Glucosamine Treatment-mediated O-GlcNAc Modification of Paxillin Depends on Adhesion State of Rat Insulinoma INS-1 Cells*§

Tae Kyoung Kwak†, Hyeonjung Kim†, Oisun Jung†, Sin-Ae Lee†, Minkyung Kang†, Hyun Jeong Kim**, Ji-Min Park‡‡, Sung-Hoon Kim§§, and Jung Weon Lee†††‡‡‡

From the †Cancer Research Institute, Department of Tumor Biology, the ‡‡Department of Molecular and Clinical Oncology, and the ‡‡‡Department of Biomedical Sciences, College of Medicine and the **Department of Dental Anesthesiology, School of Dentistry, Seoul National University, Seoul 110-799, the ‡Department of Genetic Engineering, Seoul National University, Seoul 151-742, the ¶¶Department of Pharmacy, Cell Dynamics Research Center, Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, and the §§Cancer Preventive Material Development Research Center, Kyunghee University, Seoul 131-701, Korea

Received for publication, April 1, 2010, and in revised form, September 5, 2010. Published, JBC Papers in Press, September 9, 2010, DOI 10.1074/jbc.M110.129601

Protein-protein interactions and/or signaling activities at focal adhesions, where integrin-mediated adhesion to extracellular matrix occurs, are critical for the regulation of adhesion-dependent cellular functions. Although the phosphorylation and activities of focal adhesion molecules have been intensively studied, the effects of the O-GlcNAc modification of their Ser/Thr residues on cellular functions have been largely unexplored. We investigated the effects of O-GlcNAc modification on actin reorganization and morphology of rat insulinoma INS-1 cells after glucosamine (GlcN) treatment. We found that paxillin, a key adaptor molecule in focal adhesions, could be modified by O-GlcNAc in INS-1 cells treated with GlcN and in pancreatic islets from mice treated with streptozotocin. Ser-84/85 in human paxillin appeared to be modified by O-GlcNAc, which was inversely correlated to Ser-85 phosphorylation (Ser-83 in rat paxillin). Integrin-mediated adhesion signaling induced the GlcN treatment-enhanced O-GlcNAc modification of paxillin. Adherent INS-1 cells treated with GlcN showed restricted protrusions, whereas untreated cells showed active protrusions for multiple-elongated morphologies. Upon GlcN treatment, expression of a triple mutation (S83A/S84A/S85A) resulted in no further restriction of protrusions. Together these observations suggest that murine pancreatic β cells may have restricted actin organization upon GlcN treatment by virtue of the O-GlcNAc modification of paxillin, which can be antagonized by a persistent cell adhesion process.

Unlike N-linked glycosylation of membrane or secreted proteins, O-linked N-acetylgalactosamine (O-GlcNAc) attachment to Ser and/or Thr residues occurs mainly in nuclear and cytosolic proteins (1, 2). O-GlcNAc modifications on Ser/Thr residue(s) alter the activity of the molecule(s) and can eventually regulate cell function and/or behavior (2, 3).

Pancreatic β cells sense changes in extracellular glucose concentrations, leading to O-GlcNAc modification of various proteins (4–6). Enhanced glucose flux through the hexosamine biosynthesis pathway can increase the O-GlcNAc modification of proteins responsive to extracellular glucose levels. Furthermore, the O-GlcNAc modification of certain proteins is known to be associated with β-cell apoptosis under hyperglycemic conditions (7).

The architectural integrity of cells is critical for both the function and homeostasis of epithelium. The tissue architecture is maintained by extracellular cell matrix (ECM)2 adhesions via integrin engagements with ECM proteins in the extracellular space as well as cell-cell adhesions via hemophilic interactions between E-cadherins on neighboring cells (8). Cell-ECM adhesion sites (i.e. focal adhesions) are linked to intracellular actin filaments through diverse protein-protein interactions. Therefore, cell adhesion-dependent tissue integrity and intracellular actin organization influence each other, and disruption of cell adhesions can cause aberrant actin organization.

Integrin-ECM engagement results in formation of focal adhesions that contain more than 125 signaling and adaptor proteins, including paxillin (9). Paxillin binds integrins (10), talin (11), and vinculin (12) at focal adhesion sites (9). In addition, paxillin is phosphorylated at many Ser/Thr and Tyr residues, which are important for the signaling activity of several signaling pathways (13). Focal adhesion molecules regulate morphological changes via actin reorganization in diverse cell types. Integrins function by recruiting adaptors or signaling molecules to their cytoplasmic tails and reorganizing actin filaments or intermediate filaments (14).

We investigated the significance of O-GlcNAc modification of paxillin in integrin-mediated cell adhesion in pancreatic INS-1 cells treated with glucosamine (GlcN) as a hyperglycemic

* This work was supported by Korea Science and Engineering Foundation Ministry of Education, Science, and Technology Grant R01-2006-000-10248-0, Cell Dynamics Research Center Grant R11-2007-007-10004-0, and by the Support for Senior Researchers (Leap Research) program through National Research Foundation by the Ministry of Education, Science, and Technology Grant 010-00150029 (to J. W. L.).
† The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.
‡ To whom correspondence should be addressed. Tel.: 82-2-880-2495; Fax: 82-2-872-1795; E-mail: jwl@snu.ac.kr.

