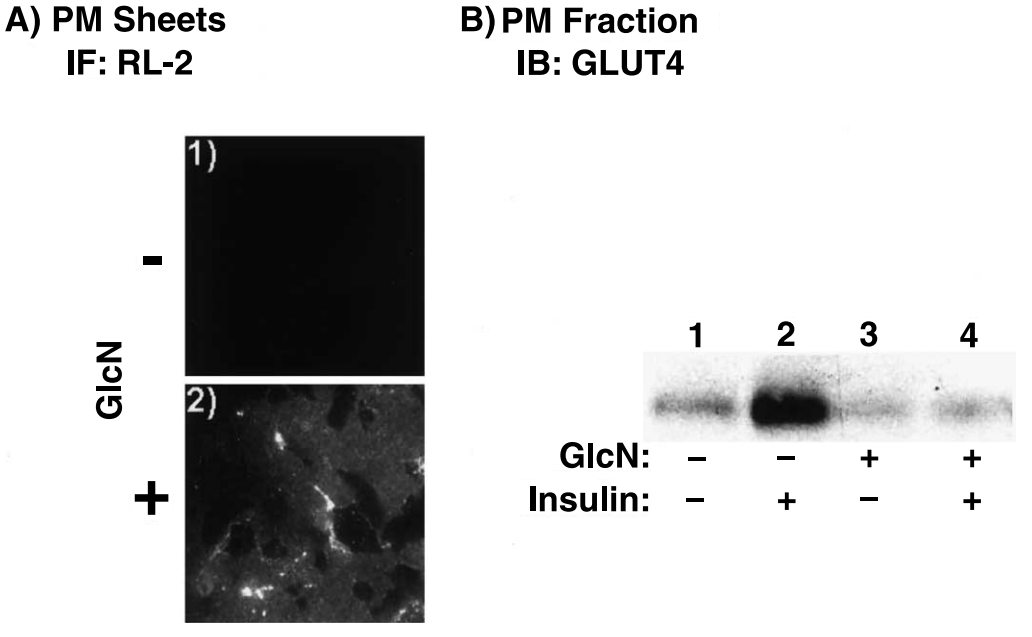

Fig. 2. O-linked glycosylated proteins are detected in highly purified plasma membrane sheets concomitant with a decrease in the insulin-stimulated plasma membrane level of GLUT4. 3T3-L1 adipocytes were incubated in the absence (−GlcN, panel 1 and lanes 1 and 2) or presence (+GlcN, panel 2 and lanes 3 and 4) of 2 mM glucosamine for 12 h as described in Section 2. A: Plasma membrane sheets were prepared and subjected to immunofluorescence microscopy with the RL2 antibody. Representative observations from three independent experiments are shown. B: Following glucosamine treatment, cells were left untreated (−insulin, lanes 1 and 3) or treated with 100 nM insulin (+insulin, lanes 2 and 4) for 15 min. Plasma membrane fractions prepared as described in Section 2 were resolved on a 12% SDS–polyacrylamide gel and subjected to Western blotting with the GLUT4 antibody. This is a representative immunoblot from three independent experiments.

