_O_-GlcNAc modulation at Akt1 Ser473 correlates with apoptosis of murine pancreatic β cells

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Abstract

O-GlcNAc transferase (OGT)-mediated modification of protein Ser/Thr residues with *O*-GlcNAc influences protein activity, similar to the effects of phosphorylation. The anti-apoptotic Akt1 is both activated by phosphorylation and modified with *O*-GlcNAc. However, the nature and significance of the Akt1 *O*-GlcNAc modification is unknown. The relationship of *O*-GlcNAc modification and phosphorylation at Akt1 Ser473 was examined with respect to apoptosis of murine β-pancreatic cells. Glucosamine treatment induced apoptosis, which correlated with enhanced *O*-GlcNAc modification of Akt1 and concomitant reduction in Ser473 phosphorylation. Pharmacological inhibition of OGT or *O*-GlcNAcase revealed an inverse correlation between *O*-GlcNAc modification and Ser473 phosphorylation of Akt1. MALDI-TOF/TOF mass spectrometry analysis of Akt1 immunoprecipitates from glucosamine-treated cells, but not untreated controls, showed a peptide containing S473/T479 that was presumably modified with *O*-GlcNAc. Furthermore, *in vitro O*-GlcNAc-modification analysis of wildtype and mutant Akt1 revealed that S473 was targeted by recombinant OGT. A S473A Akt1 mutant demonstrated reduced basal and glucosamine-induced Akt1 *O*-GlcNAc modification compared with wildtype Akt1. Furthermore, wildtype Akt1, but not the S473A mutant, appeared to be associated with

OGT following glucosamine treatment. Together, these observations suggest that Akt1 Ser473 may undergo both phosphorylation and *O*-GlcNAc modification, and the balance between these may regulate murine β-pancreatic cell fate.

Key words: apoptosis, Akt, posttranslational modification, phosphorylation, and *O*-glycosylation.

Abbreviation lists: Alloxan, 1,3-Diazinane-2,4,5,6-tetrone; BADGP, benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside; GlcN, glucosamine; HBP, hexosamine biosynthesis pathway; HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; INS-1, insulinoma cell line isolated from rat pancreas; ncOGT, nucleocytoplasmic OGT; NTA, nitroloacetic acid; *O*-GlcNAc, *O*-linked N-acetylglucosamine; *O*-GlcNAcase, *O*-GlcNAc-selective N-acetyl-β-D-glucosaminidase; OGT, *O*-GlcNAc transferase; PUGNAc, *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate.

Introduction

In contrast to *N*-linked glycosylation of membrane or secreted proteins, attachment of the monosaccharide of *O*-linked N-acetylglucosamine (*O*-GlcNAc) to Ser and/or Thr residues occurs mainly on nuclear and cytosolic proteins [1, 2]. The *O*-GlcNAc modification is dynamically catalyzed by *O*-GlcNAc transferase (OGT) [3] and removal of *O*-GlcNAc is achieved by *O*-GlcNAc-selective N-acetyl-β-D-glucosaminidase (*O*-GlcNAcase) [4]. *O*-GlcNAc modification of Ser/Thr residue(s) on a specific protein can modulate the activity of that molecule to the extent of ultimately regulating cell function or behavior [2, 4].

Many studies have indicated that activation of hexosamine biosynthesis pathway (HBP), which serves as a nutrient sensor, can lead to abnormalities in protein *O*-GlcNAc modification that may be associated with insulin resistance and diabetic complications [5]. When physiological changes in extracellular glucose concentrations are detected by β-pancreatic cells that are uniquely enriched with OGT [6], glucose is converted to glucose-6-phosphate and then to fructose-6-phosphate [7]. The rate-limiting conversion of fructose-6-phosphate to glucosamine-6-phosphate is mediated by L-glutamine:fructose-6-phosphate amidotransferase (GFAT) [8]. Glucosamine-6-phosphate can subsequently be converted to UDP-N-acetylglucosamine

(UDP-GlcNAc), the requisite substrate for *O*-GlcNAc modification of many different proteins [9]. Therefore, an enhanced glucose flux through the HBP can increase *O*-GlcNAc-modification of proteins in response to nutrient levels. Intracellular accumulation of *O*-GlcNAc-modified proteins can also be achieved by inhibition of *O*-GlcNAcase [4].

Patients with type II diabetes are characterized by reduced secretion of insulin by β-pancreatic cells in response to hyperglycemia combined with impaired ability of skeletal muscle, fat and liver cells to respond to insulin [10]. The failure of β-cells to compensate for the insulin demand results from reduced β-cell mass through their necrosis and apoptosis [11]. Thus, a loss in pancreatic β-cell mass may intensify the functional defects, and cause further impairment of the aberrant insulin secretion observed in inefficiently controlled diabetes [12]. *O*-GlcNAc modification of certain proteins is known to be associated with β-cell apoptosis in hyperglycemic conditions [11].

The anti-apoptotic protein Akt1 is activated by phosphorylation [13]; specifically, phosphorylation at Thr308 and Ser473 is required for full Akt1 activity [14]. Akt1 in turn phosphorylates diverse downstream molecules including caspase 3, Bad, forkhead transcription factor (FOXO family), and GSK3β, inhibiting their pro-apoptotic

activities and promoting cell survival [14]. Interestingly, the phosphorylation level of Ser473 is modulated by hyperglycemic conditions [12], and Akt1 may also be modified with *O*-GlcNAc [15]. However, it is currently not clear which Ser/Thr residue(s) of Akt1 are *O*-GlcNAc-modified and how the *O*-GlcNAc modification is related to its phosphorylation. Results of this study indicate that Akt1 Ser473 may be modified with *O*-GlcNAc, and that *O*-GlcNAc modification and phosphorylation of Ser473 are reciprocally regulated by hyperglycemic treatment in murine β-pancreatic cells.

Materials and Methods

Cell cultures: βTC-6 (ATCC), an insulinoma cell line isolated from mouse pancreas, was grown in RPMI-1640 containing 10% FBS (WelGENE, Daegu, Korea) in 5% CO_2 at 37°C. INS-1, an insulinoma cell line isolated from rat pancreas, was grown in RPMI-1640 culture medium containing 10% FBS, 1 mM sodium pyruvate, 10 mM HEPES and 50 μM 2-mercaptoethanol in 5% CO_2 at 37°C. INS-1 cells that stably express human nucleocytoplasmic OGT (ncOGT) were prepared by transfection with mammalian ncOGT plasmid (Gene accession number DQ893623; cDNA cloned into pcDNA3 vector at *BamH*I/*Not*I restriction sites) and selection with G418 (500 μg/ml). βTC-6 and INS-1 cells were subcultured at a 1:4 ratio once a week and maintained between passages 18 and 35. Culture media were changed every 48 h.

