Modulation of O-Linked N-Acetylg glucosamine Levels on Nuclear and Cytoplasmic Proteins \textit{in Vivo} Using the Peptide O-GlcNAc-$\beta$-N-acetylglucosaminidase Inhibitor O-(2-Acetamido-2-deoxy-\(\beta\)-glucopyranosylidene)amino-N-phenylcarbamate\textsuperscript{*}

Robert S. Haltiwanger,\textsuperscript{‡} Kathleen Grove, and Glenn A. Philipsberg

From the Department of Biochemistry and Cell Biology, Institute for Cell and Developmental Biology, State University of New York, Stony Brook, New York 11794-5215

\textit{O}-Linked \(N\)-acetylg glucosamine (O-GlcNAc) is a ubiquitous and abundant post-translational modification found on nuclear and cytoplasmic proteins and is thought to be a dynamically regulated modification much like phosphorylation. In this study we have demonstrated that O-(2-acetamido-2-deoxy-\(\beta\)-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc), a potent \textit{in vitro} inhibitor of the enzyme responsible for the removal of O-GlcNAc from proteins (peptide O-GlcNAc-$\beta$-N-acetylglucosaminidase), can be used to increase O-GlcNAc levels on nuclear and cytoplasmic proteins \textit{in vivo}. Overall, PUGNAc caused approximately a 2-fold increase in O-GlcNAc levels in the human colon cancer cells, HT29, although the effects on individual proteins varied. The increase appeared to be the result of the direct inhibition of the peptide O-GlcNAc-$\beta$-N-acetylglucosaminidase since neither the O-GlcNAc transferase nor UDP-GlcNAc levels were affected by the treatment. O-GlcNAc levels in other cell lines tested (NIH 3T3, CV-1, and HeLa) were also affected by PUGNAc, although the effects on HeLa cells were minimal. At the concentrations tested, PUGNAc was non-toxic and had no affect on the growth rate of any of the cell lines examined. Interestingly, we demonstrated that an increase in O-GlcNAc levels on the transcription factor Sp1 resulted in a reciprocal decrease in its level of phosphorylation, supporting the hypothesis that O-GlcNAc competes with phosphate on some proteins. These studies demonstrate that PUGNAc is an effective inhibitor of O-GlcNAc turnover within cells and can be used to selectively alter the extent of O-GlcNAc on cellular proteins.

\footnotetext[1]{This work was supported by National Institutes of Health Grant GM 48666. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked \textit{advertisement} in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.}

\footnotetext[2]{Recipient of an American Cancer Society Junior Faculty Research Award. To whom correspondence should be addressed. Tel.: 516-632-7336; Fax: 516-632-8575.}

\footnotetext[3]{The abbreviations used are: O-GlcNAc, O-linked \(N\)-acetylg glucosamine; PUGNAc, O-(2-acetamido-2-deoxy-\(\beta\)-glucopyranosylidene)amino-N-phenylcarbamate; O-GlcNAcase, peptide O-GlcNAc-$\beta$-N-acetylglucosaminidase; O-GlcNAc transferase, UDP-GlcNAc-peptide $\beta$-N-acetylglucosaminidase; PAG, polyacrylamide gel electrophoresis; PNGase F, peptide $N$-glycosidase F; UDP-HexNAc, uridine diphospho-$N$-acetylhexosaminase.}

\textsuperscript{*} This work was supported by National Institutes of Health Grant GM 48666. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked \textit{advertisement} in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{‡} Recipient of an American Cancer Society Junior Faculty Research Award. To whom correspondence should be addressed. Tel.: 516-632-7336; Fax: 516-632-8575.

