Glycobiology and Extracellular Matrices:

O-GlcNAc Protein Modification in Cancer Cells Increases in Response to Glucose Deprivation through Glycogen Degradation

Jeong Gu Kang, Sang Yoon Park, Suena Ji, Insook Jang, Sujin Park, Hyun Sil Kim, Sung-Min Kim, Jong In Yook, Yong-Il Park, Jürgen Roth and Jin Won Cho

J. Biol. Chem. 2009, 284:34777-34784.
doi: 10.1074/jbc.M109.026351 originally published online October 15, 2009

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 50, pp. 34777–34784, December 11, 2009

O-GlcNAc Protein Modification in Cancer Cells Increases in Response to Glucose Deprivation through Glycogen Degradation*[S]

Received for publication, May 28, 2009, and in revised form, September 24, 2009 Published, JBC Papers in Press, October 15, 2009, DOI 10.1074/jbc.M109.026351

Jeong Gu Kang[‡1], Sang Yoon Park[‡1], Suena Ji[‡1], Insook Jang[‡1], Sujin Park[‡1], Hyun Sil Kim[§], Sung-Min Kim[¶], Jong In Yook[§], Yong-Il Park[¶], Jürgen Roth[‖], and Jin Won Cho[‡‖2]

From the Departments of [‡]Biology and [§]Oral Pathology, Oral Cancer Research Institute, and [‖]World Class University Integrated OMICS for Biomedical Science, Yonsei University, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-749, Korea, and the [¶]Department of Biotechnology and Biomaterial Engineering Research Center, Catholic University of Korea, Bucheon 420-743, Korea

When cellular glucose concentrations fall below normal levels, in general the extent of protein *O*-GlcNAc modification (*O*-GlcNAcylation) decreases. However, recent reports demonstrated increased *O*-GlcNAcylation by glucose deprivation in HepG2 and Neuro-2a cells. Here, we report increased *O*-GlcNAcylation in non-small cell lung carcinoma A549 cells and various other cells in response to glucose deprivation. Although the level of *O*-GlcNAc transferase was unchanged, the enzyme contained less *O*-GlcNAc, and its activity was increased. Moreover, *O*-GlcNAcase activity was reduced. The studied cells contain glycogen, and we show that its degradation in response to glucose deprivation provides a source for UDP-GlcNAc required for increased *O*-GlcNAcylation under this condition. This required active glycogen phosphorylase and resulted in increased glutamine:fructose-6-phosphate amidotransferase, the first and rate-limiting enzyme in the hexosamine biosynthetic pathway. Interestingly, glucose deprivation reduced the amount of phosphofructokinase 1, a regulatory glycolytic enzyme, and blocked ATP synthesis. These findings suggest that glycogen is the source for increased *O*-GlcNAcylation but not for generating ATP in response to glucose deprivation and that this may be useful for cancer cells to survive.

Extracellular glucose is transferred into cells by glucose transporters (1, 2), where it is rapidly converted into glucose 6-phosphate by hexokinase. Glucose 6-phosphate is either used for glycogen synthesis or enters the glycolytic pathway after conversion to fructose 6-phosphate. Most of the transferred glucose is used for ATP synthesis through the glycolytic pathway and the tricarboxylic acid cycle. Only about 2–5% of the transferred glucose enters the hexosamine biosynthetic pathway (HBP)[3] and is converted into uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) (3). UDP-GlcNAc is transferred by *O*-GlcNAc transferase (OGT) to serine or threonine residues to yield the single *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) modification (4). UDP-GlcNAc represents a nutrient sensor being the product of various metabolic pathways such as the glucose, fatty acid, amino acid, nucleotide, and energy metabolism (5, 6). Because protein *O*-GlcNAc modification (*O*-GlcNAcylation for brevity) by OGT is highly dependent on UDP-GlcNAc concentration, *O*-GlcNAc metabolism is thought to have a critical role in nutrient homeostasis (5, 6).

Since its first description as a novel posttranslational protein modification by Torres and Hart (7), >600 proteins as diverse as transcription factors, enzymes, cytoskeletal proteins, and ribosomal proteins have been identified to carry *O*-linked GlcNAc (4–6). Thus, protein *O*-GlcNAcylation appears to be involved in many different cellular activities, which include the regulation of transcription and translation (8–12), of protein degradation (12–16), and localization (17), as well as of protein-protein interactions (13, 18). Of particular interest are reports that *O*-GlcNAcylation of nucleocytoplasmic proteins can occur under various forms of cellular stress such as heat shock, UV light, and high salt concentration (19, 20). Under such conditions, the *O*-GlcNAcylation may have a function for cell survival, but the mechanism is not clear.

Recently, it was reported that glucose deprivation increases protein *O*-GlcNAcylation in HepG2 (21) and Neuro-2a cells (22). Further studies have revealed the dynamics of *O*-GlcNAcylation in HepG2 cells during glucose deprivation and have shown that up-regulation of OGT is mediated by decreased HBP flux (23). In light of the above mentioned importance of glucose as a source for UDP-GlcNAc synthesis and thus protein *O*-GlcNAcylation, increased *O*-GlcNAcylation under glucose deprivation appears paradoxical. However, it may provide a clue for the importance of the *O*-GlcNAcylation as a nutrient sensor, in glucose homeostasis,

* This work was supported by the Korea Science and Engineering Foundation (KOSEF) funded by Ministry of Education, Science, and Technology Grant R0A-2007-000-20011-0, Korea Research Foundation Grant KRF-2006-005-J04502, and World Class University Project R31-2008-000-10086-0. This work was made possible through the use of research facilities in the Yonsei Center for Biotechnology.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures" and Figs. 1 and 2.

[1] Fellowship awardees of the Brain Korea 21 program.

[2] To whom correspondence should be addressed: Shinchon-dong, Seodaemun-gu, Seoul 120-749, Korea. Fax: 82-2-312-5657; E-mail: chojw311@yonsei.ac.kr.

[3] The abbreviations used are: HBP, hexosamine biosynthetic pathway; AMPK, AMP-activated protein kinase; DON, 6-diazo-5-oxo-L-norleucine; GFAT, glutamine:fructose-6-phosphate amidotransferase; GP, glycogen phosphorylase; *O*-GlcNAc, *O*-linked β-*N*-acetylglucosamine; OGT, *O*-GlcNAc transferase; PAS, periodic acid-Schiff; PBS, phosphate-buffered saline; sWGA, succinylated wheat germ agglutinin; UDP-GlcNAc, uridine 5′-diphospho-*N*-acetylglucosamine.

and for cell survival. We observed increased protein *O*-GlcNAcylation in glucose-deprived A549 lung cancer cells and other cell lines, similar to that reported for HepG2 and Neuro-2a cells (21, 22). However, we observe that increased *O*-GlcNAcylation in response to glucose deprivation was not due to up-regulated OGT (21, 22) and not dependent on the activation of AMP-activated protein kinase (AMPK) (22).