Cell extract preparation and Western blots: βTC-6 or INS-1 cells were incubated in the normal culture media or serum-free media with containing glucosamine in 0.5 mM HEPES, pH 7.5 (Sigma) at the indicated concentrations for different time periods prior to lysis with a modified RIPA lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM sodium pyrophosphate, 0.1% sodium deoxycholate, 1% NP-40 and protease inhibitors). In certain experiments, INS-1 cells were treated with RPMI-1640 with 11.1 (normal glucose, NG), 22.2, or 33.3 mM glucose and compensated with mannitol

for osmotic balance. When required, pharmacological inhibitor against OGT [BADGP (Calbiochem) or Alloxan (Sigma)] or *O*-GlcNAcase (PUGNAc, Toronto Research Chemical Inc., Ontario, Canada) was directly added to serum-free culture media at the indicated concentrations 1 h before treatment with glucosamine in 0.5 mM HEPES (pH 7.5). Protein concentration of lysates was quantitated by the BCA (Pierce) method, and normalized prior to analysis by standard Western blot procedures using antibodies against phospho-S^{473}Akt, phospho-T^{308}Akt, Akt, phospho-S^{9}GSK3β, GSK3β, active caspase-3 (Cell Signaling), OGT (Sigma), and O-GlcNAc (RL2 clone from Affinity BioReagent or CTD110.6 clone from Convence).

S473A and T479A Akt1 mutation: pCMV5-$(HA)_3$-Akt1 wildtype (WT) was point-mutated at Ser473 or Thr479 using the Quick-change site-directed mutagenesis system (Stratagene). In each case, mutation to Ala was confirmed by direct sequence analysis.

Immunoprecipitation: INS-1 cells were treated with glucosamine in 0.5 mM HEPES (pH 7.5) at different concentrations for 14 h in the absence of serum, then lysed with modified RIPA buffer. Lysate containing 500 μg protein was mixed with anti-*O*-GlcNAc (RL-2 clone) or anti-Akt antibody (0.5 μg/condition) and rotated for 2 h at 4°C. Protein A/G sepharose beads (30 μl of 50% slurry, Upstate) were added to the mixture before an additional 2 h rotation at 4°C. The immunoprecipitates were collected,

washed, and eluted prior to standard Western blot analysis [16].

DNA content analysis: Subconfluent INS-1 cells in serum-free media were treated without or with glucosamine in 0.5 mM HEPES (pH 7.5) at different concentrations for 22 h before propidium iodide (PI) staining for 30 min and FACS analysis as described previously [17].

Expression and elution of recombinant fusion proteins: Human ncOGT was cloned into pGEX-6p-2 vector (GE healthcare) at *BamHI/Not*I cloning sites. Mouse Akt1 WT, S473A mutant, or T479A mutant were cloned into pET-28a-c(+) vector (Novagen) at *BamHI/EcoR*I cloning sites. The sequence of each construct was confirmed by direct sequence analysis. Recombinant proteins were induced in BL21 *E.coli* strain by 0.3 mM IPTG for 3 h, prior to extraction via sonications. Bacterial extracts were incubated with glutathione-sepharose or Ni^{2+}-conjugated NTA (nitroloacetic acid) beads for 1 h and recombinant proteins were eluted from beads with GST fusion protein elution buffer (50 mM Tris-HCl, pH 8.2, 2 mM $MgCl_2$, 0.2 mM $Na_2S_2O_5$, 10% Glycerol, 20% Sucrose, 2 mM DTT, 1 mM Na_2VO_4, and protease inhibitors) or with $(His)_6$ fusion protein elution buffer (8 M Urea, 50 mM Tris-HCl, pH 8.0, and 10 mM DTT) from beads.

In vitro O-GlcNAc modification assay: Recombinant $(His)_6$-Akt1 WT, S473A, or

T479A protein bound to Ni^{2+}-NTA beads was washed with ice-cold PBS and incubated with 2 μg recombinant GST-ncOGT protein and 1 μM UDP-GlcNAc in OGT activity assay buffer (50 mM Tris-HCl, pH 7.5, 12.5 mM $MgCl_2$, and 1 mM β-mercaptoethanol) for 30 min at 22°C. In specific cases, ncOGT was inhibited by 1 mM BADGP (benzyl-2-acetamido-2-deoxy-alpha-d-galactopyranoside). After incubation, the reaction mixture was washed three times with ice-cold PBS, then 2x SDS-PAGE sample buffer was added to the mixtures prior to boiling for 5 min. Standard Western blots using anti-*O*-GlcNAc (CTD 110.6 clone) (Convence) antibody were performed.

2D-PAGE: Akt1 immunoprecipitates were prepared from control or INS-1 cells treated with 7.5 mM glucosamine in the absence of serum and solubilized in rehydration buffer (9 M urea, 2% CHAPS, 60 mM DTT, 0.5% pharmalyte, pH 4-7, 0.002% bromophenol blue). Protein samples were loaded onto IPG strips and rehydrated overnight, then IEF gels were run as described previously [18]. Briefly, pre-cast immobilized pH gradient strips (24 cm, pH 4-7, linear, Amersham Biosciences, Uppsala, Sweden) for isoelectric focusing (IEF) were equilibrated for 15 min in a reducing solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30%(v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT), and then for a further 15 min in an alkylating solution, which was identical to the reducing solution except that 2.5% (w/v) iodoacetamide was substituted for DTT. Gel

electrophoresis was performed by standard 8% SDS-PAGE, and proteins were visualized by silver staining or immunoblotted with anti-*O*-GlcNAc (CTD 110.6 clone). *MALDI-TOF/TOF*: Spots on silver-stained gels corresponding to Akt1 were excised, destained by reduction using 30 mM potassium ferricyanide/100 mM sodium thiosulfate, and washed with distilled water for MALDI-TOF/TOF analysis as described previously [19]. Briefly, gel pieces were incubated with 0.2 M NH_4HCO_3 for 20 min, dehydrated, shrunk twice with 100% acetonitrile, and dried by vacuum centrifugation. For "in-gel" trypsin digestion of Akt1 immunoprecipitates, gel pieces were rehydrated in digestion buffer containing 0.05 M NH_4HCO_3 and 10 ng/μl of modified porcine trypsin (Promega) at 4°C for 45 min. Excess supernatant was then removed, and the gel pieces were covered with 0.05 M NH_4HCO_3 buffer. Digestion was performed overnight at 37°C, then tryptic peptides were extracted from the gel particles, desalted using a GELoader tip, and packed with POROS 20R2 resin (Applied Biosystems Inc.). Peptide binding and washing were performed in 0.1% trifluoroacetic acid (TFA) in water. To produce the MALDI sample matrix, α-cyano-4-hydroxy cinnamic acid was dissolved at a concentration of 5 g/l in a solution containing 70% acetonitrile and 0.1% TFA. Elution was performed with 1 μl of sample matrix and the eluted peptides were directly spotted on the target plate.

Protein identification was carried out by peptide mass fingerprinting (PMF) using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager DE-PRO MALDITOF mass spectrometer) (Biosystems Inc.). Mass spectra were registered in reflectron positive ion mode and mass accuracy was set at 50 ppm. Database searches for PMF were performed using the MASCOT search program developed by Matrix Science Ltd. (access is available on http://www.matrix.science.com), with the NCBI database (http://www. ncbi.nlm.nih.gov/entrez), and using the ExPASy Molecular Biology Server at the SWISSPROT database (http://www.expasy.org).