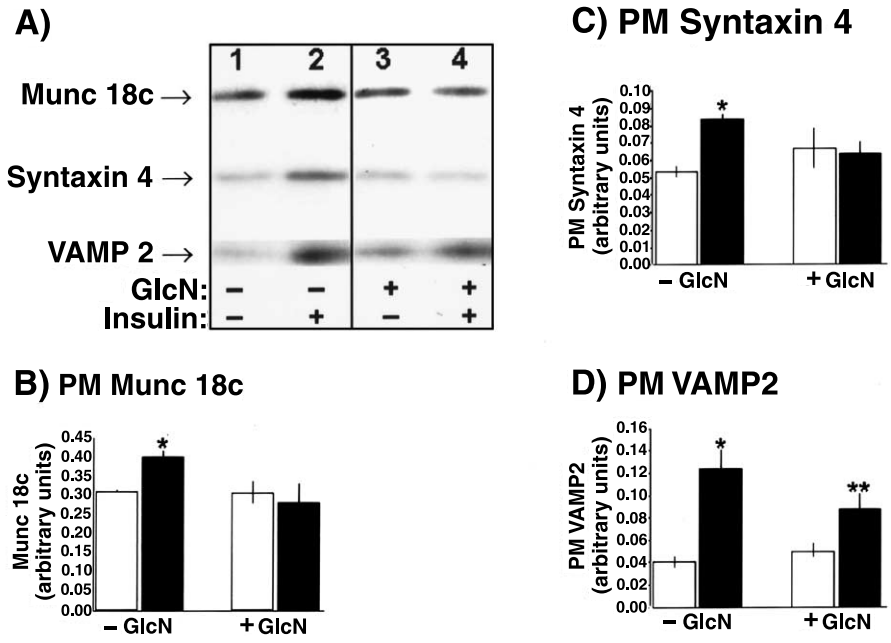

Fig. 3. Insulin-stimulated trafficking of several SNARE proteins to the plasma membrane is decreased by glucosamine. Following an overnight treatment without (−GlcN, lanes 1 and 2) or with (+GlcN, lanes 3 and 4) glucosamine, cells were either left untreated (−insulin, lanes 1 and 3,) or treated with 100 nM insulin (+insulin, lanes 2 and 4) for 15 min. Plasma membrane fractions were prepared, resolved on a 12% polyacrylamide gel and subjected to Western blotting with the Munc18c, syntaxin 4, or VAMP2 antibodies. Representative immunoblots (A) and densitometric quantitation (means ± S.E.) from three experiments (B–D) are shown. (*$P < 0.05$ versus −GlcN/−insulin; **$P < 0.05$ versus −GlcN/+insulin).

posed to glucosamine displayed a robust cell-surface immunofluorescence labeling of O-linked glycosylation (Figs. 1 and 2A), we next assessed whether syntaxin 4 and SNAP23 were modified by O-linked glycosylation following glucosamine treatment. Immunoblotting analysis of these immunoprecipitated plasma membrane-localized t-SNARE proteins with an antibody that recognizes O-linked glycoproteins (RL2) revealed that neither syntaxin 4 (Fig. 4A, compare lanes 1 and 2) nor SNAP23 (data not shown) were O-linked glycosylated. In contrast, O-linked glycosylation of Munc18c was apparent in cells treated with glucosamine (Fig. 4B, compare lanes 1 and 2). Immunoblotting of the same Western blots with syntaxin 4 (Fig. 4A, lanes 3 and 4) and Munc18c antibodies (Fig. 4B, lanes 3 and 4) indicated the presence of equal amounts of syntaxin 4 and Munc18c, respectively. In agreement with these findings, immunoblotting analysis of RL2 immunoprecipitated O-linked glycosylated proteins clearly demonstrated that Munc18c, but not syntaxin 4 was O-linked glycosylated in cells exposed to glucosamine (Fig. 5, compare lanes 1 and 2). Parallel immunoblotting of non-immunoprecipitated whole cell lysates from the same cells were resolved on the same gel to confirm the migration of Munc18c and syntaxin 4 (Fig. 5, lanes 3 and 4). In addition to these immunoprecipitation studies, we immunofluorescently labeled control and glucosamine-exposed cells with RL2 and Munc18c antibodies. Consistent with the biochemical data, this microscopic approach demonstrated a robust glucosamine-induced co-localization of Munc18c and O-linked glycosylation (data not shown).