It was reported that inhibition of glycogen phosphorylase (GP), which catalyzes glycogen degradation, suppressed the growth of A549 cells (24), but the role of glycogen in cancer biology is not well understood. We demonstrate here that glycogen is a source for protein *O*-GlcNAcylation in glucose-deprived A549 cells. Thus, protein *O*-GlcNAcylation in tumors may have a function in overcoming the effects of hypoxia and hypoglycemia. Our findings have the interesting implication that *O*-GlcNAcylation may act as an intermediary between glucose and glycogen metabolism in cancer cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Glucose Deprivation, and Inhibitor Treatments—Human non-small cell lung carcinoma cell lines A549, H1299, and H460, human lung L132 fibroblasts, human breast cancer MCF7 cells, and human embryonic kidney HEK293 cells were grown in high glucose (25 mM) Dulbecco's modified Eagle's medium (Invitrogen) containing 10% (v/v) fetal bovine serum (Lonza) and 1% (v/v) antibiotics (Invitrogen) at 37 °C in 5% CO_2. Cells were rinsed with PBS and then cultured in glucose-free Dulbecco's modified Eagle's medium (WelGENE, Inc.) containing 10% (v/v) fetal bovine serum and 1% (v/v) antibiotics for up to 36 h. For the inhibitor treatment, cells were cultured in medium containing either 1 μg/ml cycloheximide (Sigma), 20 μM 6-Diazo-5-oxo-L-norleucine (DON, Sigma), 20 μM Compound C (Calbiochem), or 20 μM glycogen phosphorylase inhibitor (Calbiochem) for 1 h. Afterward, cells were washed with PBS and incubated in the glucose-free medium containing inhibitor.

Transient Overexpression of OGT—Human full-length OGT was cloned into the p3XFLAG-CMV-7.1 expression vector (Sigma). The vector encoding FLAG-tagged OGT or empty vector was transfected using Tranfectin (Bio-Rad).

Cell Lysis, Immunoprecipitation, and WGA Precipitation—Cells were washed twice in ice-cold PBS and solubilized in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, and protease inhibitors). The lysates were centrifuged at 18,000 × *g* for 20 min at 4 °C. Protein concentrations were determined by Bradford assay (Bio-Rad). To immunoprecipitate FLAG-tagged OGT, 1 mg of a given lysate was mixed with EZview™ Red anti-FLAG M2 Affinity Gel (Sigma) overnight at 4 °C under gentle agitation. Beads were washed four times with lysis buffer. Pellet was resuspended in 2× SDS sample buffer. Samples were boiled for 5 min and subjected to Western blotting. For succinylated wheat germ agglutinin (sWGA) precipitation, 1 mg of cell lysate was incubated with 25 μl of agarose-sWGA (Vector Laboratories) overnight at 4 °C. The precipitates were washed with lysis buffer and then eluted in 2× SDS sample buffer.

Western Blotting—Soluble protein samples or precipitates were subjected to SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare) using a PROTEIN II xi cell system (Bio-Rad) and mini-TransBlot cell system (Bio-Rad). The membranes were blocked in 5% skimmed milk in TBST (20 mM Tris, pH 7.4, 150 mM sodium chloride, and 1% Tween 20) for 1 h at ambient temperature and then incubated overnight at 4 °C in 1% skimmed milk containing diluted primary antibodies. The following antibodies were used: anti-*O*-GlcNAc (CTD110.6, Covance), anti-OGT (DM17, Sigma), anti-actin, anti-glycogen phosphorylase (Santa Cruz Biotechnology), anti-FLAG (Sigma), anti-AMPK, anti-phospho-AMPK and anti-glycogen synthase (Cell Signaling). Rabbit polyclonal *O*-GlcNAcase antibodies were raised by immunizing two rabbits with human full-length *O*-GlcNAcase (AbFrontier). Rabbit polyclonal glutamine:fructose-6-phosphate amidotransferase (GFAT) antibodies were kindly provided by Dr. Pann-Ghill Suh (POSTECH, Pohang, Korea). Afterward, membranes were washed several times and incubated with horseradish peroxidase-conjugated appropriate secondary antibodies (Santa Cruz Biotechnology) for 1 h. Following several washes, the membranes were developed using ECL reagent (GE Healthcare) and then exposed to Medical x-ray Film Blue (Agfa) or Amersham Biosciences Hyperfilm™ ECL (GE Healthcare). Densitometry measurements were performed using a Multi Gauge version 2.3 program (Fuji Photo Film).

OGT and O-GlcNAcase Assay—OGT activity was measured in whole cell lysates, which were incubated with reaction buffer containing recombinant glutathione *S*-transferase-tagged p62 and 100 μM UDP-GlcNAc for 2 h (25). Recombinant glutathione *S*-transferase-tagged p62 fragment used as substrate for OGT was extracted from *Escherichia coli* and purified on glutathione-Sepharose (GE Healthcare). The reaction was stopped by the addition of 2 mM glutathione, and supernatants were mixed with SDS-PAGE sample buffer, subjected to SDS-PAGE, and immunoblotted using anti-*O*-GlcNAc antibodies.

O-GlcNAcase assays were performed as described previously with minor modifications (10). The whole cell lysates were incubated with assay buffer (50 mM sodium cacodylate, pH 6.5, 50 mM *N*-acetylgalactosamine, 2 mM *p*-nitrophenyl-*N*-acetyl-D-glucosaminide) for 1 h at 37 °C. The reaction was stopped by adding 0.5 M sodium carbonate. Hydrolyzed *p*-nitrophenol was measured spectrophotometrically at 400 nm.

GFAT Assay—The GFAT assay was performed as described previously with minor modifications (26). Cells were washed with ice-cold PBS and mechanically removed in 500 μl of GFAT buffer (50 mM Tris-HCl, 5 mM EDTA, 5 mM glucose 6-phosphate and glutathione, 5 mM glucose 6-phophate Na_2, and 5 mM KCl, pH 7.5). The harvested cells were sonicated and pelleted by centrifugation (18,000 × *g*, 20 min). Protein concentration was determined with Bio-Rad protein assay reagent. Samples were adjusted to the same protein concentration in 500 μl of GFAT buffer. Then 500 μl of reaction buffer (0.8 mM D-fructose 6-phosphate, 6.0 mM glutamine, 0.3 mM acetylpyridine adenine dinucleotide, 50 mM KCl, 0.1 mM KH_2PO_4, 6 units of glutamic acid dehydrogenase, pH 7.8) was added to the respective samples. After incubation for 2 h at 37 °C, changes of absorbance at 370 nm due to the reduction of acetylpyridine adenine dinucleotide were measured.