Statistical analysis: The relative levels of pS^{473}Akt1 and *O*-GlcNAc-modified Akt1 were calculated after normalization against Akt1 band intensities measured by a densitometry and presented as mean ± standard deviation. Mean values were compared using Student's *t*-test; p values < 0.05 were considered significant.

Results

Glucosamine treatment induced apoptosis of murine β-pancreatic cells.

To confirm that glucosamine-mediated hyperglycemic conditions induced cell death, rat INS-1 β-pancreatic cells were treated with glucosamine dissolved in 0.5 mM HEPES (pH 7.5) at different concentrations. Cells were treated with glucosamine under osmotic control by mannitol in the absence of serum, and viable cells were counted after trypan blue staining. Glucosamine treatment of INS-1 cells induced cell death (Fig. 1A), activated caspase 3 (Fig. 1B) and significantly increased the sub-G1 population in a dose-dependent manner (Fig.1C). Treatment with 7.5 mM glucosamine for less than 6 h showed a less significant apoptotic rate of ~ 9.0% (data not shown), therefore cells were generally treated for 14 h in the following experiments. Treatment with glucosamine also induced cell death in mouse βTC-6 pancreatic cells (Fig. 1D). These observations confirm that glucosamine treatment of murine β-pancreatic cells caused caspase-dependent apoptosis.

Hyperglycemic conditions increased *O*-GlcNAc modification and concomitantly decreased Ser473 phosphorylation of Akt1.

When murine β-pancreatic cells were treated with glucosamine (7.5 mM GlcN), the overall *O*-GlcNAc modification level of proteins increased with a particularly obvious

modification of an approximately 60 kD protein that may correspond to Akt1 protein involved in cell survival (Fig. 2A). It is thus likely that *O*-GlcNAc modification of certain molecules, such as Akt1, was increased in glucosamine-mediated hyperglycemic conditions to regulate cell viability [10]. We next tested the level of *O*-GlcNAc-modified Akt1 in murine β-pancreatic cells without or with glucosamine treatment by immunoblotting using anti-Akt1 antibody for *O*-GlcNAc immunoprecipitates or anti-*O*-GlcNAc antibody for Akt1 immunoprecipitates. The *O*-GlcNAc modification level of Akt1 increased in a glucosamine dose-dependent manner, whereas Akt1 Ser473 phosphorylation (pS^{473}Akt1) was concomitantly decreased (Fig. 2B). Consistent with these findings, treatment with higher concentrations of glucose also increased *O*-GlcNAc modification of Akt1 and decreased pS^{473}Akt1 (Fig. 2C). Furthermore, phosphorylation of GSK3β, a substrate of Akt1, was reduced following glucosamine treatment, indicating that glucosamine treatment decreased Akt1 activity (Fig. 2B). These observations indicate that Akt1 Ser473 phosphorylation, and thus activity, might be mutually exclusive with *O*-GlcNAc modification, although it is not clear whether the modified residues are the same.

OGT or *O*-GlcNAcase inhibitor reciprocally regulated Akt1 *O*-GlcNAc modification and pS^{473}Akt1.

To further confirm the reciprocal relationship between *O*-GlcNAc modification and phosphorylation of Akt1, pharmacological inhibitors against OGT (BADGP or alloxan) or *O*-GlcNAcase (PUGNAc) were added to the culture media before glucosamine treatment. When INS-1 cells were treated with either BADGP or alloxan in the absence or presence of 7.5 mM glucosamine, the basal and glucosamine-mediated Akt1 *O*-GlcNAc modifications were decreased, whereas pS^{473}Akt1 levels were increased (Fig. 3A and B). On the other hand, when removal of *O*-GlcNAc was blocked by PUGNAc treatment, the basal pS^{473}Akt1 level was slightly reduced, whereas glucosamine-mediated pS^{473}Akt1 levels were not observed regardless of whether PUGNAc was treated or not (Fig. 3C). Interestingly, pT^{308}Akt1 levels were not significantly changed by PUGNAc treatment, although they were slightly reduced by treatment with 7.5 mM GlcN (Fig. 3C).

The Akt1 Ser473 residue appeared to be modified with *O*-GlcNAc following glucosamine treatment.

It is reasonable to predict that *O*-GlcNAc modification and phosphorylation of Akt1 both occur at the Ser473 residue in a reciprocal manner. Based on analysis by the YinOYang 1.2 program (http://www.cbs.dtu.dk/services/YinOYang/) [20], we found that Akt1 may be modified with *O*-GlcNAc at Ser122, Thr430, Ser473, and/or Thr479

residues, with the highest potential value of *O*-GlcNAc modification at Ser473 (Table 1). Thus, to map the *O*-GlcNAc modification site(s) of Akt1, we performed MALDI-TOF/TOF analysis with tryptic-digested peptides of Akt1 immunoprecipitates. Whole cell extracts from INS-1 cells without or with 7.5 mM glucosamine treatment were immunoprecipitated with anti-Akt1 antibody, and the immunoprecipitates were separated by 2D electrophoresis prior to immunoblotting with anti-*O*-GlcNAc antibody (CTD 110.6 clone) (Fig. 4A). Akt1 was excised from silver-stained 2D electrophoresis gels and digested with trypsin prior to MALDI-TOF/TOF analysis. The unmodified 466-480 peptide of Akt1 at $[M+H]^+$ *m/z* 1652.72 was observed among the tryptic-digested peptides prepared from control and glucosamine-treated cells (Fig. 4B). When the Ser or Thr residue is modified with *O*-GlcNAc the mass would be predicted to increase by ~ 203 dalton, therefore we screened for tryptic-digested peptide(s) of Akt1 with an increased mass of ~ 203 dalton. Interestingly, the presumed *O*-GlcNAc-modified 466-480 peptide of Akt1 was observed at $[M+H]^+$ *m/z* 1854.22 among the tryptic-digested peptides prepared from the glucosamine-treated cells, but not from control cells (Fig. 4B, bottom). This observation indicates that glucosamine treatment of INS-1 cells causes *O*-GlcNAc modification of Akt1, presumably at either Ser473 or Thr479.

Akt1 WT and T479A mutant, but not S473A mutant, were *O*-GlcNAc-modified by recombinant OGT.

Since Thr479 of Akt1 showed a tendency for *O*-GlcNAc modification in the YinOYang analysis (Table 1) and the *O*-GlcNAcylated peptide of Akt1 contained Thr479, we examined whether Ser473 and/or Thr479 were targeted by OGT using an *in vitro O*-GlcNAc modification assay. Bacterial extracts of GST-ncOGT were incubated with recombinant $(His)_6$-Akt1 WT, S473A mutant, or T479A mutant bound to Ni^{2+}-NTA beads as the substrate. Compared with a mock control substrate of plain $(His)_6$-vector alone without Akt1 insert, *O*-GlcNAc modification of WT Akt1 was increased (Fig. 5A, lanes 1 and 2), whereas the Akt1 S473A mutant was not modified (Fig. 5A, lane 3). Interestingly, the T479A mutant was robustly modified with *O*-GlcNAc, even more than WT Akt1 (Fig. 5A), indicating that Thr479 was not an *O*-GlcNAc modification site, and further suggesting that the T479A mutation might have caused structural changes that increased access of ncOGT to other Ser/Thr residue(s) on Akt1, including Ser473. The *in vitro O*-GlcNAc modification of $(His)_6$-Akt1 by recombinant GST-ncOGT occurred in a dose-dependent manner (Fig. 5B), and was blocked by treatment with the OGT inhibitor BADGP (Fig. 5C), indicating that the *O*-GlcNAc modification observed in the *in vitro* assay was indeed mediated by OGT

activity.