3.3. *Effect of glucosamine on insulin-stimulated VAMP2 and syntaxin 4 association*

Finally, we assessed whether the extent of insulin-stimulated

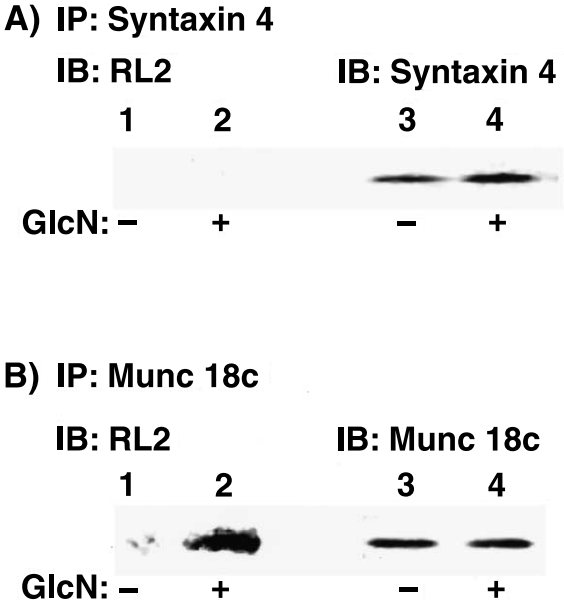

Fig. 4. Glucosamine induces O-linked glycosylation of Munc18c, but not syntaxin 4. Cells were incubated in the absence (−GlcN, lanes 1 and 3) or presence (+GlcN, lanes 2 and 4) of 2 mM glucosamine for 12 h. Whole cell detergent extracts were prepared, immunoprecipitated with (A) syntaxin 4 or (B) Munc18c antibodies, resolved on a 12% polyacrylamide gel and subjected to Western blotting with RL2 antibody (lanes 1 and 2). These same membranes were stripped and reprobed with (A) syntaxin 4 or (B) Munc18c antibodies. These are representative immunoblots from three independent experiments.

association of VAMP2 with syntaxin 4 was diminished in glucosamine-exposed cells (Fig. 6). Insulin stimulation resulted in a characteristic increase in the amount of VAMP2 co-immunoprecipitated with anti-syntaxin 4 antibody (Fig. 6, compare lanes 1 and 2). The ability of insulin to stimulate this VAMP2–syntaxin 4 association was completely blocked in cells exposed to glucosamine (Fig. 6, compare lanes 3 and 4). Densitometry analysis of three separate independent experiments clearly shows that the insulin-stimulated association of VAMP2 with syntaxin 4 was prevented by glucosamine (Fig. 6B).

4. Discussion

The main finding of this study is that Munc18c, a key mediator of membrane fusion events facilitating insulin-stimulated GLUT4 translocation, is modified by O-linked glycosylation in the glucosamine-induced insulin-resistant state. Consistent with impaired insulin-stimulated GLUT4 translocation, the insulin-stimulated translocation of VAMP2 to the plasma membrane and its subsequent association with syntaxin 4 were blocked in cells exposed to glucosamine. Since previous work from this and other laboratories demonstrates that intermediates of the PI3K signal and the newly described Cbl signal of insulin action are not altered by glucosamine treatment, the data from the present study suggest that the glucosamine-induced defect in the insulin signal occurs at the level of SNARE protein function. While this study was in progress, Nelson et al. [32] reported that glucosamine treatment of 3T3-L1 adipocytes disrupted the ability of insulin to promote the translocation of Munc18c to the plasma membrane. Importantly, the present studies extended upon this observation and