2 The abbreviations used are: ECM, extracellular cell matrix; LN, laminin I; WGA, wheat germ agglutinin; STZ, streptozotocin; CTD, COOH-terminal domain; OGT, O-linked GlcNAc transferase; OGA, O-GlcNAcase.
O-GlcNAc Modification of Paxillin

stimulant (15), although it may cause O-GlcNAc modification by other cellular oxidative and/or endoplasmic reticulum stress responses independent of the hexosamine biosynthesis pathway (16, 17). We hypothesized that depending on cell adhesion signaling, the posttranslational modification of focal adhesion molecules may be affected by GlcN treatment. We found that hyperglycemia-mediated O-GlcNAc modification of proteins, especially paxillin at focal adhesion sites, could be differentially modulated by cell adhesion state (suspension versus adhesion), leading to the regulation of actin-reorganization, which is critical for cell spreading and protrusion.

EXPERIMENTAL PROCEDURES

Cell Cultures—INS-1 (ATCC, Manassas, VA), an insulinoma β-cell line isolated from rat pancreas, or INS-1 cells stably transfected with human nOGT were maintained as described previously (15).

Cell Extracts Preparation and Western Blots—In case, INS-1 cells were transiently microinjected with pSuper-ncoGT (targeting 5′-ttttgcacctgacccagagctgtt-3′ of DQ893623 ncoGT ORF) using Microinjection MP-100 (Digital Bio, Korea). INS-1 cells were trypsinized, washed twice with PBS, and pelleted. Cell pellets were resuspended in 1% BSA-containing RPMI 1640 and rolled over (60 rpm) at 37 °C for 1 h to negate basal signaling activity. Resuspended cells were either maintained in suspension or replated onto fibronectin, collagen I, or laminin 1 (LN) (10 μg/ml, Trevigen, Gaithersburg, MD)-precoated dishes or coverslips with or without 75 mM glucosamine (GlcN) in 0.5 mM HEPES (pH 7.5, Sigma) or with 0.1 mM PUG-NAc (Toronto Research Chemicals Inc. Ontario, Canada) for the indicated times. After incubation, cells grown on dishes or coverslips were processed for whole cell extracts or actin staining. Whole cell lysates or pancreatic tissue extracts were prepared using radioimmune precipitation assay buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM sodium pyrophosphate, 0.1% SDS, 0.1% sodium deoxycholate, 1% Nonidet P-40, and protease inhibitors). Lysate protein was quantified using the bicinchoninic acid (BCA) method (Thermo Scientific Pierce), normalized, and used in standard Western blots with antibodies against phospho-Ser-83-paxillin (for rat paxillin, but Ser-85-paxillin for human paxillin, (18)), phospho-Ser-178-paxillin (ECM Biosciences, Versailles KY), Tyr(P)-577-focal adhesion kinase (paxillin (BD Biosciences), phospho-Tyr-31/118-paxillin, phosphotyrosine, α-tubulin (Cell Signaling Technology, Danvers, MA), focal adhesion kinase, c-Src, Tyr(P)-416-c-Src (Santa Cruz Biotechnology, Santa Cruz, CA), and O-GlcNAc (RL2 clone or CTD110.6 clone from GeneTex, Inc., Irvine, CA).

Paxillin Mutagenesis—pCMV-(HA)3-human paxillin wild type (WT) was mutated at Ser-74, Ser-83, Ser-84, Ser-85, or Ser-83/Ser-84/Ser-85 using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA). Alanine mutations were confirmed by direct sequence analyses. Immunoprecipitation—After treatment with 7.5 mM GlcN in 0.5 mM HEPES (pH 7.5) in the absence of serum, INS-1 cells were incubated on LN for 2 or 6 h, and then lysed with radioimmune precipitation assay buffer. Lysates containing 500 μg proteins were mixed with either anti-HA, anti-paxillin antibodies, or normal mouse IgG (0.5 μg/condition) and rotated for 2 h at 4 °C in the absence or presence of 10 mM GlcNAc (Sigma). Protein A/G-Sepharose beads (30 μl of a 50% slurry, Upstate, Billerica, MA) were added to the mixture and rotated for an additional 2 h at 4 °C. The immunoprecipitates were collected, washed, and eluted before standard Western blot analysis using either anti-O-GlcNAc (CTD110.6) or -paxillin antibodies.

Wheat Germ Agglutinin (WGA) Pulldown—Cell extracts (300 μg of proteins) were mixed and incubated with WGA-agarose beads (Sigma) by rotation (60 rpm) at 4 °C overnight before washing with lysis buffer. Beads with attached proteins were mixed with 4× SDS-PAGE sample buffer and boiled for 5 min before immunoblotting using either anti-O-GlcNAc (CTD110.6) or -paxillin antibodies.