Intracellular *O*-GlcNAc modification of the S473A Akt1 mutant was not observed following glucosamine treatment or ncOGT overexpression.

We next examined whether *O*-GlcNAc-modification of the S473A Akt1 mutant occurred in an *in vivo* cellular system. INS-1 cells were transfected with a mock control vector, $(HA)_3$-Akt1 WT, or S473A mutant 24 h before treatment with control vehicle or glucosamine (7.5.mM) for 14 h. Cell extracts were immunoblotted or immunoprecipitated with anti-*O*-GlcNAc antibody prior to Akt1 immunoblot analysis. In the absence of glucosamine, exogenous expression of Akt1 WT, but not S473A mutant, increased levels of pS^{473}Akt1 (Fig. 6A, lanes 1 to 3). However, pS^{473}Akt1 was not detected following glucosamine treatment, regardless of whether Akt1 WT or S473A mutant was expressed (Fig. 6A, lanes 4 to 6). *O*-GlcNAc modification of Akt1 occurred when the cells overexpressed Akt1 WT, but not S473A mutant protein, and was further increased when the cells were treated with glucosamine, compared with the untreated control (Fig. 6A, bottom). Cell viability assays showed that viability was approximately 10% lower in cells transfected with the S473A Akt1 mutant than in cells transfected with WT Akt1 following treatment with control vehicle ($5.7 \pm 0.2 \times 10^5$ versus $6.3 \pm 0.3 \times 10^5$ cells, respectively) or GlcN (7.5 mM for 14 h) condition (3.3 ±

0.4 x 10^5 and 3.6 ± 0.4 x 10^5 cells, respectively) (data not shown). This reduced viability of S473A Akt1-transfected cells, compared with WT transfected cells, was observed with a transfection efficiency of around 30-40%. Furthermore, glucosamine treatment of cells transfected with WT or S473A Akt1 mutant resulted in a similar decrease in viability of 42-43%, indicating that *O*-GlcNAc-modified Akt1 (in the WT Akt1-transfected condition) or non-phosphorylated Akt1 (in the S473A Akt1-transfected condition) have a similar effect on cell viability.

We further examined whether exogenous overexpression of ncOGT would regulate Akt1 *O*-GlcNAc modification and pS^{473}Akt1 even in the absence of glucosamine treatment (Fig. 6B). Cells that stably overexpress control vector or ncOGT were transiently transfected with either WT or S473A Akt1 and cell extracts were immunoblotted, or immunoprecipitated with anti-*O*-GlcNAc antibody before immunoblotting for Akt1. When cells were transfected with Akt1 WT, ncOGT-overexpressing cells showed a much higher level of Akt1 *O*-GlcNAc modification than control cells (Fig. 6C, lanes 1 and 3). However, when the cells were transfected with S473A mutant Akt1, *O*-GlcNAc modifications were abolished in both control and ncOGT-overexpressing cells (Fig. 6C, lanes 2 and 4), further indicating that Ser473 is targeted by OGT. Reciprocally, pS^{473}Akt1 levels were partially reduced by

transfection with the S473A mutant, presumably due to incomplete transfection efficiency, and pS^{473}Akt1 levels were lower in the ncOGT-overexpressing cells than in control cells (Fig. 6C). Our observations that the Akt1 S473A mutation abolished *O*-GlcNAc-modification, and that *O*-GlcNAc modification of Akt1 WT inversely correlated with pS^{473}Akt1, suggest that *O*-GlcNAc modification at Akt1 Ser473 might compete with phosphorylation at Akt1 Ser473.

OGT associated with Akt1 WT, but not S473A mutant, following glucosamine treatment.

We next examined the effect of hyperglycemic conditions on the localization of OGT and Akt1 via a coimmunoprecipitation approach. Immunoprecipitates using anti-HA antibody from cells transfected with either WT Akt1 or S473A mutant Akt1 tagged with $(HA)_3$ were immunoblotted for OGT. When treated with glucosamine, Akt1 WT co-immunoprecipitated OGT (presumably ncOGT of 116 kD), whereas S473A mutant did not (Fig. 7). This observation suggests that modification of Akt1 Ser473 with *O*-GlcNAc could be mediated by physical association between Akt1 and OGT, following a hyperglycemic treatment of INS-1 cells.

Discussion

Observations from this study indicated that Ser473 of Akt1 was modified with *O*-GlcNAc when murine β-pancreatic cells were treated with glucosamine, a direct precursor of the downstream product of the hexosamine biosynthesis pathway (HBP). We found that a tryptic-digested peptide of Akt1 (amino acids 466 to 480 including Ser473 and Thr479) was modified with *O*-GlcNAc, consistent with theoretical predictions by the YinOYang 1.2 program that both Ser473 and Thr479 would be *O*-GlcNAc-modified (http://www.cbs.dtu.dk/services/YinOYang/) (Table 1). Furthermore, the calculated potential for Ser473 to be *O*-GlcNAc-modified is higher than that for Thr479 (Table 1), and we observed that a T479A mutant of Akt1, but not a S473A mutant, maintained robust *O*-GlcNAc-modification in an *in vitro* *O*-GlcNAc modification assay. Treatment with glucosamine or exogenous overexpression of mammalian ncOGT increased *O*-GlcNAc-modification of Akt1 WT, but not of S473A mutant, with a concomitant decrease in pS^{473}Akt1. Furthermore, Akt1 S473A mutant did not associate with OGT following treatment with glucosamine, whereas Akt1 WT did. Together, these observations indicate that S473 Akt1 might be targeted by OGT in a glucosamine treatment-dependent manner. The reciprocal relationship between increased *O*-GlcNAc-modification and decreased phosphorylation of Akt1 Ser473 upon

glucosamine treatment correlated with enhanced apoptotic death of murine β-pancreatic cells. Since phosphorylation of Akt1 Ser473 is critical for its anti-apoptotic activity (14), we speculate that modification of Akt1 Ser473 plays an important role in determining the cell fate of murine β-pancreatic cells under conditions of extracellular hyperglycemia.

Since *O*-GlcNAc modification occurs at Ser/Thr residues, it is likely that *O*-GlcNAc modification and phosphorylation are reciprocally regulated under certain circumstances, leading to regulation of the protein's activity and stability [21, 22]. Dynamic regulation of *O*-GlcNAc modification turnover in response to extracellular fluctuations in glucose levels may allow modulation of the signaling activities of certain intracellular proteins in β-pancreatic cells [4, 23, 24]. In particular, in diabetes mellitus, increased *O*-GlcNAc modification of many proteins under extracellular hyperglycemic conditions appears to be correlated with β-pancreatic cell death [11, 25].