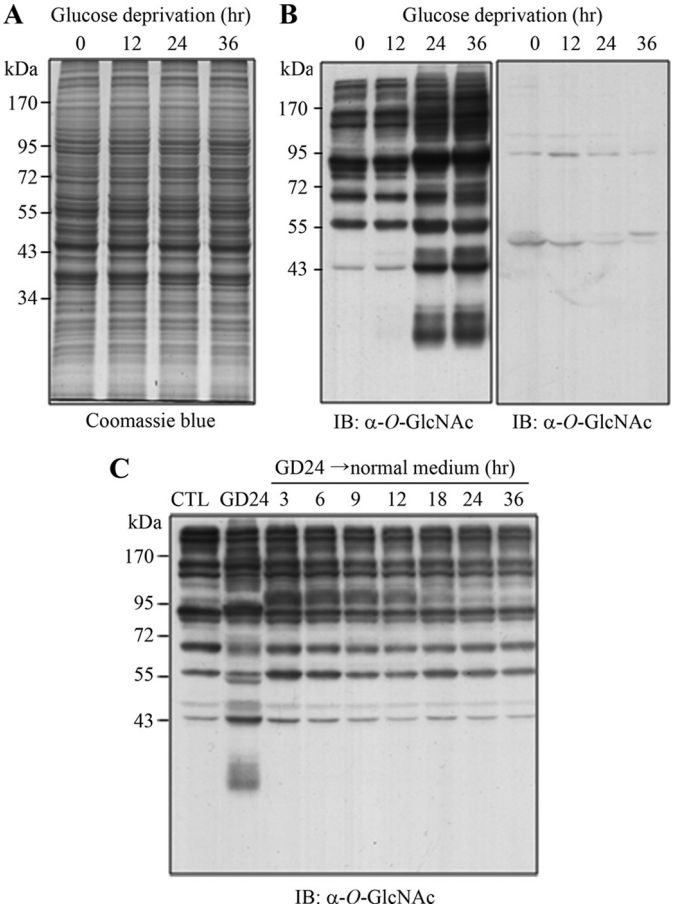

FIGURE 1. **Glucose deprivation increases protein *O*-GlcNAcylation in A549 cells which is reversible upon glucose replenishment.** *A* and *B*, lysates from A549 cells cultured in glucose-free medium for the indicated time points were stained with Coomassie Blue G-250 (*A*) and immunoblotted either with *O*-GlcNAc antibody or *O*-GlcNAc antibody in 10 mM GlcNAc (*B*). *C*, A549 cells were cultured in glucose-free medium for 24 h, rinsed with PBS, and subsequently incubated in medium containing 25 mM glucose for the indicated time points. Total cellular extract (30 μg/lane) was separated by SDS-PAGE, and *O*-GlcNAc residues were detected by immunoblotting (*IB*). *CTL*, control; *GD24*, glucose deprivation for 24 h.

Periodic Acid-Schiff (PAS) Staining and ATP Content Determination—The cell plates were treated with 0.5% periodic acid (Janssen, Geel, Belgium) solution for 10 min. The plates were rinsed with PBS. Then the cell plates were incubated with Schiff solution (Muto Pure Chemicals) overnight, washed in tap water, and counterstained with Harris hematoxylin. ATP concentrations were determined using the ATP Bioluminescent assay kit (Sigma).

Statistical Analysis—Statistical significance of enzyme activities or ATP level was determined by unpaired Student's *t* test using Microsoft Excel-based application. Results are expressed as mean ± S.E.

RESULTS

Glucose Deprivation Increases Protein O-GlcNAcylation in A549 Cells—Under glucose deprivation, no significant change of cellular protein level in A549 cells was detected in Coomassie Brilliant Blue-stained gels (Fig. 1*A*). Glucose deprivation was reported to increase protein *O*-GlcNAcylation in HepG2 and Neuro-2a cells (21, 22), and we could confirm this effect for A549 cells and several other cell lines. As shown in Fig. 1*B*, protein *O*-GlcNAcylation increased under glucose deprivation to reach a maximum after 24 h and remained at this level for 36 h, the longest time period analyzed. Specificity of the immunoblot was verified by competition with 10 mM GlcNAc (Fig. 1*B*). When glucose-free culture medium was replaced by medium containing 25 mM glucose, protein *O*-GlcNAcylation gradually decreased to reach control levels after 24 h (Fig. 1*C*).

Increase of O-GlcNAcylation under Glucose Deprivation Is Not Accompanied by an Increase in OGT or O-GlcNAcase Protein—The recent studies on HepG2 and Neuro-2a cells showed that the increase of protein *O*-GlcNAcylation in response to glucose deprivation was due to increased OGT (21, 22). We, however, observed no significant change in OGT and *O*-GlcNAcase protein in A549 cells by glucose deprivation (Fig. 2*A*). This is supported by increased *O*-GlcNAcylation observed under cycloheximide treatment (Fig. 2*B*). Thus, *O*-GlcNAcylation increased by glucose deprivation seems to be independent of the induction of OGT and *O*-GlcNAcase.

Glucose Deprivation Enhances OGT Activity and Reduces O-GlcNAcase Activity—To investigate whether glucose deprivation affects the activities of OGT and *O*-GlcNAcase, we performed OGT and *O*-GlcNAcase assays with total lysates of A549 cells. The OGT activity toward the p62 peptide substrate was increased about 2-fold following 24 h glucose deprivation (Fig. 3*A*). However, *O*-GlcNAcase activity decreased gradually and was only 60% of control after 36 h of glucose deprivation (Fig. 3*B*). Together, these data indicate that increased *O*-GlcNAcylation by glucose deprivation is due to increased OGT activity and decreased *O*-GlcNAcase activity.

O-GlcNAcylation on OGT Is Decreased by Glucose Deprivation—Because OGT is known to be *O*-GlcNAcylated, we studied the effect of glucose deprivation on *O*-GlcNAcylation of FLAG-tagged OGT expressed in A549 cells. As observed for endogenous OGT (Fig. 2*A*), the amount of FLAG-OGT remained unchanged under glucose deprivation (Fig. 4*A*). However, *O*-GlcNAcylation of OGT was greatly reduced (Fig. 4*A*). In agreement, pull-down of OGT with sWGA, which binds preferentially to terminal GlcNAc (27), showed greatly reduced OGT compared with control (Fig. 4*B*). Furthermore, we observed differences in the pattern of protein *O*-GlcNAcylation between OGT-overexpressing and control cells under glucose deprivation (Fig. 4*C*). This indicates that changes of *O*-GlcNAcylation following glucose deprivation are OGT-specific. It has been reported that OGT substrate specificity is regulated by transiently interacting proteins and depends on various cellular conditions (22, 28). In agreement, our results show that glucose deprivation not only enhances OGT activity but in addition affects its substrate specificity.

Hexosamine Biosynthetic Pathway and O-GlcNAc Modification in Response to Glucose Deprivation—GFAT is the first and rate-limiting enzyme for entry of glucose into the HBP. To reveal a relation between the HBP and increased *O*-GlcNAcylation by glucose deprivation, we measured both the amount of GFAT protein and its activity. Both GFAT protein and activity, measured by the glutamate dehydrogenase method (26), increased during glucose deprivation (Fig. 5*A*). Treatment of cells with the GFAT inhibitor DON completely blocked *O*-Glc-