Immunofluorescence—INS-1 cells were transiently transfected with either pCMV-(HA)3-human paxillin WT or mutants for 48 h. The cells were replated on coverslips precoated with LN, collagen I, or fibronectin in the absence of serum as above before immunostaining using anti-HA antibody (Santa Cruz Biotechnology) at 4 °C overnight. Cells were stained for actin using phalloidin-conjugated rhodamine (Molecular Probes, Eugene, OR) or fluorescein-conjugated phalloidin (Invitrogen) or for nuclei using DAPI for 1 h at room temperature. Cells were washed three times with PBS and mounted with a mounting solution (DakoCytomation). Visualization was performed using a fluorescent microscope (BX51TR, Olympus, Japan). Protrusions in multiple directions beyond the bi-spindle shape were counted and recorded as the number of protrusions in each cell. Each protrusion longer than one-third of the cell length (i.e. the longest length along the bi-spindle direction) was considered a protrusion.

In Vitro O-GlcNAc Modification Assay—Recombinant GST-paxillin (WT, S74A, or S85Aa mutant) and GST-ncoGT (human nucleocytoplasmic ncoGT) were prepared from iso-propyl 1-thio-β-d-galactopyranoside-induced bacterial cultures (0.1 mM for 2 h). The reaction mixtures included 5 μg of GST-paxillin, 2 μg of GST-ncoGT, and 1 μM UDP-GlcNAc in OGT assay buffer (50 mM Tris-HCl (pH 7.5), 12.5 mM MgCl2, and 1 μM β-mercaptoethanol) for 30 min at 22 °C as described previously (15). In parallel, control reactions without either GST-ncoGT or UDP-GlcNAc were performed. The reaction was terminated by centrifugation (13,000 × g for 10 min at 4 °C) to precipitate GST-beads, which were then washed 3 times with ice-cold radioimmune precipitation assay buffer. The beads were mixed with 2× SDS-PAGE sample buffer, boiled for 5 min, and used in standard Western blots using anti-O-GlcNAc antibody (CTD110.6 clone).

Streptozotocin-induced Diabetic Mouse Preparation—ICR mice (Orient Co. Korea) were handled according to the Seoul National University Laboratory Animal Maintenance Manual and Institutional Review Board agreement. To induce diabetes (19), 20 animals (10 for control and 10 for streptozotocin (STZ)-induced diabetes) were intraperitoneally injected with 200 μl of either citrate buffer (pH 4.5, Sigma) or streptozotocin (40 mg/kg in citrate buffer (pH 4.5)) every day for 5 consecutive days. Two weeks later, blood glucose levels were analyzed every day for 1 week. Animals were sacrificed, and the pancreas was used to prepare extracts for standard Western blot analysis.
Immunohistochemistry—Pancreatic tissue from mice injected with control citrate buffer or STZ was prepared for immunohistochemistry as described previously (20). Anti-O-GlcNAc (CTD110.6 clone, 1:100) or active caspase 3 (Abcam, Cambridge, UK, 1:50) antibodies were used for immunohistochemistry.

Statistical Analysis—The relative intensities of O-GlcNAc proteins under diverse experimental conditions were calculated for fold difference (mean ± S.D.) after normalization of a band (i.e. of a band slightly smaller than 83- or 175-kDa molecular markers as indicated in each figure) over either paxillin or α-tubulin band intensities measured by densitometry. Student’s t test was performed for comparison of mean values to determine whether the difference was significant. p values less than 0.05 or greater than 0.05 were considered significant (depicted as *) or insignificant (depicted as **), respectively.

RESULTS

Cell Adhesion-dependent O-GlcNAc Modification of Proteins in Rat Pancreatic β INS-1 Cells—To study how integrin signaling may regulate the O-GlcNAc modification of proteins after GlcN treatment, we used insulinoma INS-1 cells isolated from rat pancreas that were either maintained in suspension or adhered on diverse ECM proteins with or without 7.5 mM GlcN treatment, as previously described (15). We did not observe any differences in adhesion of INS-1 cells on ECM proteins in the absence or presence of GlcN (data not shown). We observed that cells in suspension had increased O-GlcNAc modification of proteins, whereas cells adherent on different ECM proteins for 6 h showed less O-GlcNAc modifications, as shown by immunoblotting with anti-O-GlcNAc antibody (CTD110.6 clone) (Fig. 1A). Interestingly, cells adherent on laminin I did not show increased O-GlcNAc modification even after GlcN treatment (Fig. 1A, last two lanes). This pattern was also confirmed using another anti-O-GlcNAc antibody (RL2 clone, Fig. 1B). These observations suggest that laminin I receptor-mediated adhesion may efficiently transduce intracellular signaling to inhibit the GlcN treatment-mediated O-GlcNAc modifica- tion. Furthermore, cell adhesion on laminin I for longer periods (10 h) resulted in more obvious cell adhesion-mediated negative effects on O-GlcNAc modification (Fig. 1C). Next we examined the effects of O-GlcNAc modification when cells were replated on different ECM proteins for shorter times. When INS-1 cells were incubated for 2 h either in suspension or on laminin I-precoated dishes, GlcN treatment resulted in efficient O-GlcNAc modifications of proteins under both conditions (Fig. 1D), indicating that signaling from a shorter or earlier adhesion might not be sufficient to inhibit the O-GlcNAc modification of proteins. Furthermore, the differential O-GlcNAc modification of proteins depending on cell adhesion were not correlated with different expression levels of OGA or OGT, as their expression levels were similar no matter whether cells were in suspension or adherent for 2 or 6 h (Fig. 1E).

