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N-glycosylation modulates the membrane sub-domain distribution and activity of glucose transporter 2 in pancreatic beta cells

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ABSTRACT

The glucose transporter isoform, GLUT2, -mediated glucose sensing is essential for maintaining normal glucose-stimulated insulin secretion in pancreatic beta cells. We previously reported that GnT-IVa glycosyltransferase is required for the production of an *N*-glycan structure that acts as a ligand for galectins to form the glycan–galectin lattice that maintains the stable cell surface expression of GLUT2, and cellular glucose transport activity, although the functional relevance of the *N*-glycosylation of GLUT2 to its membrane sub-domain distribution is not fully understood. In the present study, we demonstrated that disruption of the GLUT2 *N*-glycan–galectin lattice by the genetic inactivation of GnT-IVa, or by treatment of pancreatic beta cells with competitive glycan mimetics, induced the re-distribution of GLUT2 into the lipid-raft microdomain. This subsequently resulted in the binding of Stomatin to GLUT2 and an attenuation of cellular glucose transport activity. Moreover, disruption of the lipid-raft microdomain by treatment with methyl-β-cyclodextrin caused the GLUT2 to be released from lipid-rafts and reactivation of the cellular glucose transport activity in GnT-IVa deficient beta cells. These results indicate that the membrane sub-domain distribution of GLUT2 is associated with the glucose transport activity of beta cells, in which the GnT-IVa-dependent formation of the *N*-glycan–galectin lattice plays an important role. This provides a novel pathophysiological insight into the mechanism of beta cell failure in the pathogenesis of type 2 diabetes.

1. Introduction

In mammalian cells, glucose is a major source for energy production in the form of ATP and for the synthesis of proteins, lipids, and nucleotide-sugars through cellular metabolism and homeostasis. In mammals, blood glucose levels are maintained within a narrow range by endocrinological regulation. Cells uptake extracellular glucose from interstitial fluid by a passive and facilitative transport process along with the downward gradient concentration of glucose across the cellular plasma membrane [1]. The plasma membrane possesses a glucose transport system facilitating diffusion either into or out of the cells. This system referred to as the "glucose transporter" (GLUT). GLUT consists of 13 members, of which 11 are specific for sugar transport without any energy-requirements, such as ATP hydrolysis or a H+ gradient [2,3]. The GLUT-dependent cellular glucose uptake is a physiologically mandatory process. GLUT2 is involved in glucose sensing for insulin secretion in pancreatic beta cells, the net glucose release into the blood stream by hepatic gluconeogenesis, and transepithelial glucose transport in the kidney and intestine. GLUT4 is involved in the acute insulin-responsive glucose absorption in adipose tissue and muscle for rapid removal of blood glucose into energy stores such as glycogen and triacylglycerol. GLUT1 is ubiquitously expressed and is involved in whole-body glucose homeostasis [4]. Members of the GLUT family proteins are structurally conserved and share a structure that includes 12 membrane-spanning regions and a single *N*-glycan attached in either the first or the fifth extracellular loop domain [3,5]. Previous studies have reported that *N*-glycosylation is

Abbreviations: ATP, adenosine tri-phosphate; GLUT, glucose transporter; GnT, *N*-acetylglucosaminyltransferase; PBS, phosphate buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KRH, Krebs–Ringer HEPES buffer; DTSSP, 3,3'-dithiobis[sulfosuccinimidylpropionate]; DMSO, dimethyl sulfoxide; DSP, dithiobis[succinimidyl propionate]; HBSS, Hank's balanced salt solution; PKA, protein kinase A; MβCD, methyl-β-cyclodextrin; 2-NBDG, 2-[*N*-(7-nitrobenz-2-oxadiazol-4-yl)amino]-2-deoxy D-glucose.

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required for achieving stable cell surface expression and for the transporter activity of GLUT1 and GLUT2 [6–11]. Generally, newly synthesized plasmalemmal proteins and secretory proteins are transported to the Golgi apparatus, where they undergo maturation of their *N*-glycans by sequential enzymatic reactions of Golgi-residing glycosyltransferases. This process consequently produces diverse *N*-glycan structures and confers various functions on proteins. The biological significance of protein *N*-glycosylation has been recently established. *N*-acetylglucosaminyltransferase (GnT)-IV and GnT-V are essential glycosyltransferases cooperatively catalyzing the formation of multi-antennary *N*-glycans (Supplemental Fig. 1), bearing various glycan epitopes that enable the carrier proteins to interact with other molecules on the cell surface. Galectins are a family of lectins that selectively bind to the Gal-GlcNAc moieties of *N*-glycan branches with relatively weak affinities [12]. The apparent binding affinity depends on the intrinsic multivalency, and oligomeric state of the galectin, and on the multivalency of the glycan ligands. As a result, interactions among oligomers of galectins and multivalent glycans promote the formation of glycan–galectin lattice structures on the cell surface that have a great influence on the functions of membrane proteins [13]. It has recently been established that multi-antennary *N*-glycan production facilitates *N*-glycan–galectin lattice formation that restricts the diffusive mobility of the cell surface membrane proteins and is involved in their conversion into clusters, a process consequently regulates molecular and cellular functions [14,15]. Cell surface membrane proteins form homotypic and heterotypic clusters in various membrane sub-domains, are dynamically exchanged and are redistributed among them in response to signaling reflecting cellular microenvironments [16–21]. It has been established that the glucose transport activity of GLUT1 is regulated by membrane sub-domain localization [22,23], as well as an *N*-glycosylation step, which implies the presence of a functional linkage between membrane sub-domain distribution and *N*-glycosylation for controlling glucose transport activity.