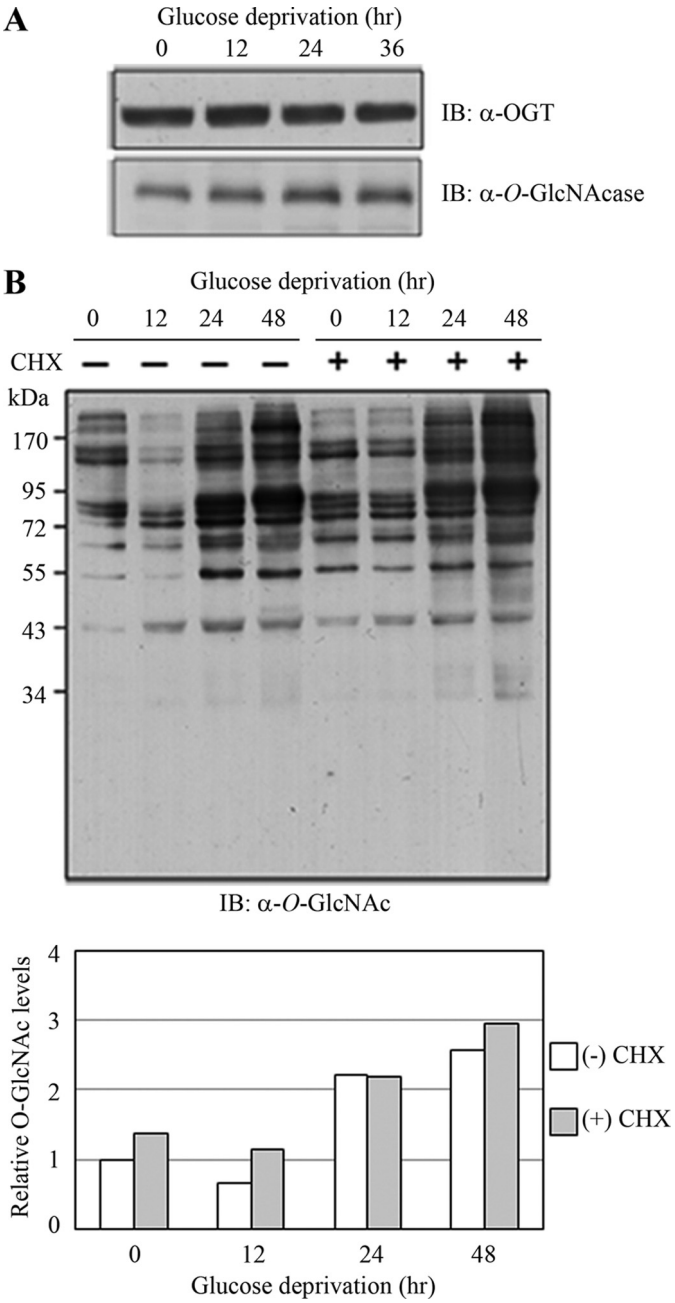

FIGURE 2. **Increase of *O*-GlcNAcylation by glucose deprivation is not accompanied by OGT induction but by *O*-GlcNAcase reduction and is independent of *de novo* protein synthesis.** *A*, lysates from A549 cells cultured in glucose-free medium for the indicated times were resolved by SDS-PAGE, and membranes were immunoblotted (*IB*) for OGT and *O*-GlcNAcase. *B*, cells were cultured in the presence of cycloheximide (*CHX*; 1 μg/ml) for 1 h, washed with PBS, and subsequently incubated in the glucose-free medium containing cycloheximide (1 μg/ml) for the indicated times. Total cellular extract (30 μg/lane) was resolved SDS-PAGE, and *O*-GlcNAc was determined by immunoblotting. Densitometry measurements were performed using a Multi Gauge version 2.3 program (Fuji Photo Film).

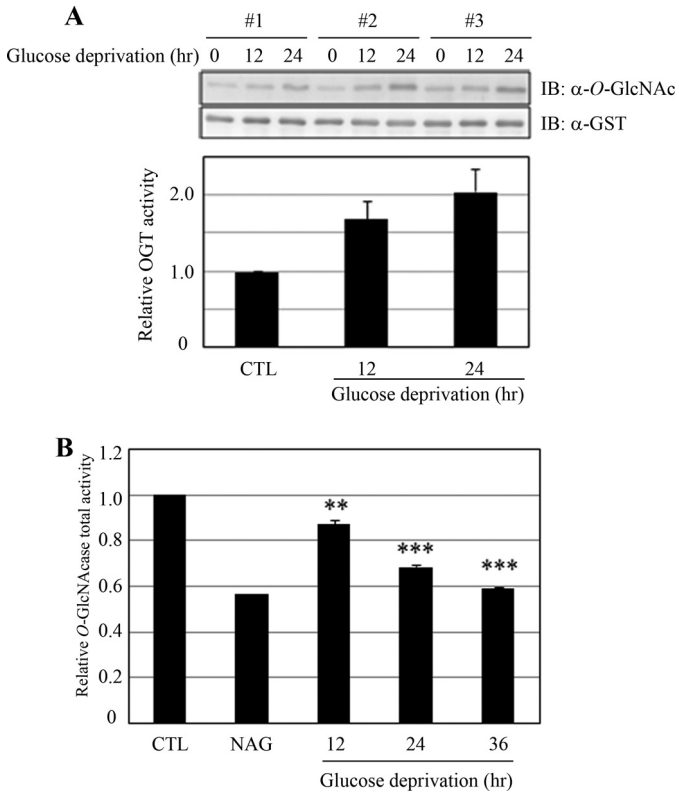

FIGURE 3. **Glucose deprivation increases OGT activity and reduces *O*-GlcNAcase activity.** *A*, whole cell lysates from glucose-deprived A549 cells were assayed for OGT activity at the indicated time points as described under "Experimental Procedures." The *O*-GlcNAcylation level of p62 was taken as measure for OGT activity and normalized to the amount of p62. *IB*, immunoblot. *B*, whole cell lysates from glucose-deprived A549 cells were assayed for *O*-GlcNAcase activity as described under "Experimental Procedures" for the indicated times. Hydrolyzed *p*-nitrophenol was measured spectrophotometrically at 400 nm. **, $p < 0.01$ compared with control; ***, $p < 0.001$ compared with control. *CTL*, control; *NAG*, 1,2-dideoxy-2′-methyl-α-D-glucopyranoso-[2,1-*d*]-Δ2′-thiazoline.

NAcylation under glucose deprivation (Fig. 5*B*). These data indicate a relation between the HBP and increased *O*-GlcNAcylation under conditions of glucose deprivation. However, glucose deprivation reduced the amount of phosphofructokinase 1 (Fig. 5*C*) that converts fructose 6-phosphate into fructose 1,6-bisphosphate under consumption of ATP. Under our experimental conditions, we also observed that generation of ATP was blocked (Fig. 5*D*). Because ATP is not only a substrate but also al allosteric inhibitor of phosphofructokinase 1 (for review, see ref. 29), a possible relationship between the reduction of phosphofructokinase 1 and the exhaustion of ATP remains to be clarified. Despite, we conclude that glucose deprivation abolishes the glycolytic pathway in A549 cells.

Increase of O-GlcNAcylation in Response to Glucose Deprivation Occurs through Glycogen Degradation—Because A549 and H1299 cells contain glycogen (24), we considered glycogen as a possible source for fructose 6-phosphate in glucose-deprived cells. By PAS staining, both A549 and H1299 cells contained glycogen (Fig. 6*A*) and became PAS-negative after 24 h of glucose deprivation (Fig. 6*A*). Subsequently, we analyzed GP, which catalyzes the phosphorylation of glycogen to glucose 1-phosphate. Inhibition of GP prevented the effect of glucose deprivation on *O*-GlcNAcylation (Fig. 6*B*). GP protein in A549 cells remained unchanged during glucose deprivation (Fig. 6*C*). When cells were grown in medium containing 25 mM glucose, inhibition of GP had no effect on *O*-GlcNAcylation (data not shown). Together, these data indicate a relation between increased *O*-GlcNAcylation by glucose deprivation and glycogen degradation, the latter providing a source for UDP-GlcNAc. To analyze the relationship between UDP-GlcNAc levels