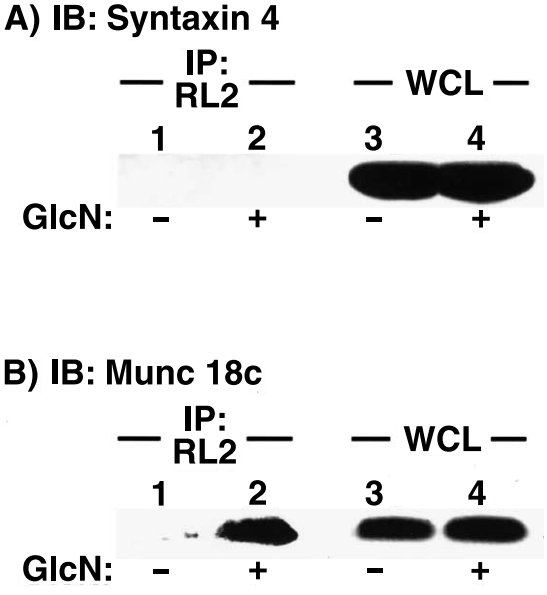

Fig. 5. O-linked glycosylation of Munc18c is detected in RL2 immunoprecipitates. Following an overnight treatment without (−GlcN, lanes 1 and 3) or with (+GlcN, lanes 2 and 4) glucosamine whole cell detergent extracts were prepared and immunoprecipitated with RL2 antibody, resolved on a 12% polyacrylamide gel (lanes 1 and 2). Non-immunoprecipitated lysates was resolved on the same gel (lanes 3 and 4). The membrane was then subjected to Western blotting with either (A) syntaxin 4 or (B) Munc18c antibodies. These are representative immunoblots from three independent experiments.

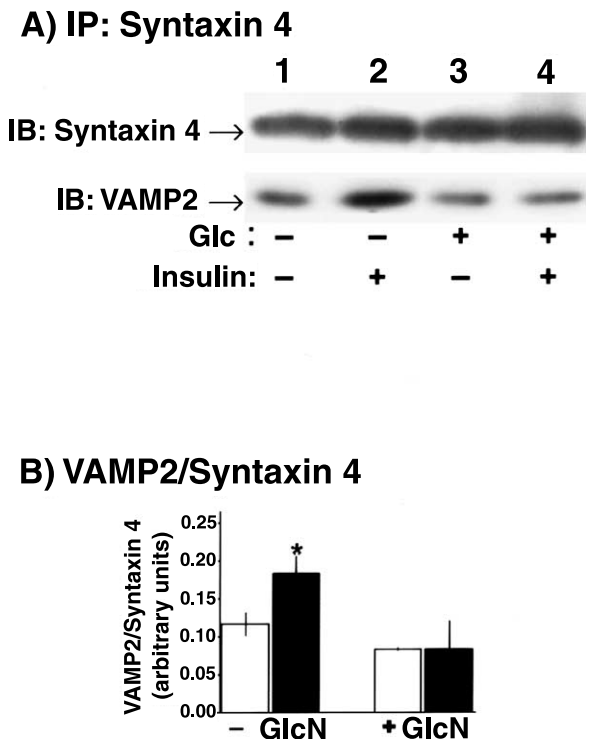

Fig. 6. Stimulation of VAMP2 and syntaxin 4 association by insulin is abolished in cells exposed to glucosamine. Whole cell detergent extracts were prepared from non- (−GlcN, lanes 1 and 2) or glucosamine-exposed (+GlcN, lanes 3 and 4) cells that were either left untreated (−insulin, lanes 1 and 3) or treated with 100 nM insulin (+insulin, lanes 2 and 4) for 15 min. Extracts were immunoprecipitated with syntaxin 4 antibody, resolved on a 12% polyacrylamide gel, and subjected to Western blotting with either syntaxin 4 or VAMP2 antibodies. Representative immunoblots (A) and densitometric quantitation (means ± S.E.) from three experiments (B) are shown. (*$P < 0.05$ versus −GlcN/−insulin).

demonstrated that Munc18c is a target of O-linked glycosylation. Furthermore, we show that the Munc18c-mediated event of VAMP2–syntaxin 4 association is blocked in the glucosamine-induced insulin-resistant state. Thus, the present study provides a potential mechanism by which glucosamine prevents insulin-stimulated GLUT4 translocation.

Studies in both skeletal muscle and 3T3-L1 adipocytes provide evidence against the insulin receptor being a plasma membrane target of glucosamine action [7,8,33–36] and our recent work supports a role at the more distal GLUT4-containing vesicle and plasma membrane docking and fusion step [7]. Consistent with this, our immunofluorescence data as determined by immunolabeling the cell (Fig. 1) and highly purified plasma membrane sheets (Fig. 2A) with RL2 antibody indicates that plasma membrane proteins are targets of O-linked glycosylation in glucosamine-induced insulin-resistant 3T3-L1 adipocytes. With regards to the GLUT4-containing vesicle membrane, immunoisolation studies have revealed that the rate of labeled glucosamine incorporation was approximately 100-fold greater following glucosamine compared with saline infusions [37]. More recent work has shown that there is enhanced O-linked glycosylation of GLUT4 in insulin-resistant GLUT1-overexpressing muscles [38]. Although studies demonstrate that neuron-specific synaptic vesicle-associated phosphoproteins synapsin I and synapsin II contain