Glucosamine treatment of human islets of Langerhans and RIN rat β-pancreatic cells increases apoptosis due to serum-deprivation through impaired activation of the insulin receptor (IR)/IR substrate (IRS)/PI3-K/Akt survival pathway [10]. However, in this previous study, glucosamine treatment of β-pancreatic cells appeared to cause *O*-GlcNAc modification of IR/IRS, upstream of PI3-K/Akt [10]. Although the *O*-

GlcNAc modification residue was not determined, another report showed that Akt1 was shown to be *O*-GlcNAc-modified and phosphorylated in SH-SY5Y cells treated with insulin-like growth factor, and that PUGNAc-mediated accumulation of Akt1 *O*-GlcNAc modification did not attenuate Akt1 phosphorylation with translocation of Akt1 into the nucleus [15]. Therefore, Akt1 modification under hyperglycemic stimulations such as glucosamine treatment may be dependent on the signaling context and/or the cell type, which may have different expression levels of the proteins involved in the HBP pathway and *O*-GlcNAc modification turnover. In this study, treatment with glucosamine increased *O*-GlcNAc modification of Akt1 Ser473, and concomitantly decreased phosphorylation of Akt1 Ser473 and its substrate, GSK3β, correlating with increased death of mouse βTC-6 and rat INS-1 β-pancreatic cells. Our observations suggest that the balance between *O*-GlcNAc-modification and phosphorylation of Akt1 Ser473 plays a role in the determination of apoptosis or survival of murine β-pancreatic cells, respectively, in response to hyperglycemic conditions induced by glucosamine treatment.

O-GlcNAc-modified proteins are typically phosphoproteins that play diverse roles in carbohydrate metabolism, signaling, gene regulation, and stress response [4]. This fact indicates that protein activity can depend on the nature of posttranslational

modifications that may ultimately regulate cell function or behavior, especially when the same Ser/Thr residue(s) on a specific protein may be modified with *O*-GlcNAc or *O*-phosphate. Similar to our study for Akt1, estrogen receptor β (Ser16), c-Myc (Thr58), and endothelial nitric oxide synthase (eNOS, Ser1177) are also reciprocally *O*-GlcNAc-modified or phosphorylated [26-28]. In the case of RNA polymerase II, multiple Ser/Thr residues in the COOH-terminal domain (CTD) are shown to be modified with *O*-GlcNAc or *O*-phosphate in a mutually exclusive manner, resulting in a reciprocal regulation of transcriptional elongation [29]. *O*-GlcNAc modification at Ser149 of p53 decreases the phosphorylation at Thr155 thereby stabilizing p53 by blocking ubiquitin-dependent proteolysis [21], indicating that these reciprocal modifications can also occur between adjacent residues. For many proteins involved in signaling pathways, activity is determined by their phosphorylation status. Since *O*-GlcNAc modification at a particular Ser or Thr may sterically affect phosphorylation of the same or nearby residues, the activity of such proteins may also be regulated through *O*-GlcNAc modification. Further investigation into the nature of *O*-GlcNAc modification may aid in the design and development of therapeutic reagents against diseases involving abnormal signaling activity such as type II diabetes and cancer.

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Figure legends

Fig. 1. Glucosamine treatment caused death of murine β-pancreatic cells. (A and D) Rat or mouse β-pancreatic INS-1 (A) or βTC-6 (D) cells, respectively, seeded into 6 well culture plates. One day after, serum was deprived and treatment of glucosamine (GlcN) in 0.5 mM HEPES (pH 7.5) was done at the indicated concentrations for 22 h. Mannitol and glucose were parallelly used to compensate osmotic balance. After incubation, viable cells were counted with a hemocytometer following trypan blue stain. (B) INS-1 cells treated without or with 7.5 mM glucosamine (GlcN), as above, were harvested and immunoblotted for active caspase 3 and α-tubulin. (C) INS-1 cells were manipulated as in (A), before FACS analysis of PI-stained cells for DNA contents. Data shown represent at least three independent experiments.

Fig. 2. Hyperglycemic conditions increased *O*-GlcNAc modification and concomitantly reduced Ser473 phosphorylation of Akt1. (A) INS-1 or βTC-6 cells were treated without or with 7.5 mM glucosamine (GlcN) for the indicated periods in the absence of serum. Whole cell lysates were prepared after incubation and used for standard Western blots to see *O*-GlcNAc-modified proteins. Arrows indicate

molecular weights of protein markers. (B) INS-1 (upper) or βTC-6 (lower) cells were treated with glucosamine at the indicated concentrations for 14 h in the absence of serum. Both mannitol and glucose were included into the treatment for osmotic compensation, as in Fig. 1. After incubation, cell lysates were prepared and used for standard Western blots for pS^{473}Akt1, pS^{9}GSK3β and Akt1 (lysates) or immunoprecipitated with anti-*O*-GlcNAc or anti-Akt antibody for immunoblots against Akt or *O*-GlcNAc (CTD 110.6 clone), respectively, to reveal *O*-GlcNAc modified Akt. (C) INS-1 cells were treated with glucose (11.1. mM normal glucose in RPMI-1640) at 11.1, 22.2 or 33.3. mM for 3 days at osmotic balance with mannitol, before whole cell lysates preparation. The lysates were immunoblotted or immunoprecipitated with anti-Akt prior to immunoblots against Akt or *O*-GlcNAc (CTD 110.6 clone). The relative pS^{473}Akt1 and *O*-GlcNAc-modified Akt1 levels under diverse experimental conditions were calculated for graphic presentation (mean ± standard deviation) after normalization of them over Akt1 band intensities measured by a densitometry. Data shown represent at least three independent experiments.

Fig. 3. OGT or *O*-GlcNAcase inhibitor regulated reciprocally Akt1 *O*-GlcNAc modification and pS^{473}Akt1. (A to C) OGT inhibitor, BADGP (A) or alloxan (B), or

O-GlcNAcase inhibitor, PUGNAc (C), was added to serum-deprived culture media. One hour later, cells were treated without or with 7.5 mM glucosamine (GlcN) for 14 h, prior to lysate harvests for standard Western blots (lysates in A, B and C) or immunoprecipitation (IP) with anti-*O*-GlcNAc antibody (RL2 clone) for Akt immunoblots. Both mannitol and glucose were included for osmotic compensation (not depicted), as in Fig. 1. The relative pS^{473}Akt1 and/or *O*-GlcNAc-modified Akt1 levels under diverse experimental conditions were calculated for graphic presentation (mean ± standard deviation) after normalization of them over Akt1 band intensities measured by a densitometry. * indicates p values < 0.05 for significant differences. Data shown represent three different experiments.