Differential Morphology of INS-1 Cells Adherent on Laminin I with or without Glucosamine Treatment—Integrin-mediated cell adhesion on ECM proteins results in morphological changes through integrin-mediated signaling for actin reorganization (21). It is also known that the O-GlcNAc modification of proteins can affect the phosphorylation of proteins important for numerous signaling pathways (2, 3, 15). Therefore, we investigated whether GlcN treatment of INS-1 cells adherent on laminin I would alter their morphologies, presumably through antagonistic relationships between integrin/ECM engagement-mediated adhesion signaling and GlcN treatment-mediated O-GlcNAc modification of proteins. Interestingly, when INS-1 cells were newly replated onto laminin I in the absence of glucosamine for 2 or 6 h, they formed protrusions. Cells that were incubated for 2 h with glucosamine treatment showed a less protrusive phenotype, but cells incubated for 6 h

![Image of Figure 1](image-url)
modified by O-GlcNAc (Fig. 2, C and D). Negative effects on INS-1 cell protrusions mediated by GlcN indicate that O-GlcNAc modification may antagonize the cell adhesion-dependent phosphorylation of proteins that are critically important for integrin-mediated cell adhesion and spreading.

**Differential O-GlcNAc Modification of Paxillin in Suspended or Laminin I-adherent INS-1 Cells after Glucosamine Treatment**—Integrin/ECM engagement is known to mediate the formation of protein complexes at focal adhesions, where diverse signaling and/or adaptor molecules interact and are phosphorylated in a cell adhesion-dependent manner (14, 22). Thus, in GlcN-treated INS-1 cells, O-GlcNAc modifications of component(s) of the focal adhesion protein complex may lead to inefficient activities and/or roles in adhesion and spreading. Therefore, we performed immunoblot analysis of WGA-pulldown assays prepared from INS-1 cells either in suspension or adherent on laminin I for 6 h. The primary antibodies for the immunoblots included those diverse against focal adhesion molecules including paxillin. Interestingly, the WGA pulldown assays showed a clear responsiveness to anti-paxillin antibody (Fig. 3A), suggesting that paxillin or a tightly paxillin-binding protein might undergo distinct O-GlcNAc modification and increased adhesion signaling in a GlcN-independent manner compared with a 2-h incubation on laminin I. These results

on laminin I with glucosamine treatment showed obvious protrusions (Fig. 2A). However, incubation for 6 h on other ECM proteins such as collagen I or fibronectin resulted in less protrusive phenotypes in INS-1 cells after 7.5 mM GlcN treatment (supplemental Fig. S1), consistent with O-GlcNAc modification under the same experimental conditions shown in Fig. 1A. The number of protrusive cells and protrusions in each cell significantly decreased after GlcN treatment of cells adherent on laminin I for 2 h, but not for 6 h (Fig. 2B). Because a specific inhibitor for OGA, PUGNAc, may allow the accumulation of proteins modified by O-GlcNAc similar to GlcN, we tested whether PUGNAc treatment in the absence of GlcN led to less protrusive cells, mimicking GlcN treatment and suggesting that treatment with the OGA inhibitor was sufficient to allow the accumulation of proteins...
strongly suggest that O-GlcNAc modification of paxillin or its binding protein may occur after GlcN treatment of INS-1 cells and that the degree of O-GlcNAc modification may be antagonized by a persistent adhesion process that affects cell morphology.

To confirm that paxillin is modified with O-GlcNAc, whole cell extracts were immunoprecipitated with anti-paxillin antibody before immunoblotting with either anti-O-GlcNAc or paxillin antibody. Suspended INS-1 cells treated with GlcN obviously showed O-GlcNAc-modified paxillin, but cells adherent on laminin I showed insignificant O-GlcNAc modification of paxillin (Fig. 3D). However, upon depletion of anti-O-GlcNAc (CTD110.6 clone) using O-GlcNAc, the band on the anti-O-GlcNAc immunoblot was not observed (Fig. 3E). Furthermore, immunoprecipitates using normal mouse IgG did not show any bands on the anti-O-GlcNAc immunoblot (Fig. 3F). Meanwhile, it was found that OGA or OGT bound paxillin similarly in cells both adherent on LN and in suspension, indicating that differential binding of paxillin to either OGA or OGT is not different in suspended or LN-adherent cells (Fig. 3G). Overexpression of ncOGT in Xenopus oocytes has previously been shown to increase O-GlcNAc modification activity (23). When ncOGT cDNA or siRNA against ncOGT was stably or transiently transfected, O-GlcNAc modification of proteins and paxillin increased or decreased, respectively (Fig. 3H). These observations indicate that the bands shown in the anti-O-GlcNAc immunoblot of the immunoprecipitates using anti-paxillin antibody are quite specifically O-GlcNAc-modified paxillin or paxillin-binding protein.