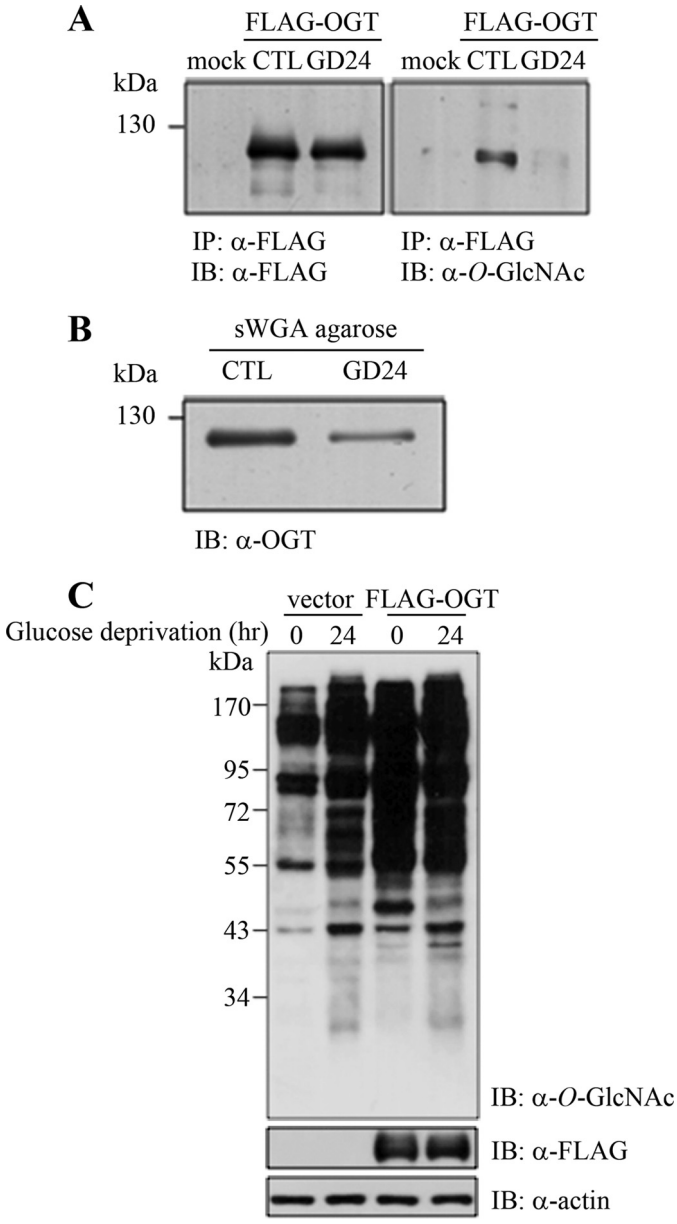

FIGURE 4. ***O*-GlcNAcylation on OGT is decreased by glucose deprivation.** *A*, whole cell lysates from A549 cells transfected with either FLAG vector or FLAG-tagged OGT were immunoprecipitated (*IP*) with FLAG antibodies and immunoblotted (*IB*) for FLAG and *O*-GlcNAc. *B*, whole cell lysates from control and glucose-deprived A549 cells were precipitated with sWGA-agarose. The precipitates were separated by SDS-PAGE and immunoblotted for OGT. *C*, whole cell lysates from A549 cells transfected with FLAG vector or FLAG-OGT immunoblotted for *O*-GlcNAc, FLAG, and α-actin. *CTL*, control; *GD24*, glucose deprivation for 24 h.

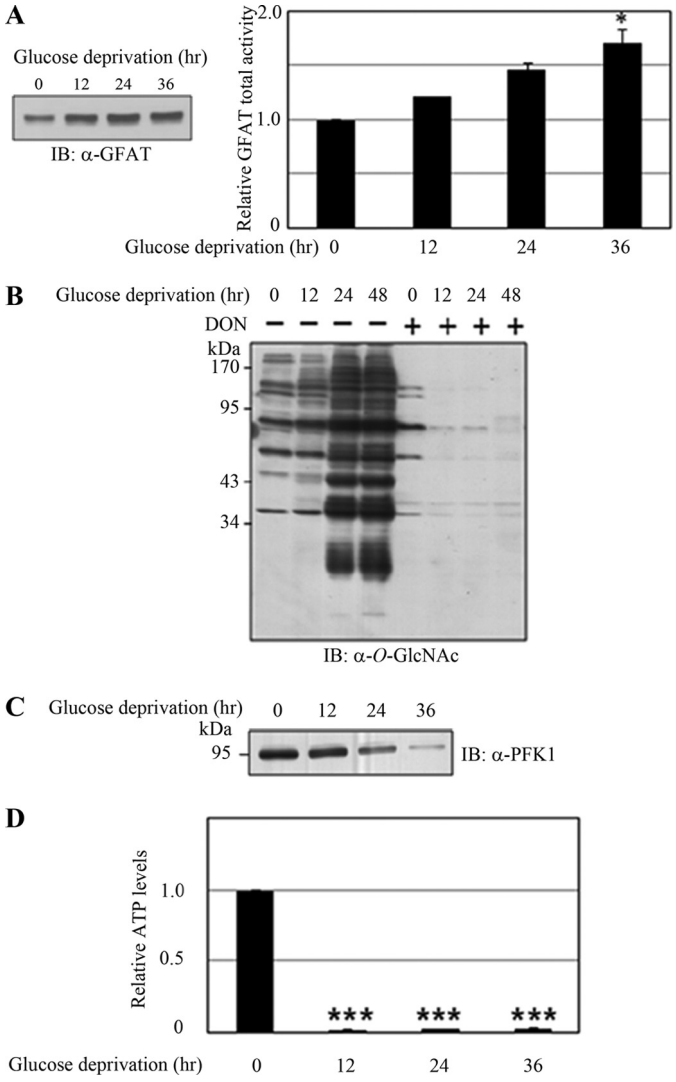

FIGURE 5. **HBP is activated in response to glucose deprivation.** *A*, whole cell lysates from glucose-deprived A549 cells were assayed for GFAT activity as described under "Experimental Procedures" at indicated time points. *IB*, immunoblot. *, $p < 0.05$ compared with control. *B*, cells were treated with 20 μM DON, washed with PBS, and then incubated with glucose-free medium containing 20 μM DON for the indicated time points. Total cellular extract (30 μg/*lane*) was resolved by SDS-PAGE, and *O*-GlcNAc was detected by immunoblotting (*IB*). *C*, whole lysates from glucose-deprived A549 cells were immunoblotted for phosphofructokinase 1 (*PFK1*) at the indicated time points. *D*, ATP content of glucose-deprived A549 cells was determined at the indicated time points as described under "Experimental Procedures." ***, $p <$ 0.001 compared with control.

and glycogen breakdown, we measured the intracellular UDP-GlcNAc levels of glucose-deprived cells under GP inhibitor treatment. Although UDP-GlcNAc could be detected in control A549 cells, we were unable to measure UDP-GlcNAc following 24 h of glucose deprivation whether or not GP was inhibited (supplementary Fig. 1). Most probably, all UDP-GlcNAc was rapidly used for *O*-GlcNAcylation.