Fig. 4. The Akt1 Ser473 residue modified with *O*-GlcNAc following glucosamine treatment. (A) INS-1 cells were seeded and treated without or with 7.5 mM glucosamine (GlcN) in the absence of serum for 14 h, prior to cell harvests. Both mannitol and glucose were included into the treatment for osmotic compensation (not depicted), as in Fig. 1. Normalized lysates were immunoprecipitated with anti-Akt antibody. Akt immunoprecipitates were processed for standard 2-D electrophoresis before immunoblots using anti-*O*-GlcNAc antibody (CTD 110.6 clone), as explained in

the Materials and Methods. (B) The Akt spots on silver-stained gels, corresponding to circles of the anti-*O*-GlcNAc immunoblots as in (A), were cut and trypsinized. The digests were processed for MALDI-TOF/TOF mass spectrometry to examine the existence of *O*-GlcNAc-modified Akt1 peptide including Ser473. Note that hyperglycemic condition resulted in a peak corresponding to a peptide (amino acids 466 to 480) including Ser473 with an *O*-GlcNAc modification, which causes an increase in mass (201.5 dalton) from 1652.72 to 1854.22 dalton.

Fig. 5. Akt1 WT, but not the S473A mutant, was *O*-GlcNAc-modified by recombinant ncOGT. (A) Recombinant GST-human ncOGT was prepared from bacterial cultures, and recombinant mock, $(His)_6$-Akt1 WT, S473A, or T479A proteins were purified and bound to Ni^+-NTA beads. Both recombinant GST-ncOGT and $(His)_6$-Akt1 proteins were mixed with 1 μM UDP-GlcNAc, and the mixtures were incubated for 30 min at 22°C while shaking. After incubation, the reaction mixture was diluted abundantly with the reaction buffer and spun down for washings. SDS-PAGE sample buffer (2 x) was added to the washed beads and boiled prior to recovery of proteins for standard Western blots using *O*-GlcNAc antibody (CTD 110.6 clone). (B) Different amount of recombinant mock, $(His)_6$-Akt1 WT, or S473A proteins on Ni^+-

NTA beads were mixed with recombinant GST-human ncOGT for *in vitro* assay for *O*-GlcNAc modification, as above. (C) During the *in vitro* assay for mock, $(His)_6$-Akt1 WT or S473A mutant, as in (B), one set of reactions was untreated and the other set was treated with 3.3 mM BADGP, an OGT inhibitor. In parallel, recombinant proteins on gels were stained with coomassie blue (A) or immunoblotted with either anti-GST (B) or anti-$(His)_6$ antibody (C) for their equal loadings. The relative *O*-GlcNAc-modified Akt1 levels under diverse experimental conditions were calculated for graphic presentation (mean ± standard deviation) based on band intensities measured by a densitometry. * indicates p values < 0.05 for significant differences. Data shown represent at least three isolated assays.

Fig. 6. No *O*-GlcNAc modification of S473A Akt1 mutant by glucosamine treatment or ncOGT overexpression. (A) INS-1 cells were transiently transfected with mock, Akt1 WT or S473A mutant. One day later, the cells were treated without (Control) or with 7.5 mM glucosamine (GlcN) in the absence of serum for 14 h. After incubation, cell lysates were prepared and normalized for standard Western blots using anti-phospho-S^{473}Akt1 or anti-HA antibody (lysate) or immunoprecipitated (IP) with anti-*O*-GlcNAc antibody (RL2 clone) prior to Akt1 immunoblots (bottom panel). (B)

Mock (Con) or stably OGT (human ncOGT)-expressing INS-1 cells were prepared. Whole cell lysates were prepared from a subconfluent condition and blotted for ncOGT and mitochondrial OGT (mOGT) expression levels. (C) Mock (Con) or ncOGT stable INS-1 cells were transiently transfected with Akt1 WT or S473A mutant. Two days after transfection, lysates were prepared and used for standard Western blots for the indicated molecules (lysate) or immunoprecipitated (IP) with anti-*O*-GlcNAc antibody (RL2 clone) prior to Akt1 immunoblots (bottom panel). Both mannitol and glucose were included into the treatment for osmotic compensation (not depicted), as in Fig. 1. The relative pS^{473}Akt1 and *O*-GlcNAc-modified Akt1 levels under diverse experimental conditions were calculated for graphic presentation (mean ± standard deviation). * indicates p values < 0.05 for significant differences. Data shown represent at least three different assays.

Fig. 7. Akt1 WT, but not the S473A mutant, associates with OGT following glucosamine treatment. INS-1 cells were transiently transfected with $(HA)_3$-Akt1 WT or S473A mutant. One day later, the cells were treated with 7.5 mM glucosamine (GlcN) in the absence of serum for 14 h. Both mannitol and glucose were included into the treatment for osmotic compensation (not depicted), as in Fig. 1. After

incubation, cells were harvested for an immunoprecipitation with anti-HA antibody prior to OGT immunoblots. Data shown represent at least three independent assays.

Table 1. Predicted *O*-GlcNAc sites of Akt1 protein sequence by YinOYang 2.1[1].

SeqName	Residue	*O*-GlcNAc result	Potential[2]	Thresh. (1)	Thresh (2)
Akt1	**122 S**	++	**0.5080**	**0.4017**	**0.4918**
Akt1	**430 T**	++	**0.5647**	**0.4071**	**0.4992**
Akt1	**473 S**	++	**0.6456**	**0.4484**	**0.5548**
Akt1	**479 T**	+	**0.3882**	**0.3791**	**0.4614**

[1]) (http://www.cbs.dtu.dk/services/YinOYang/)

[2]) The higher potential value is, the higher the tendency to be modified with *O*-GlcNAc is.

[3]) '++' indicates a higher possibility of *O*-GlcNAcylation than '+'.