**Differential O-GlcNAc Modification and Phosphorylation of Paxillin by Glucosamine Treatment**—Paxillin phosphorylation is important for its function as an adaptor molecule in focal adhesions (24, 25). Therefore, we examined how GlcN treatment affects the phosphorylation status of paxillin. Paxillin is phosphorylated on both Ser-83 by p38MAPK during nerve growth factor-induced neurite extension of PC-12 cells (26) and on Thr-317 by Src kinase during integrin signaling (27). We therefore measured the phosphorylation levels of paxillin at both Ser-83 and Thr-317 in suspended and adherent INS-1 cells treated with GlcN. As shown in Fig. 4A, treatment with GlcN caused a decrease in Ser-83 phosphorylation and an increase in Thr-317 phosphorylation in adherent cells compared with suspended cells. These data indicate that glucosamine treatment differentially affects the phosphorylation of paxillin, suggesting that O-GlcNAc modification may regulate the phosphorylation of paxillin in adherent cells.
Ser-83/84/85 Residues of Human Paxillin Are Important for the Regulation of Cell Morphology after Glucosamine Treatment—Rat paxillin (NCBI accession number of NP_001012147) has the amino acid sequence Ser-83—Pro-84—Ser-85, whereas human paxillin (NP_001074324) has the sequence Ser-83—Ser-84—Ser-85. In addition, Ser-83 phosphorylation of rat paxillin by p38MAPK is correlated with Ser-85 phosphorylation of human paxillin by Erk1/2 (18). Furthermore, mass spectrometry of human paxillin immunoprecipitates prepared from extracts of HEK cells treated with phosphatase inhibitors (e.g. 1 mM sodium peroxovanadate and 10 mM calyculin) revealed that Ser-74 is modified with -GlcNAc, as in Fig. 1, before harvests of whole cell lysates and then standard Western blots (WB) for anti-phospho-Ser-83 paxillin (pSer83-paxillin) (A and D), -phospho-Ser-178 paxillin (pSer178-paxillin) (B), or -phospho-Tyr-31118 paxillin (pY31/118-paxillin) (C) and -paxillin antibody. D, PUGNAC at 0.1 mM or vehicle DMSO (Con) was directly treated into the replicating media, as explained under “Experimental Procedures.” * or ** depicts statistically significant (p < 0.05) or insignificant (p ≥ 0.05) values, respectively. Data shown are representative of three isolated experiments.

and Ser-178 by JNK during the migration of fish keratocytes and rat bladder tumor epithelial cells (27). Therefore, we examined whether GlcN treatment might affect the Ser phosphorylation status of paxillin when INS-1 cells adherent on laminin I were treated with or without GlcN. Interestingly, we observed that Ser-83 phosphorylation (Ser(P)-83-paxillin) was inversely correlated to -GlcNAc modification under our experimental conditions (Fig. 4C). As shown in Fig. 4C, adherent INS-1 cells showed higher Tyr(P)-31118-paxillin levels that were slightly dependent on GlcN treatment, whereas cells in suspension did not show any Tyr(P)-31118-paxillin that correlated with treatments. More interestingly, treatment with the specific OGA inhibitor PUGNAC even without GlcN treatment increased -GlcNAc protein modification and decreased Ser(P)-83-paxillin levels, similar to GlcN alone (Fig. 4D), indicating that Ser(P)-83-paxillin is affected by intracellular -GlcNAc protein modification. These results indicate that Tyr(P)-31118-paxillin is primarily affected by the process of cell adhesion but not by GlcN treatment alone and that GlcN treatment slightly reduces Tyr(P)-31118-paxillin levels only in adherent cells. These observations together suggest that -GlcNAc modification negatively affects the phosphorylation of Ser-83, but not of Ser-178, presumably leading to less efficient paxillin activity required for cell spreading.

Ser-83/84/85 -GlcNAc antibody (clone RL2). Expression of either S84A or S85A mutant inhibited the GlcN-induced -GlcNAc protein modification, whereas the S83A mutation slightly reduced O-GlcNAc modifications, but the S74A mutation did not alter the GlcN-mediated effects (Fig. 5A). When anti-HA immunoprecipitates were immunoblotted using CTD110.6 anti-O-GlcNAc antibodies, the control vector-transfected cells did not show any O-GlcNAc modifications of the anti-HA immunoprecipitates (Fig. 5B). However, WT paxillin-expressing cells showed GlcN treatment-dependent O-GlcNAc modification after 2 h on laminin I, whereas expression of the S74A mutant slightly inhibited the GlcN treatment-dependent O-GlcNAc modification, suggesting that additional O-GlcNAc modifications of other residues may be possible (Fig. 5B, first four lanes). Interestingly, expression of the S83A mutant resulted in GlcN treatment-dependent O-GlcNAc modifications, suggesting the O-GlcNAc modification of residues other than Ser 83 (Fig. 5B, fifth and sixth lanes). However,
neither S84A nor S85A mutant paxillin showed GlcN treatment-enhanced O-GlcNAc modification, indicating that the Ser residues at 84/85 might be O-GlcNAc-modified after GlcN treatment (Fig. 5B, seventh through tenth lanes). Finally, expression of the S83A/S84A/S85A triple mutant paxillin resulted in no GlcN treatment-dependent O-GlcNAc modification (Fig. 5B, eleventh and twelfth lanes), confirming that Ser-84/85 residues can be modified with O-GlcNAc after GlcN treatment.