Increased O-GlcNAcylation in Response to Glucose Deprivation Is Independent of Activation of AMPK—Next, we investigated the correlation between AMPK and increased *O*-GlcNAcylation by glucose deprivation because AMPK is known to be a nutrient sensor and activated by glucose depletion (30–32). Previously, Cheung *et al.* (22) demonstrated that glucose deprivation induces *O*-GlcNAcylation by OGT induction in an AMPK-dependent manner in Neuro-2a cells. However, we obtained no evidence for AMPK dependence of increased *O*-GlcNAcylation by glucose deprivation in A549 cells. AMPK was already phosphorylated in control conditions, and its phosphorylation rather decreased than increased following glucose deprivation (Fig. 7*A*). In line with this, treatment with the AMPK inhibitor Compound C did not significantly affect the patterns of *O*-GlcNAcylation following glucose deprivation, albeit the increase of *O*-GlcNAcylation was less prominent (Fig. 7*B*, compare *left two lanes* with *left two lanes*) which may be

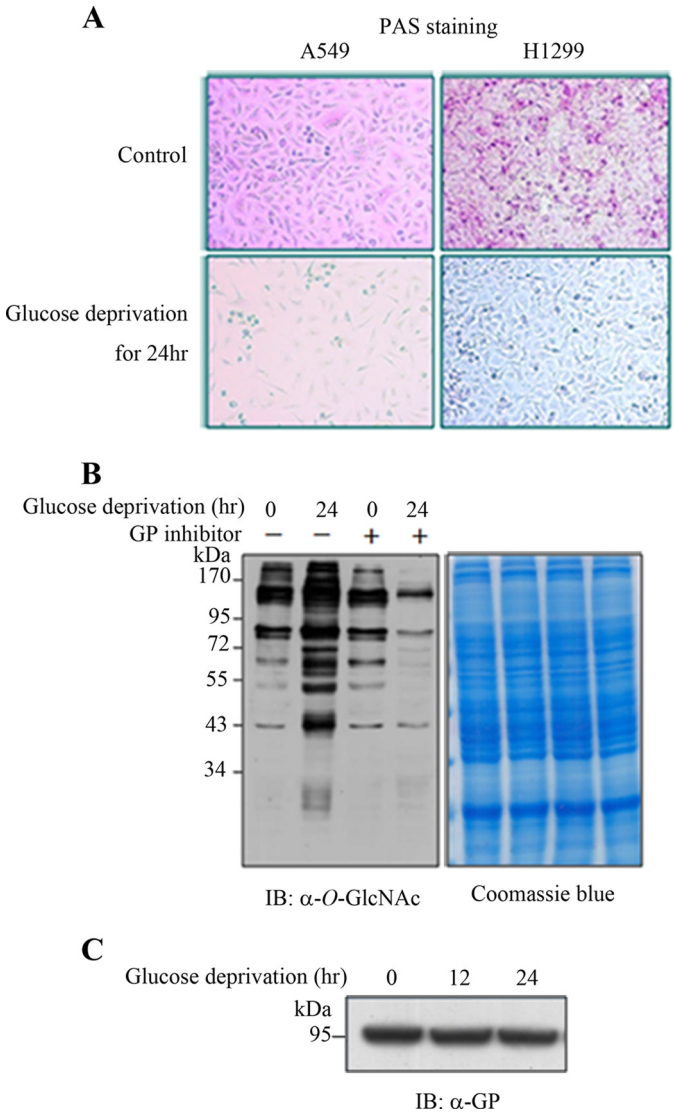

FIGURE 6. **Increase of *O*-GlcNAcylation in response to glucose deprivation occurs through glycogen degradation.** *A*, control and glucose-deprived A549 and H1299 cells were stained by PAS reagent as described under "Experimental Procedures." PAS-stained cells appear in *pink*. *B*, GP inhibitor (20 μM) treatment. Total cell lysates were separated by SDS-PAGE and immunoblotted (*IB*) for *O*-GlcNAc or stained with Coomassie Blue G-250. *C*, whole cell lysates from glucose-deprived A549 cells were immunoblotted for GP.

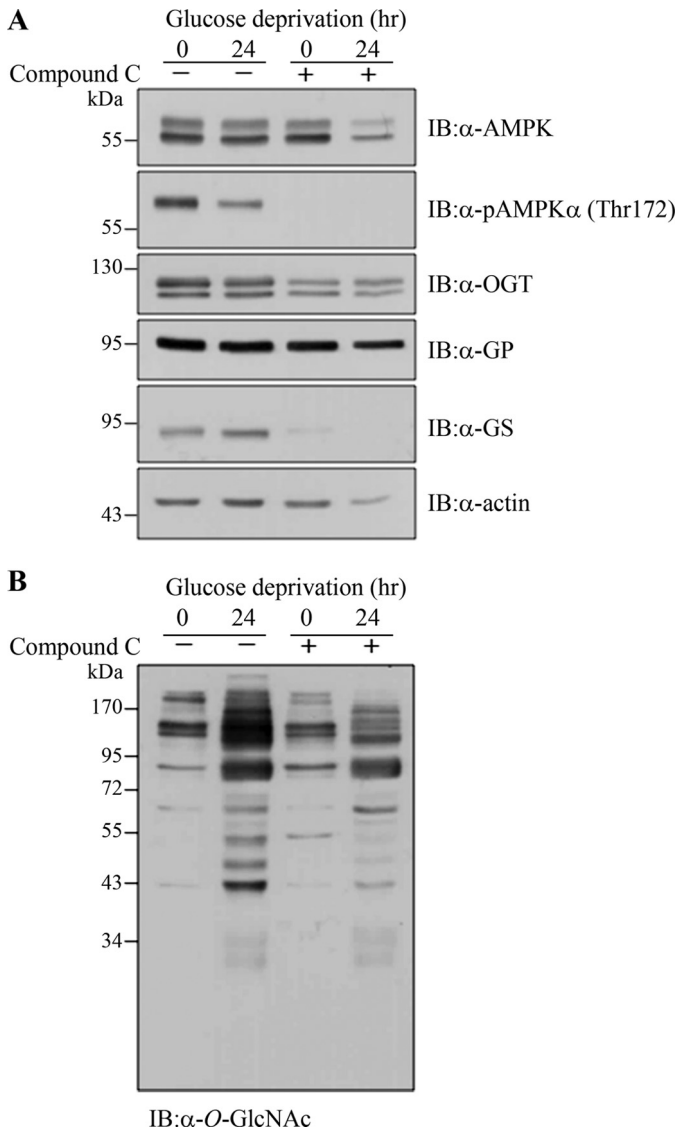

FIGURE 7. **Increased *O*-GlcNAcylation in response to glucose deprivation is not wholly dependent on activation of AMPK.** Cells were cultured in presence of 20 μM Compound C for 1 h, washed with PBS, and incubated in glucose-free medium containing 20 μM Compound C for 24 h. Total cellular extract (30 μg/*lane*) was resolved by SDS-PAGE and immunoblotted (*IB*) for AMPK, pAMPK, OGT, GP, and glycogen synthase (*GS*) (*A*) and *O*-GlcNAc (*B*).

related to reduced OGT protein expression under Compound C treatment (Fig. 7*A*). Recently, Taylor *et al.* (23) reported no increase in AMPK pathway activation in glucose-deprived HepG2 cells. Furthermore, we observed that Compound C reduced the amount of glycogen synthase but not of GP (Fig. 7*A*). From this, we speculate that the glycogen metabolism in A549 cells may be influenced by AMPK through its effect on glycogen synthase.