Fig. 1

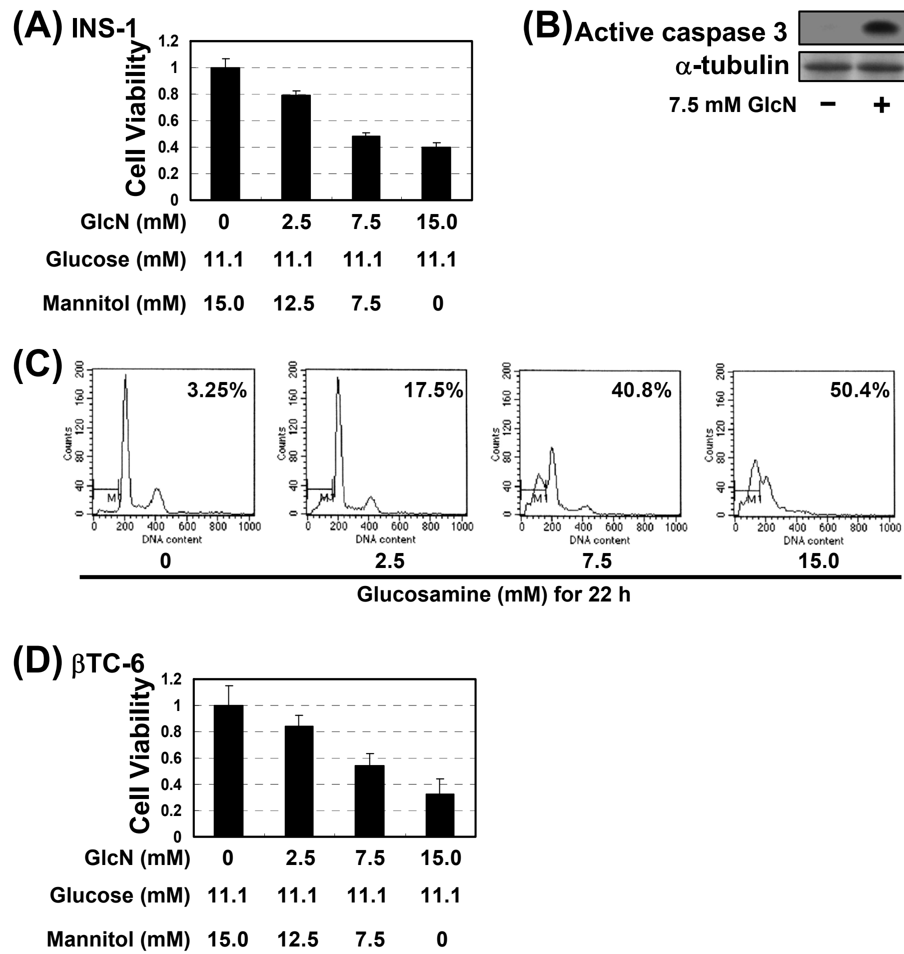


Fig 2

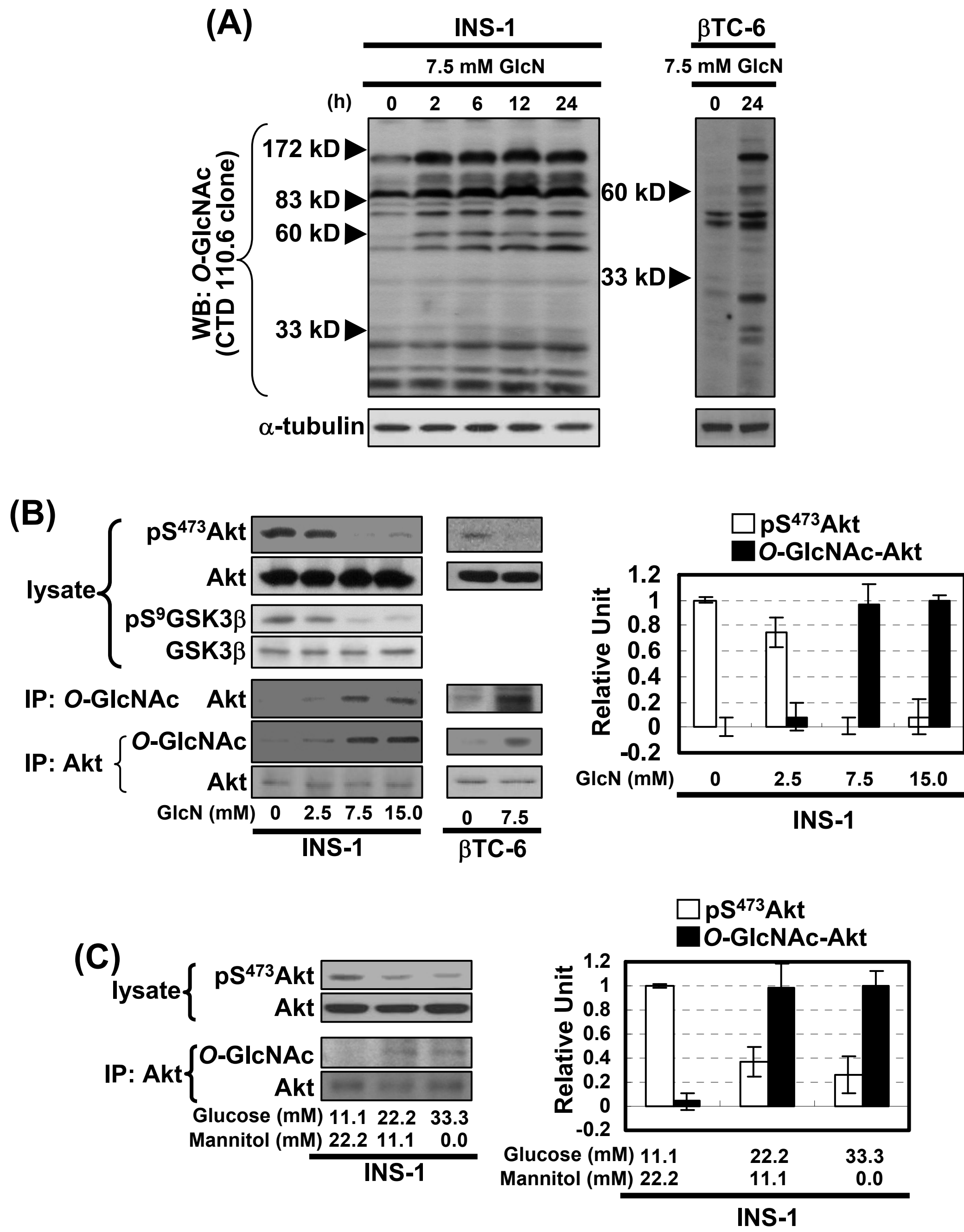
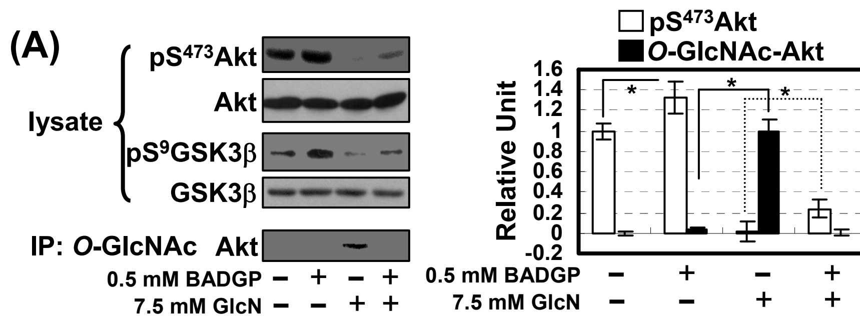
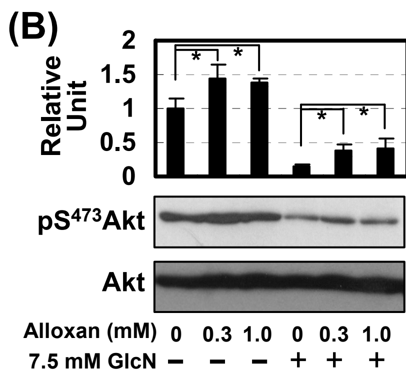


Fig 3





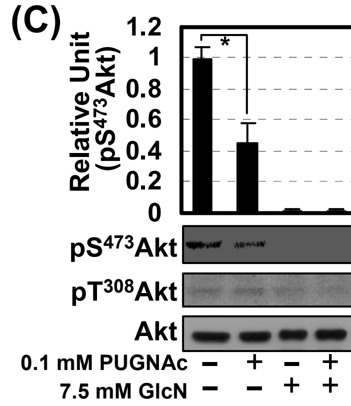
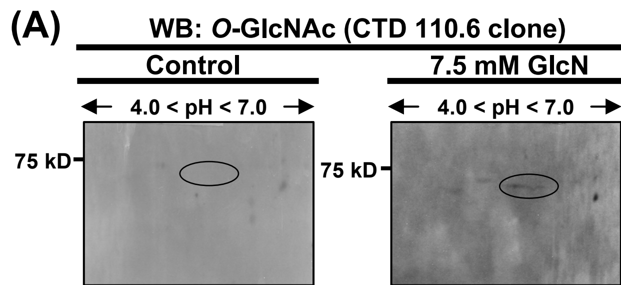