\textsuperscript{1} The abbreviations used are: O-GlcNAc, O-linked \(N\)-acetylg glucosamine; PUGNAc, O-(2-acetamido-2-deoxy-\(\beta\)-glucopyranosylidene)amino-N-phenylcarbamate; O-GlcNAcase, peptide O-GlcNAc-$\beta$-N-acetylglucosaminidase; O-GlcNAc transferase, UDP-GlcNAc-peptide $\beta$-N-acetylglucosaminidase; PAG, polyacrylamide gel electrophoresis; PNGase F, peptide $N$-glycosidase F; UDP-HexNAc, uridine diphospho-$N$-acetylhexosaminase.

with the modification (1–3), including RNA polymerase II (4), several RNA polymerase II transcription factors (5), nuclear pore proteins (6), the tumor suppressor protein p53 (7), and c-Myc (8). Sites of glycosylation have been identified on a number of O-GlcNAc-modified proteins, and although a strict consensus sequence cannot be discerned, most sites contain a proline residue amino-terminal to the modified serine or threonine (1). About half of the sites contain the motif PX(S/T), where X is valine, serine, or threonine and (S/T) is the modified serine or threonine.

Accumulating evidence suggests that O-GlcNAc is a regulated modification much like phosphorylation (1–3). In several instances it has been shown that the sugar turns over more rapidly than the protein it modifies (9, 10), implying that O-GlcNAc is dynamically added to and removed from proteins. In addition, conditions of growth stimulation (11) and of growth arrest (12) have been demonstrated to alter the level of O-GlcNAc in cells. Enzymes for the addition (O-GlcNAc transferase) (13–16) and removal (O-GlcNAcase) (17) of the sugar are known to exist in the cytoplasm of most eukaryotic cells. Thus, a system capable of dynamic regulated addition and removal of the sugar analogous to a kinase/phosphatase system is present in cells.

A major function of O-GlcNAc may be to compete with phosphorylation for sites on proteins (1–3). Since GlcNAc is neutral, it is possible that it would have different effects on protein function than a strongly negatively charged phosphate group. Thus, competition between GlcNAc and phosphate for similar sites would add an extra level of control to signal transduction cascades in cells. Alternatively, O-GlcNAc or phosphate could affect protein activity in a similar manner, although the modifications would be the result of different signaling cascades. In either case, only one modification could occur at an individual site at any one time. Evidence indicating that this type of competition occurs has been obtained by demonstration that the sites of glycosylation on both RNA polymerase II (4) and c-Myc (8) coincide with known phosphorylation sites. Even so, direct modulation of the level of phosphate on a protein by changing the level of glycosylation on the same protein has not yet been demonstrated \textit{in vivo}.

Selective inhibitors of protein kinases and phosphatases have been used extensively to modulate the level of phosphate on proteins as an approach to determine the functional implications of phosphorylation. By analogy to the phosphorylation system, specific inhibitors of either the O-GlcNAcase or O-GlcNAc transferase would be valuable tools in the study of the function of the O-GlcNAc modification. In the present study, we sought to address two goals. First, we wanted to examine the feasibility of using a known \textit{in vitro} inhibitor of the O-GlcNA-
case for modulation of O-GlcNAc levels on proteins in vivo. Second, if such modulation was feasible, we wanted to test the hypothesis that O-GlcNAc and phosphate compete on proteins by determining whether alterations in the level of O-GlcNAc on a given protein would result in changes in the level of phosphorylation of the same protein. In these studies, we have utilized the potent in vitro inhibitor of O-GlcNAcase, PUGNAc (17), to address these goals.