Glucose Deprivation Increases O-GlcNAcylation in Various Cell Lines—To verify that increased *O*-GlcNAcylation by glucose deprivation is not a particular feature of A549 cells, other cell lines were studied. As observed for A549 cells, *O*-GlcNAcylation was increased in glucose-deprived H460 and H1299 cells as well as in HEK293 and MCF7 cells (Fig. 8). Both H460 cells, another non-small cell lung carcinoma line, and MCF7 cells, human breast cancer cells, contained glycogen and became PAS-negative after 24 h of glucose deprivation (supplementary Fig. 2). In contrast, *O*-GlcNAcylation was decreased in glucose-deprived L132 lung fibroblasts (Fig. 8*A*, *right panel*). Thus, glucose deprivation resulted in increased *O*-GlcNAcylation in various carcinoma cell lines and in kidney-derived embryonic epithelia, but not in lung fibroblasts. Other glycogen-containing carcinoma cell lines also showed increased *O*-GlcNAcylation following glucose deprivation (data not shown).

DISCUSSION

An increase of *O*-GlcNAcylation by glucose deprivation was reported by Taylor *et al.* (21) for HepG2 cells and by Cheung *et al.* (22) for Neuro-2a cells and could be confirmed in the present study for several other carcinoma cell lines and embryonic kidney epithelia. We have made the following new observations: (i) the increased *O*-GlcNAcylation in A549 cells was due to an

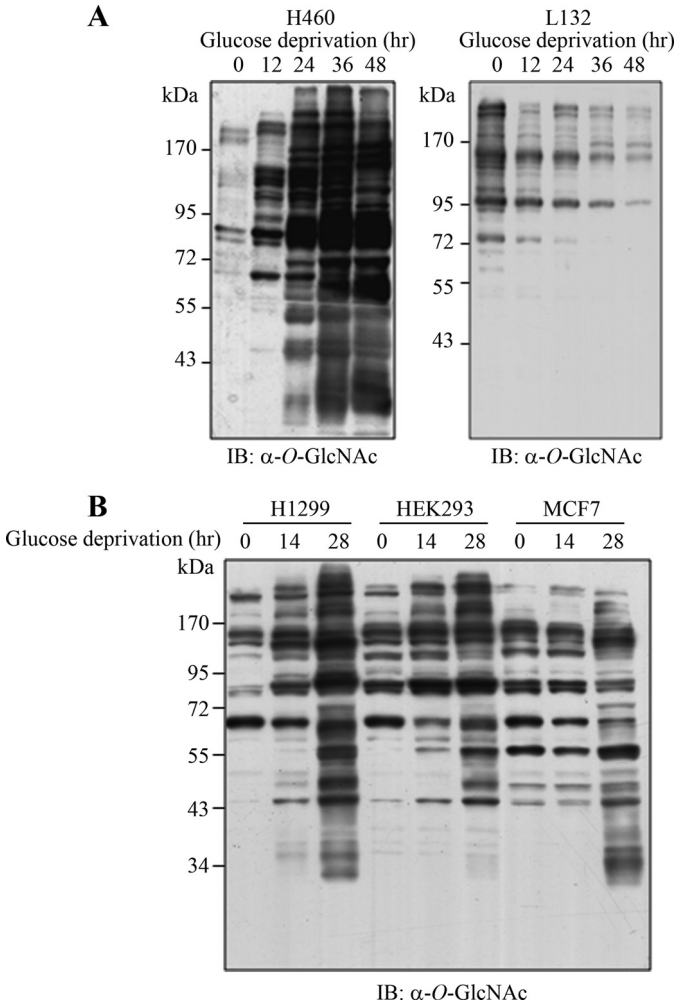

FIGURE 8. **Glucose deprivation increases protein *O*-GlcNAcylation of other cell lines except L132 lung fibroblasts.** Whole lysates from glucose-deprived H460, L132, H1299, HEK293T, and MCF7 cells were immunoblotted (*IB*) for *O*-GlcNAc following glucose deprivation at the indicated time points.

increase of OGT activity rather than OGT protein and a concurrent decrease of *O*-GlcNAcase activity, and (ii) the increased *O*-GlcNAcylation under conditions of glucose deprivation in the studied cell lines is apparently due to glycogen degradation.

In previous studies by others (21, 22), *O*-GlcNAcylation increased by glucose deprivation was found to be paralleled by induction of OGT. Our analysis of A549 lung carcinoma cells showed no change in the amount of OGT protein during glucose deprivation but a significant increase in OGT activity as well as decrease of *O*-GlcNAcase protein and activity. This 2-fold effect may explain the observed increase of *O*-GlcNAcylation. Interestingly, and in contrast to the increase in *O*-GlcNAcylation of cellular proteins, we detected a decrease of *O*-GlcNAcylation of OGT itself. This was accompanied not only by an increase of its activity but also a change in substrate specificity. Based on these observations, we propose that the reduced *O*-GlcNAcylation of OGT has an effect on both its catalytic activity and substrate specificity. It is known that the substrate specificity of OGT is regulated by transiently interacting proteins and depends on various cellular conditions (28). It can be speculated that the *O*-GlcNAcylation state of OGT may influence its interaction with such regulatory proteins, which potentially suggests a regulatory role of *O*-GlcNAcylation for OGT. This proposal should be tested in future experiments.

Also, we showed that *O*-GlcNAcase activity decreased gradually under glucose deprivation without simultaneous decreases of *O*-GlcNAcase proteins. We speculate that post-translational regulation of *O*-GlcNAcase probably influences *O*-GlcNAcase activity under glucose deprivation.

Although certain details of the mechanism leading to increased *O*-GlcNAcylation upon glucose deprivation are now established (21, 23, 28), the origin of the UDP-GlcNAc required for increased *O*-GlcNAcylation is unknown. Our results indicate that glycogen provides an important source for the needed glucose because the inhibition of GP, the rate-limiting enzyme for glycogenolysis, prevented increased *O*-GlcNAcylation under glucose deprivation. Furthermore, we demonstrate the involvement of the HBP by showing an increased amount of GFAT protein under glucose deprivation and that GFAT inhibition blocked the increased *O*-GlcNAcylation. Glycogen is a polymeric form of glucose, which functions as the short term energy storage in animal cells. It is most abundant in liver hepatocytes and skeletal muscle fibers, but most other cell types synthesize some glycogen as well. Muscle glycogen is used to generate ATP by rapid anaerobic glycolysis, and liver glycogen is used as a source of glucose that is released into the blood during fasting (for review, see ref. 33). However the role of glycogen metabolism in other cell types, especially cancer cells, is not well understood, even though the carbohydrate metabolism has long been associated with cancer cells (34). Because of the limited supply with nutrients and oxygen in rapidly growing carcinomas, glycogen may be used by carcinoma cells as source for energy. As our present study show, cancer cells in culture use glycogen for *O*-GlcNAcylation in response to glucose deprivation and not for generating ATP. How can this unexpected phenomenon be explained? Under glucose deprivation, energy-consuming gene transcription and protein synthesis might be reduced, and protein stability might be increased until glucose stress is overcome. We hypothesize that the general increase of *O*-GlcNAcylation might induce the transient block of gene transcription and protein synthesis. This is based on the finding that the highly *O*-GlcNAcylated CTD domain of RNA polymerase II may inhibit transcriptional elongation induced by phosphorylation of CTD (9, 35). Also, *O*-GlcNAc modification probably protects proteins from proteasomal degradation via association by HSP70 (13–15, 19). HSP70 has a lectin-like activity toward *O*-GlcNAc (13), and several heat shock proteins including HSP70 are modified by *O*-GlcNAc, too (36–38). A direct link between increased *O*-GlcNAcylation and the regulation of protein degradation has emerged from studies of proteosomes because it was demonstrated that *O*-GlcNAcylation is inhibitory for the 19 S cap of the proteosome (16, 39). Taken together, we suggest that *O*-GlcNAcylation may protect proteins from damage under glucose deprivation and therefore, may help cells to survive as proposed by others (19, 20, 40).