Fig 4



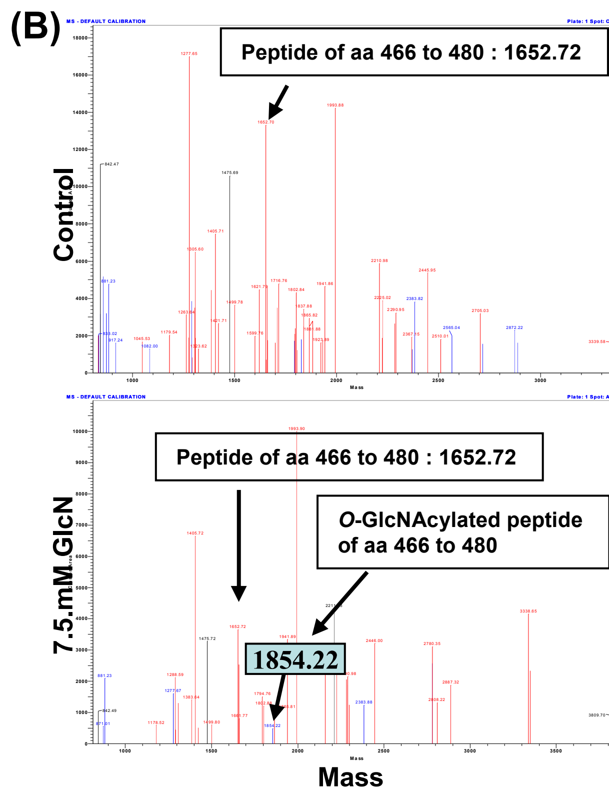


Fig 5

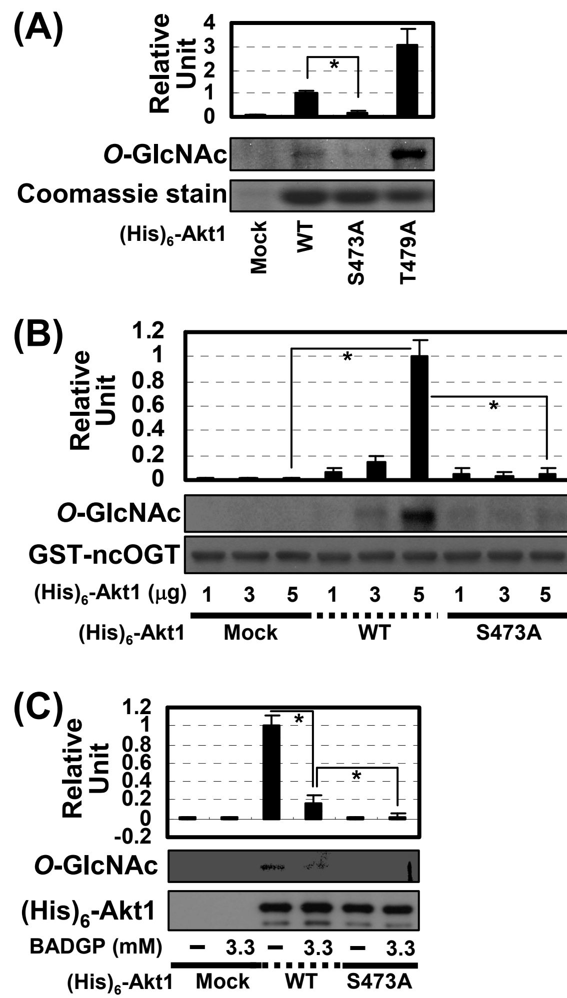


Fig 6

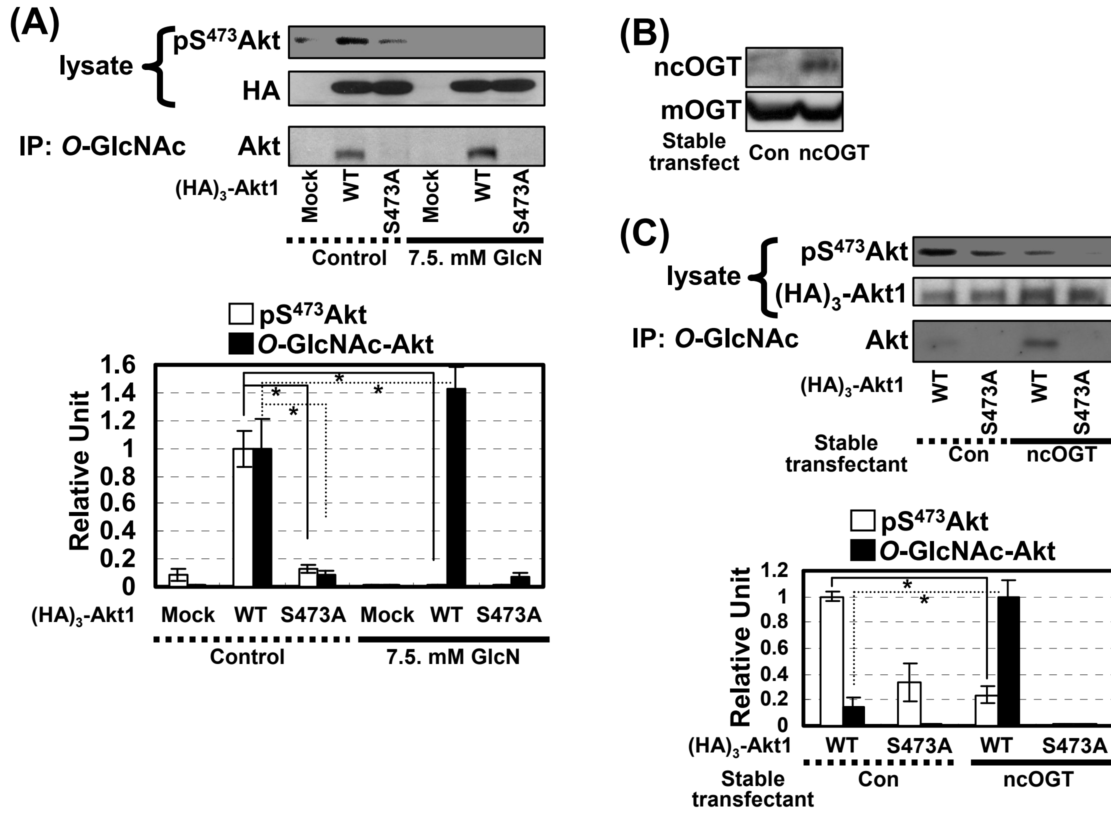


Fig 7