EXPERIMENTAL PROCEDURES

Materials—Bovine milk galactosyltransferase, Protein G-agarose, disaccharides (galactose-β-1,3-N-acetylgalactosamine, galactose-β-1,3-
N-acetylglucosamine, and galactose-β-1,4-N-acetylglucosamine), and ovalbumin were from Sigma. The alditol derivatives of the disaccharides were prepared by reduction with sodium borohydride as described (15). Peptide N-glycosidase F (PNGase F) was purified from the culture filtrate of Flavobacterium meneghinianum as described (18). Anti-SP1 antibodies (rabbit polyclonal PEP2 with competing peptide and mouse monoclonal 1C6) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). HT29 (human colon cancer) and CV-1 (green monkey kidney) cells were obtained from American Type Culture Collection (Rockville, MD). NIH 3T3 (mouse embryo) and HeLa (human cervical cancer) cells were obtained from the Department of Microbiology Tissue Culture Facility (SUNY, Stony Brook). Dulbecco’s modified Eagle’s medium (low glucose), minimal essential medium (with and without glucose), and Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (dialyzed) in the presence or absence of 40 μM PUGNAc. Cells were treated for 24 h with 0, 20, or 40 μM PUGNAc. The cells were removed from the plates using trypsin/EDTA, counted, and extracted with perchloric acid as described (20). The chromogenic procedure used to separate the nucleotides does not resolve UDP-GlcNAc from UDP-GalNAc, but we have previously shown that the ratio of UDP-GlcNAc to UDP-GalNAc in HT29 cells is approximately 2:1, as it is in most cells (20, 21). Mammalian cells contain an epimerase which maintains this ratio. Thus, the amount of UDP-GlcNAc was calculated from the values obtained for uridine diphospho-N-acetylgalactosamine (UDP-GlcNAc plus UDP-GalNAc) using this ratio.

Other—Galactosyltransferase labeling after denaturing proteins with 1% SDS was performed as described previously (20). Immunoprecipitations, Western blots, and carbohydrate analysis (digestion with PNGase F, alkaline-induced β-elimination, size fractionation, and high pH anion exchange chromatography) were all performed as described previously (20). Densitometric scans of Western blots and fluorographs were performed on a Bio-Rad model GS-670 Imaging Densitometer. All gels were 10% SDS-polyacrylamide gels as described by Laemmli (22). Protein was estimated using the BCA assay (Pierce).

RESULTS

PUGNAc Increases O-GlcNAc Levels on Proteins in HT29 Cells—To examine the effects of PUGNAc on O-GlcNAc levels in cells, we treated HT29 cells with varying concentrations of PUGNAc overnight (Fig. 1A) and with 40 μM PUGNAc for varying lengths of time (Fig. 1B). The cells were lysed at the indicated times, and the level of terminal GlcNAc moieties per unit of protein was determined using galactosyltransferase and UDP-[3H]galactose. Both a concentration- and time-dependent increase in the level of terminal GlcNAc moieties resulted from PUGNAc treatment (Fig. 1, A and B). The maximal increase appeared at 40 μM PUGNAc. No further increase in GlcNAc levels was seen in cells treated with higher PUGNAc concentrations (up to 400 μM, data not shown). Increases could be seen after 2 h of treatment and seemed to plateau after approximately 8 h. The increased level of terminal GlcNAc moieties could be maintained for several days without changing media (Fig. 1B), indicating that the inhibitor was not being significantly metabolized by the cells. Interestingly, the level of terminal GlcNAc in the absence of PUGNAc was already substantially higher than that of untreated cells, indicating that the O-GlcNAc levels in untreated cells are constitutively high. Finally, the effects of the inhibitor could be reversed fairly rapidly (Fig. 1C) by simply replacing the inhibitor-containing media with inhibitor-free media.