The recent reports (21, 23, 28) and the present study about increased *O*-GlcNAcylation under glucose deprivation are interesting in a more general sense because they demonstrate a high degree of diversity. The mechanism leading to increased

O-GlcNAcylation under glucose deprivation not only seems to vary in a cell type but also in a metabolism-related manner, which suggests that further studies on the regulation *O*-GlcNAcylation are warranted because of its importance for cellular function.

Acknowledgments—We thank Dr. Pann-Ghill Suh (POSTECH, Pohang, Korea) for providing the glutamine:fructose-6-phosphate amidotransferase antibody.

REFERENCES

1. Hruz, P. W., and Mueckler, M. M. (2001) *Mol. Membr. Biol.* **18,** 183–193
2. Mueckler, M. (1994) *Eur. J. Biochem.* **219,** 713–725
3. McClain, D. A., and Crook, E. D. (1996) *Diabetes* **45,** 1003–1009
4. Wells, L., Vosseller, K., and Hart, G. W. (2001) *Science* **291,** 2376–2378
5. Love, D. C., and Hanover, J. A. (2005) *Sci. STKE* 2005, re13
6. Zachara, N. E., and Hart, G. W. (2004) *Biochim. Biophys. Acta* **1673,** 13–28
7. Torres, C. R., and Hart, G. W. (1984) *J. Biol. Chem.* **259,** 3308–3317
8. Gao, Y., Miyazaki, J., and Hart, G. W. (2003) *Arch. Biochem. Biophys.* **415,** 155–163
9. Comer, F. I., and Hart, G. W. (2001) *Biochemistry* **40,** 7845–7852
10. Kang, H. T., Ju, J. W., Cho, J. W., and Hwang, E. S. (2003) *J. Biol. Chem.* **278,** 51223–51231
11. Kuo, M., Zilberfarb, V., Gangneux, N., Christeff, N., and Issad, T. (2008) *Biochimie* **90,** 679–685
12. 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
13. Guinez, C., Lemoine, J., Michalski, J. C., and Lefebvre, T. (2004) *Biochem. Biophys. Res. Commun.* **319,** 21–26
14. Guinez, C., Losfeld, M. E., Cacan, R., Michalski, J. C., and Lefebvre, T. (2006) *Glycobiology* **16,** 22–28
15. Zhang, F., Su, K., Yang, X., Bowe, D. B., Paterson, A. J., and Kudlow, J. E. (2003) *Cell* **115,** 715–725
16. Zachara, N. E., and Hart, G. W. (2004) *Trends Cell Biol.* **14,** 218–221
17. Andrali, S. S., Qian, Q., and Ozcan, S. (2007) *J. Biol. Chem.* **282,** 15589–15596
18. Yang, W. H., Park, S. Y., Nam, H. W., Kim do, H., Kang, J. G., Kang, E. S., Kim, Y. S., Lee, H. C., Kim, K. S., and Cho, J. W. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 17345–17350
19. Zachara, N. E., and Hart, G. W. (2006) *Biochim. Biophys. Acta* **1761,** 599–617
20. Zachara, N. E., O'Donnell, N., Cheung, W. D., Mercer, J. J., Marth, J. D., and Hart, G. W. (2004) *J. Biol. Chem.* **279,** 30133–30142
21. Taylor, R. P., Parker, G. J., Hazel, M. W., Soesanto, Y., Fuller, W., Yazzie, M. J., and McClain, D. A. (2008) *J. Biol. Chem.* **283,** 6050–6057
22. Cheung, W. D., and Hart, G. W. (2008) *J. Biol. Chem.* **283,** 13009–13020
23. Taylor, R. P., Geisler, T. S., Chambers, J. H., and McClain, D. A. (2009) *J. Biol. Chem.* **284,** 3425–3432
24. Schnier, J. B., Nishi, K., Monks, A., Gorin, F. A., and Bradbury, E. M. (2003) *Biochem. Biophys. Res. Commun.* **309,** 126–134
25. Konrad, R. J., Zhang, F., Hale, J. E., Knierman, M. D., Becker, G. W., and Kudlow, J. E. (2002) *Biochem. Biophys. Res. Commun.* **293,** 207–212
26. Ye, F., Maegawa, H., Morino, K., Kashiwagi, A., Kikkawa, R., Xie, M., and Shen, Z. (2004) *J. Biochem. Biophys. Methods* **59,** 201–208
27. Monsigny, M., Sene, C., Obrenovitch, A., Roche, A. C., Delmotte, F., and Boschetti, E. (1979) *Eur. J. Biochem.* **98,** 39–45
28. Cheung, W. D., Sakabe, K., Housley, M. P., Dias, W. B., and Hart, G. W. (2008) *J. Biol. Chem.* **283,** 33935–33941
29. Rodicio, R., Strauss, A., and Heinisch, J. J. (2000) *J. Biol. Chem.* **275,** 40952–40960
30. Culmsee, C., Monnig, J., Kemp, B. E., and Mattson, M. P. (2001) *J. Mol. Neurosci.* **17,** 45–58
31. McCullough, L. D., Zeng, Z., Li, H., Landree, L. E., McFadden, J., and Ronnett, G. V. (2005) *J. Biol. Chem.* **280,** 20493–20502
32. Carling, D. (2009) *Cell Metab.* **9,** 7–8
33. McBride, A., and Hardie, D. G. (2009) *Acta Physiol.* **196,** 99–113
34. Mathupala, S. P., Rempel, A., and Pedersen, P. L. (1995) *J. Biol. Chem.* **270,** 16918–16925
35. Kelly, W. G., Dahmus, M. E., and Hart, G. W. (1993) *J. Biol. Chem.* **268,** 10416–10424
36. Roquemore, E. P., Chevrier, M. R., Cotter, R. J., and Hart, G. W. (1996) *Biochemistry* **35,** 3578–3586
37. Walgren, J. L., Vincent, T. S., Schey, K. L., and Buse, M. G. (2003) *Am. J. Physiol. Endocrinol. Metab.* **284,** E424–E434
38. Wells, L., Vosseller, K., Cole, R. N., Cronshaw, J. M., Matunis, M. J., and Hart, G. W. (2002) *Mol. Cell. Proteomics* **1,** 791–804
39. Liu, K., Paterson, A. J., Zhang, F., McAndrew, J., Fukuchi, K., Wyss, J. M., Peng, L., Hu, Y., and Kudlow, J. E. (2004) *J. Neurochem.* **89,** 1044–1055
40. Ngoh, G. A., Facundo, H. T., Hamid, T., Dillmann, W., Zachara, N. E., and Jones, S. P. (2009) *Circ. Res.* **104,** 41–49