Furthermore, paxillin WT or mutants did not differentially bind to OGA or OGT, despite differential O-GlcNAc modification of paxillin proteins (Fig. 5C). This observation suggests that the differential O-GlcNAc modification of paxillin dependent on cell adhesion status was caused by presumably differential OGT or OGA activity rather than its binding amount to paxillin. OGT might have a higher activity in the earlier adhesion time points (i.e. 2 h) but decline afterward (i.e.
both in the presence and absence of GlcN treatment (Fig. 6, right panels and graph), indicating that the Ser stretch (Ser-83/84/85) in human paxillin is important for the regulation of cell protrusions, especially after GlcN treatment. Cells transfected with WT paxillin showed cells with restricted protrusions (79 ± 2.6%), whereas untransfected cells also showed a similar phenotype with fewer protrusions (82 ± 3.8%) after GlcN treatment. When transfected with S83A/S84A/S85A paxillin, 75 ± 4.2% of transfection-positive cells showed multiple-elongated protrusions, whereas 21 ± 2.6% of the untransfected cells showed protrusions after GlcN treatment (data not shown). These cell morphology observations suggest that the Ser-83/84/85 stretch in human paxillin may be additionally responsive to extracellular GlcN treatment together with Ser-74.

**Streptozotocin-induced Diabetic Mice Showed O-GlcNAc Modification of Paxillin**—We next investigated whether pancreatic cells prepared from mice in which diabetes was induced by STZ injections showed O-GlcNAc-modified paxillin. In STZ-injected animals, pancreatic islets showed positive staining for anti-O-GlcNAc antibody, compared with control buffer-injected animals (n = 3) (Fig. 7A). Blood glucose levels in STZ-injected mice were significantly enhanced compared with control mice (supplemental Fig. S2), although body weights normally increased in control and STZ-injected mice (supplemental Fig. S3). Immunoblots of STZ-induced pancreatic tissue extracts using anti-O-GlcNAc antibody showed higher O-GlcNAc-modified proteins at ~70 kDa (Fig. 7B). When immunoprecipitates from pancreatic extracts using anti-paxillin antibodies were immunoblotted, O-GlcNAc modification of paxillin was found to increase in extracts from STZ-injected mice, whereas citrate buffer-injected control pancreatic tissue extracts did not show an increase in the O-GlcNAc modification of paxillin (Fig. 7C, upper panels). Such an increase in the O-GlcNAc modification of paxillin was inversely correlated to Ser(PT)-83-paxillin levels (Fig. 7C). However, O-GlcNAc depletion during anti-O-GlcNAc immunoblottting and immunoprecipitation using normal mouse IgG did not yield the appearance of any O-GlcNAc-modified protein bands (Fig. 7C). As expected, pancreatic/islet tissue from STZ-induced mice was positive for active caspase 3, but tissue from control buffer-injected mice was not (Fig. 7, A and C), indicating that STZ mediates cell death in islet/pancreatic cells.

**DISCUSSION**

Integrin-mediated cell adhesions to ECM link the extracellular space to intracellular actin filaments, allowing communication between these regions through focal adhesions for both signal transduction and actin reorganization (8). Thus, communication of cells with their microenvironment allows the dynamic adaptation and response to numerous extracellular cues including neighboring cells, soluble factors, and matrix proteins. Membrane receptors aid in both communication by sensing cues from ligand binding and transduction of intracellular signals by conformational changes and activation. Different cell surface membrane receptors can either function alone or collaborate with other types of receptors in synergistic or antagonistic ways.
This study revealed an antagonistic relationship between two different signaling mechanisms; that is, the process of cell adhesion-mediated signaling and O-GlcNAc modification of proteins by extracellular GlcN treatment. GlcN treatment of rat insulinoma INS-1 cells resulted in O-GlcNAc modification of paxillin, presumably at Ser-84/85 based on mutagenesis studies (7, 30, 31). Therefore, O-GlcNAc modifications may lead to apoptosis due to loosened cell attachments to the ECM (i.e. anoikis). As a response, INS-1 cells may increase cell adhesion-mediated signaling to lessen the effects of O-GlcNAc modification of paxillin, leading to efficient spreading/protrusion of rat INS-1 cells.