O-glycosidically bound GlcNAc [26,39], like syntaxin 4 and SNAP23, we did not detect O-linked glycosylation of VAMP2 in the present work (data not shown). In contrast, O-linked glycosylation was clearly detected in anti-Munc18c immunoprecipitates prepared from cells treated with glucosamine. Moreover, we isolated Munc18c from glucosamine treated cells via immunoprecipitation with the RL2 antibody and additionally observed co localization immunofluorescently. Collectively, these findings importantly show that a key accessory protein of insulin-stimulated VAMP2–syntaxin 4 association is O-linked glycosylated in cells exposed to glucosamine.

Munc proteins share homology with two vesicle-trafficking regulators Sec1 in *Saccharomyces cerevisiae* and unc-18 in *Caenorhabditis elegans*. Munc18a is expressed only in neuronal and neuroendocrine tissues [40]. Munc18b and c are expressed in adipocytes, but only the latter regulates GLUT4 trafficking [21,23,41]. Overexpression of Munc18c inhibited GLUT4 translocation in response to insulin [12,21,24]. This inhibitory effect was removed by co-expression of syntaxin 4 [23]. In cells that were co-transfected with tagged Munc18c and syntaxin 4, a 30-min insulin exposure decreased the localization of transfected Munc18c to the PM and the association between Munc18c and syntaxin 4. Interestingly, microinjection of Munc18c peptide fragments, which compete with endogenous Munc18c for binding to syntaxin 4, resulted in insulin-stimulated GLUT4 vesicle clustering at but not fused with the plasma membrane [23]. In vivo, Munc18c overexpression causes skeletal muscle and adipose tissue insulin resistance as a result of ablated insulin-stimulated GLUT4 translocation to the cell surface [42]. Taking these data into account, Munc18c apparently plays a positive fusogenic role in insulin-stimulated GLUT4 translocation.

In a very recent model presented by Nelson et al. [32], glucosamine-induced insulin resistance is associated with the disruption of insulin-regulated Munc18c trafficking. In that work, a subcellular fractionation method that further separates each fraction into Triton X-100-soluble and -insoluble components permitted the detection of Munc18c translocation. With this approach we also observed that insulin increases the plasma membrane content of Munc18c. As discussed by Nelson et al. [32] the docking of GLUT4 vesicles may promote the association of Munc18c with the plasma membrane, possibly facilitating fusion. Alternatively, acute insulin stimulation may cause post-translational modification(s) of Munc18c. Although there are no data relevant to Munc18c, Munc18a function can be regulated by protein kinase C and by cyclin-dependent kinase 5-mediated phosphorylation [43,44]. Insulin-regulated trafficking of Munc18c may represent a link between the proximal insulin signaling cascade and GLUT4 vesicle docking and fusion. In this regard, O-linked glycosylation is a process that enzymatically attaches single GlcNAc moieties to serine and threonine residues of cytosolic and nuclear proteins [45,46], and Munc18c is a cytosolic protein that is found associated with syntaxin 4 at the plasma membrane [21].

The present work suggests that increases in hexosamine biosynthesis, sufficient to induce insulin resistance, occur simultaneously with O-linked glycosylation of Munc18c. One mechanism by which O-linked glycosylation of Munc18c could block insulin-stimulated VAMP2–syntaxin 4 interaction may involve physical blockage of syntaxin 4–Munc18c interaction. Alternatively, the putative ability of insulin to regulate the fusogenic properties of Munc18c may be perturbed by potential interplay between O-linked glycosylation and phosphorylation. Future work will be necessary to determine whether these or other mechanisms are involved in impaired GLUT4 vesicle fusion in the glucosamine-induced insulin-resistant state.

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