Since galactosyltransferase will modify GlcNAc moieties found on both N-glycans as well as O-GlcNAc (19), we needed to determine which of these forms of glycosylation were being affected by the PUGNAc treatment. Samples treated with or without 40 μM PUGNAc for 24 h were galactosyltransferase-labeled as above and subjected to digestion with PNGase F. The PNGase F-released material (N-glycans) was separated from the PNGase F-resistant material (O-glycans, see below), and both were quantified (Fig. 2A). As we have seen before (20), the majority of the radioactivity appeared to be O-linked (i.e. was resistant to PNGase F digestion), indicating that O-linked GlcNAc moieties in HT29 cells are O-linked. The O-linked material showed an approximately 1.5- to 2-fold decrease in terminal GlcNAc moieties following PUGNAc treatment. An increase in terminal GlcNAcs on N-glycans was also observed, although it was not as statistically significant. To confirm that the PNGase F-resistant fraction consisted of O-linked sugars, it was subjected to alkaline-induced β-elimination (Fig. 2B). In both cases, essentially all of the radiolabel was released from the
proteins, indicating that it was O-linked to the protein. Size fractionation of the released O-glycans (Fig. 2C) showed that all of the radiolabel was in the form of a disaccharide. Finally, to confirm that the disaccharide was the result of O-GlcNAc, high pH anion exchange chromatography analysis (Fig. 2D) demonstrated that the disaccharide was Galβ1,4GlcNAcitol, the expected product of β-eliminated, galactosyltransferase-labeled O-GlcNAc. Thus, the majority of the increase in terminal GlcNAc moieties caused by PUGNAc appeared to be due to an increase in the total O-GlcNAc in the cell. In HT29 cells, the increase was approximately 2-fold.

We next examined whether the increase in O-GlcNAc levels caused by PUGNAc was a result of changes in the selective glycosylation of a subset of proteins or if the changes were more general. Galactosyltransferase-labeled samples from HT29 cells treated with increasing concentrations of PUGNAc were digested with PNGase F and subjected to SDS-PAGE (Fig. 3A).

An increase in [3H]galactose labeling was seen on a number of proteins. Since we had demonstrated that essentially all of the [3H]galactose remaining after PNGase F treatment was O-GlcNAc (Fig. 2), we concluded that PUGNAc induces a generalized increase in O-GlcNAc levels on many proteins. This suggests that the effects of PUGNAc are specific for the enzyme responsible for deglycosylation of most O-GlcNAc-modified proteins. Densitometric scans (Fig. 3B) of individual bands from the fluorograph in Fig. 3A showed that a similar increase in O-GlcNAc levels was seen on all of the proteins analyzed, although the extent of the change varied slightly from protein to protein (from approximately 1.5- to 2.5-fold increase in intensity). Consistent with the data shown in Fig. 1A, the majority of the increase in O-GlcNAc was seen between 0 and 20 μM PUGNAc, with a smaller increase occurring between 20 and 40 μM. Thus, PUGNAc appears to induce a generalized increase in O-GlcNAc levels on numerous proteins in HT29 cells, although the extent of the increase is somewhat protein-dependent.

Effect of PUGNAc on the O-GlcNAc Glycosylation Machinery—Although PUGNAc is known to be a competitive inhibitor of the O-GlcNAcase in vitro, we wanted to determine whether the change in O-GlcNAc levels we had observed in HT29 cells was in fact due to inhibition of this enzyme. There are three components that determine the level of O-GlcNAc in cells as follows: the O-GlcNAc transferase, O-GlcNAcase, and UDP-GlcNAc levels (1, 20). We assayed the effect of PUGNAc on each of these components. As expected, PUGNAc has a dramatic effect on the activity of the O-GlcNAcase in HT29 cells (Fig. 4A). The concentration dependence of inhibition correlates very well with the concentration required to achieve maximal effects in cells (40–50 μM, see Fig. 1A). In contrast, PUGNAc had no effect on the O-GlcNAc transferase activity from HT29 cells. In addition, treatment of HT29 cells with 20 or 40 μM PUGNAc for 24 h (concentrations which increase O-GlcNAc levels, Fig. 1A) caused no increase in UDP-GlcNAc levels (Fig. 4B). If anything, a slight decrease was observed. Consistent with these findings, we have shown that overnight treatment of HT29 cells with media containing 5 mM glucosamine (which results in more than a 10-fold increase in UDP-GlcNAc levels) has no effect on the amount of terminal GlcNAc in the cells. Thus, increasing the concentration of UDP-GlcNAc is not a mechanism for increasing O-GlcNAc levels in HT29 cells. These results strongly suggest that the PUGNAc-induced increase in O-GlcNAc levels is due to a direct inhibition of the O-GlcNAcase within these cells.