Abnormalities in the O-GlcNAc modification of various proteins causes insulin resistance and diabetic complications through the hexosamine biosynthesis pathway, which functions as a nutrient sensor (6). When physiological changes in extracellular glucose concentrations occurs, pancreatic β cells uniquely enriched with OGT (5) induce intracellular signaling to modify certain proteins with O-GlcNAc monomer (29), leading to altered signaling and/or protein-protein interaction activities. These changes could result in the abnormal regulation of cell functions including maintenance of cell morphology and thereby cell death (29). The pancreatic β cells in patients with type II diabetes cannot efficiently produce insulin because of necrosis and apoptosis of the β cells (7, 30, 31). Therefore, efficient and persistent cell adhesion may be a way to lessen the effects of hyperglycemia-mediated O-GlcNAc protein modification, facilitating cell survival.

O-GlcNAc-modified proteins are mostly phosphoproteins that play important roles in various cellular functions, including stress responses (3). O-GlcNAc modification or phosphorylation at Ser/Thr residues can be reciprocally regulated to modulate protein activity and stability (32, 33). This study showed that the O-GlcNAc modification of Ser-84/Ser-85 in human paxillin (presumably of Ser-85 in rat
O-GlcNAc Modification of Paxillin

paxillin (18). Previous reports show that Akt1 (Ser-473), estrogen receptor β (Ser-16), c-Myc (Thr-58), and endothelial nitric-oxide synthase (Ser-1177) are reciprocally O-GlcNAc-modified or phosphorylated (15, 34–36). Multiple Ser/Thr residues in the COOH-terminal domain (CTD) of RNA polymerase II are modified with O-phosphate or O-GlcNAc in a mutually exclusive manner, resulting in the reciprocal regulation of transcriptional elongation (37). O-GlcNAc modification leads to a negative relationship with Tyr-31/118 phosphorylation of paxillin in adherent INS-1 cells, presumably via a structural relay between Tyr-31/118 and Ser-84/85 residues. In suspended cells, however, paxillin O-GlcNAc modification occurs, but Tyr-31/118 phosphorylation of paxillin does not. O-GlcNAc modification of p53 Ser-149 decreases Thr-155 phosphorylation, leading to p53 stabilization via the blockade of ubiquitin-dependent proteolysis (32). Thus, O-GlcNAc modification of Ser/Thr can sterically affect phosphorylation of adjacent residues. Alternatively, it is likely that Ser-83/85 phosphorylation of rat paxillin (or Ser-83/84/85 of human paxillin) may compete with the addition of O-GlcNAc to these Ser residues or may sterically regulate the addition of O-GlcNAc to Ser-74. O-GlcNAc modification of paxillin was antagonized by integrin/ECM engagement-mediated cell adhesion, thereby maintaining efficient cellular protrusions even after GlcN treatment. Therefore, in INS-1 cells, paxillin O-GlcNAc modification of Ser-74 and Ser-85 may lead to reduced Ser-83 phosphorylation. Ser-83 phosphorylation in rat paxillin is important for the morphological protrusions that form neurite extensions of PC-12 cells (26). For human paxillin, Ser-85 rather than Ser-83 was suggested to be the site of phosphorylation by Erk1/2, which is important for hpactic growth factor-mediated paxillin-focal adhesion kinase association that leads to local cytoskeletal rearrangements and the regulation of cell morphology (18). Therefore, these previous studies suggested that Ser-85 phosphorylation in human paxillin might be important for morphological regulation and competition with O-GlcNAc modification after GlcN treatment.

Integrin-mediated engagement to ECM proteins enables proficient signaling activities by protein phosphorylation (14). Upon cell adhesion, recruitment of and protein-protein complex formation between diverse signaling and adaptor molecules including paxillin, vinculin, and talin, which are shown to be modified with O-phosphate or O-GlcNAc (3) at focal adhesions, are critical for signal transduction leading to dynamic changes in cellular morphology (38). As a focal adhesion-associated adaptor protein (24), paxillin binds to vinculin, which is a linker between focal adhesion proteins and actin filaments (39). Paxillin is also involved in adhesion disassembly (40), which may result from restricted spreading or reduced protrusions under stressful conditions, as shown in this study.

Because cell adhesion signaling mediates antagonistic effects on the O-GlcNAc modification of paxillin after GlcN stimulation, the survival of pancreatic β cells may depend on the avail-ability of integrin/ECM-mediated signaling activity. Given that phosphorylation is important for protein activity, O-GlcNAc modifications are important targets for the design and development of therapeutic reagents against diseases, such as type II diabetes and cancer.