The present study was undertaken to elucidate the regulation of GLUT2 activity by its membrane sub-domain localization and *N*-glycosylation using primary isolated pancreatic beta cells that must contribute to better understand the physiological regulation of the glucose sensor function of pancreatic beta cells and furthermore provide a novel molecular insight into the pathogenesis of type 2 diabetes.

2. Materials and methods

2.1. Reagents and antibodies

Collagenase type-V from clostridium histolyticum and DNase were purchased from Sigma. Complete proteinase inhibitor cocktails (EDTA+, or free) were purchased from Roche. Hank's balanced salt solution (HBSS) with 5.6 mM glucose was purchased from Invitrogen. Dispase was purchased from Calbiochem. Ficoll PM400 was purchased from GE healthcare. 3,3′-Dithiobis[sulfosuccinimidyl propionate] (DTSSP) and Dithiobis[succinimidyl propionate] (DSP, Loman's reagent) were purchased from Thermo scientific. GLUT2 antibody was purchased from Millipore, and Galectin9 and Stomatin antibodies were purchased from Santa Cruz Biotechnology.

2.2. Isolation of primary mouse islets

Primary pancreatic islet cells were prepared as previously described [10]. Briefly, 1 ml of collagenase solution (3 mg/ml collagenase type-V, 5 μg/ml DNase, 2× complete proteinase inhibitor (EDTA free) in HBSS with 5.6 mM glucose) was injected into the distally ligated pancreatic duct of a euthanized mouse, and the excised pancreas was then incubated with additional 2 ml of collagenase solution at 37 °C with shaking (200 strokes/min) until a homogenous mixture was obtained. Following centrifugation of the digestive solution at 200*g* for 5 min, the pellet was resuspended in 3 ml of 25% Ficoll PM400/HBSS solution, which was thenoverlaid with 3 ml of 23%, and 2 ml of 20% and 2 ml of 11% Ficoll PM400/HBSS solution to form discontinuous gradients. Following centrifugation at 800*g* for 15 min, islets were collected from the top two boundaries of gradients. To obtain higher purity of islets, islets were hand-picked under a stereomicroscope using a micropipette. Single cell suspension was obtained by disruption of islets by incubation with dispase solution (0.5 U/ml dispase, and 5 μg/ml DNase in HBSS with 5.6 mM glucose) at 37 °C and consecutive washing with HBSS.

2.3. Membrane fractionation

Lipid rafts and non-lipid raft (detergent-soluble) proteins were separated by sucrose-gradient centrifugation. In subsequent steps, solutions and samples were kept at 4 °C. Islet cells were washed twice with PBS and lysed in KRH (Krebs–Ringer HEPES buffer; 136 mM NaCl, 20 mM HEPES, 4.7 mM KCl, 1.25 mM $MgSO_4$, and 1.25 mM $CaCl_2$, pH 7.4) supplemented with 5 mM EDTA, 0.5% Triton X-100, and 1× complete proteinase inhibitor cocktail, by passage through a 21 gauge needle. The lysed cells were centrifuged at 800*g* for 10 min to remove unbroken cells and nuclear debris. 500 μl of the cell homogenate was mixed with an equal volume of 80% sucrose/KRH containing 5 mM EDTA and place on the bottom of an ultra-clear centrifuge tube (Beckman, 344057). On the resulting 40% sucrose homogenate solution, 2 ml each of 35% and 5% sucrose/KRH containing 5 mM EDTA was overlaid. The gradient was then centrifuged at 200,000*g* (42,000 rpm, in a Beckman SW55Ti rotor) for 18 h and 10 fractions (500 μl each) were collected from the gradient. 1 ml of ice-cold ethanol was added to each of the samples, and the mixtures were then centrifuged at 13,000*g* for 30 min. The resultant protein precipitates were dried and dissolved in 50 μl of 1× SDS–PAGE sample buffer. Non-solubilized membrane proteins, including lipid rafts, float toward the lighter surface fractions, and are thus separated from the soluble proteins remaining in the bottom-loading zone.

2.4. Glucose uptake assay

Cellular glucose transport activity was measured as previously described [10,24]. Briefly, islet cells were pre-incubated in KRH for 20 min at 37 °C and then incubated with 200 μM of 2-NBDG in KRH containing 10 mM glucose at 37 °C for 5 min. Glucose transport was terminated by addition of 2 mM cytochalasin B in KRH then cells were washed with KRH twice. Cellular uptaken 2-NBDG was measured by cell fluorescence using fluorescent plate microplate reader (MTP-650FA, Corona electric).

2.5. Cell surface protein cross-link, and immunoprecipitation

Islet cell surface proteins were cross-linked by DTSSP as previously described [10] and intracellular proteins were cross-linked by DSP. To analyze the glycan-mediated membrane sub-domain distribution of GLUT2 and galectin binding, islet cells were washed twice with HBSS and then incubated with glycans in RPMI 1640 medium for 2 h at 4 °C. The cells were washed twice with ice-cold PBS, and then incubated with 2 mM DTSSP in PBS for 2 h on ice. Crosslinking was terminated by the addition of 1 M Tris–HCl (pH 7.5) to a final concentration of 10 mM. To evaluate the interaction between GLUT2 and Stomatin, DSP was used instead of DTSSP. Briefly, DSP was first dissolved in DMSO at 20 mM and then mixed

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