REFERENCES

1. Zachara, N. E., and Hart, G. W. (2006) Biochim. Biophys. Acta 1761, 599–617
2. Vosseller, K., Sakabe, K., Wells, L., and Hart, G. W. (2002) Curr. Opin. Chem. Biol. 6, 851–857
3. Love, D. C., and Hanover, J. A. (2005) Sci. STKE 2005, re13
4. Kornfeld, R. (1967) J. Biol. Chem. 242, 3135–3141
5. Hanover, J. A., Lai, Z., Lee, G., Lubas, W. A., and Sato, S. M. (1999) Arch Biochem. Biophys. 362, 38–45
6. Cooksey, R. C., and McClain, D. A. (2002) Ann. N.Y. Acad. Sci. 967, 102–111
7. Liu, K., Paterson, A. J., Chin, E., and Kudlow, J. E. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 2820–2825
8. Thiery, J. P. (2003) Curr. Opin. Cell Biol. 15, 740–746
9. Zaidel-Bar, R., Milo, R., Kam, Z., and Geiger, B. (2007) J. Cell Sci. 120, 137–148
10. Hynes, R. O. (1992) Cell 69, 11–25
11. Geiger, B., Tokuyasu, K. T., Dutton, A. H., and Singer, S. J. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4127–4131
12. Brown, M. C., and Turner, C. E. (2004) Physiol. Rev. 84, 1315–1339
13. Vicente-Manzanares, M., Choi, C. K., and Horwitz, A. R. (2009) J. Cell Sci. 122, 199–206
14. Kang, E. S., Han, D., Park, J., Kwak, T. K., Oh, M. A., Lee, S. A., Choi, S., Park, Z. Y., Kim, Y., and Lee, J. W. (2008) Exp. Cell Res. 314, 2238–2248
15. Lin, Y., Berg, A. H., Iyengar, P., Lam, T. K., Giacca, A., Comb, T. P., Rajala, M. W., Durnall, B., Li, W., Hawkins, M., Barzilai, N., Rhodes, C. J., Fantus, I. G., Brownlee, M., and Scherer, P. E. (2005) J. Biol. Chem. 280, 4617–4626
16. Wurstuck, G. H., Khan, M. I., Femina, G., Kim, A. J., Tedesco, V., Trigatti, B., and Shi, Y. (2006) Diabetes 55, 93–101
17. Ishibe, S., Joly, D., Liu, Z. X., and Cantley, L. G. (2004) Mol. Cell 16, 257–267
18. McEvoy, R. C., Andersson, J., Sandler, S., and Hellerström, C. (1984) J. Clin. Invest. 74, 715–722
19. Lee, S. A., Lee, S. Y., Cho, I. H., Oh, M. A., Kang, E. S., Kim, Y. B., Seo, W. D., Choi, S., Nam, J. O., Tamamori-Adachi, M., Kitajima, S., Ye, S. K., Kim, S., Hwang, Y. J., Kim, I. S., Park, K. H., and Lee, J. W. (2008) J. Clin. Invest. 118, 1354–1366
20. Lee, J. W., and Juliano, R. (2004) Mol. Cells 17, 188–202
21. Harburger, D. S., and Calderwood, D. A. (2009) J. Cell Sci. 122, 159–163
22. Dehennault, V., Hanoulle, X., Bodart, F. J., Vilain, J. P., Michalski, J. C., Landrieu, L., Lippens, G., and Lefebvre, T. (2008) Biochem. Biophys. Res. Commun. 369, 539–546
23. Schaller, M. D. (2001) Oncogene 20, 6459–6472
24. Turner, C. E. (2000) Nat. Cell Biol. 2, E231–E236
25. Huang, C., Borchers, C. H., Schaller, M. D., and Jacobson, K. (2004) J. Cell Biol. 164, 593–602
26. Huang, C., Rajfur, Z., Borchers, C., Schaller, M. D., and Jacobson, K. (2003) Nature 424, 219–223
27. Schroeder, M. J., Webb, D. J., Shabanowitz, J., Horwitz, A. F., and Hunt, D. F. (2005) J. Proteome Res. 4, 1832–1841
28. Zachara, N. E., and Hart, G. W. (2004) Biochim. Biophys. Acta 1673, 13–28
29. Sesti, G. (2002) Ann Med. 34, 444–450
30. D’Alessandria, C., Andreozzi, F., Federici, M., Cardellini, M., Brunetti, A., Ranalli, M., Del Guerra, S., Lauro, D., Del Prato, S., Marchetti, P., Lauro, R., and Sesti, G. (2004) FASEB J. 18, 959–961
31. Yang, W. H., Kim, J. E., Nam, H. W., Ju, J. W., Kim, H. S., Kim, Y. S., and...
Cho, J. W. (2006) Nat. Cell Biol. 8, 1074–1083
33. Wells, L., Vosseller, K., and Hart, G. W. (2001) Science 291, 2376–2378
34. Cheng, X., Cole, R. N., Zaia, J., and Hart, G. W. (2000) Biochemistry 39, 11609–11620
35. Du, X. L., Edelstein, D., Dimmeler, S., Ju, Q., Sui, C., and Brownlee, M. (2001) J. Clin. Invest. 108, 1341–1348
36. Chou, T. Y., Hart, G. W., and Dang, C. V. (1995) J. Biol. Chem. 270, 18961–18965
37. Kelly, W. G., Dahmus, M. E., and Hart, G. W. (1995) J. Biol. Chem. 268, 10416–10424
38. Lock, J. G., Wehrle-Haller, B., and Strömblad, S. (2008) Semin Cancer Biol. 18, 65–76
39. Turner, C. E., Glenney, J. R., Jr., and Burridge, K. (1990) J. Cell Biol. 111, 1059–1068
40. Webb, D. J., Donais, K., Whitmore, L. A., Thomas, S. M., Turner, C. E., Parsons, J. T., and Horwitz, A. F. (2004) Nat. Cell Biol. 6, 154–161