Effects of PUGNAc on Other Cell Lines—We have also examined the effect of PUGNAc on O-GlcNAc levels in several other cell lines (NIH-3T3, HeLa, and CV-1). PUGNAc caused an increase in terminal GlcNAc in all of the cells examined, although the level of increase seen in HeLa cells was significantly less than that seen in the others (Fig. 5A). PNGase F treatment of the galactosyltransferase-labeled samples from each of the different cell types showed that the majority of the PUGNAc-induced increase was due to O-GlcNAc (data not shown). Therefore, PUGNAc appears to increase the level of O-GlcNAc on proteins in all cell lines examined, although the level of increase is cell line-dependent. PUGNAc did not appear to have any toxic effects on the cells tested, so we sought to determine whether long term exposure to PUGNAc affected cell growth. Each of the cell lines examined above was grown in the presence or absence of 40 μM PUGNAc for up to a week (Fig. 5B). The media were changed every other day to replenish the PUGNAc. The level of O-GlcNAc remained elevated in the PUGNAc-treated cells throughout the treatment (data not

2 G. A. Philipsberg and R. S. Haltiwanger, unpublished observations.
Experimental Procedures

We next investigated the effects of PUGNAc on the phosphorylation of Sp1. A protein known to be modified with both O-GlcNAc and glycosylation machinery (1–3). Our main goal in the studies described here was to determine whether inhibitors could be used to modulate O-GlcNAc levels on proteins in cells. We have examined the feasibility of using a known in vitro inhibitor of O-GlcNAcase, PUGNAc, on cultured mammalian cells in vitro. Our data demonstrate that we can in fact increase the level of O-GlcNAc on numerous proteins in a variety of cell types using this inhibitor. The increase in O-GlcNAc appears to be due to a specific inhibition of the O-GlcNAcase, since PUGNAc had no effect on the other components of the O-GlcNAc glycosylation machinery (O-GlcNAc transferase or UDP-GlcNAc). The increase in O-GlcNAc is the result of a true increase in the sugar to protein ratio and not just accumulation of O-GlcNAc-modified proteins, since we saw no increase in the amount of Sp1 protein in PUGNAc-treated cells. In addition, PUGNAc was non-toxic, indicating that at the levels tested it did not have negative effects on cell growth. These results indicate that PUGNAc can be used to inhibit the appearance of O-GlcNAc-modified proteins, since we saw no increase in the amount of Sp1 protein in PUGNAc-treated cells. In addition, PUGNAc was non-toxic, indicating that at the levels tested it did not have negative effects on cell growth. These results indicate that PUGNAc can be used to inhibit the

Effects of PUGNAc on Phosphorylation State of Proteins—One of the major hypotheses describing a potential function for O-GlcNAc is that it may compete with phosphate for individual hydroxyl groups (1–3). Now that we could reproducibly increase O-GlcNAc levels in cells, we could directly test whether this competition occurs in vivo. HT29 cells were treated with or without 40 μM PUGNAc (conditions known to approximately double O-GlcNAc levels in these cells, see Figs. 1 or 5) and metabolically labeled in vivo with [32P]orthophosphate. Proteins from crude lysates of these cells were separated by SDS-PAGE, and the radiolabeled proteins were analyzed on a PhosphorImager. Fig. 6A shows that the presence or absence of PUGNAc had no noticeable effect on the intensity of phosphorylation of any proteins that could be detected in these crude samples. Thus, PUGNAc does not cause dramatic changes in the overall phosphorylation state of major proteins in the cell. We next investigated the effects of PUGNAc on the phosphorylation of Sp1, a protein known to be modified with both phosphate (23) and O-GlcNAc (5). Sp1 was immunoprecipitated from [32P]phosphate-labeled HT29 cells that had been treated with and without 40 μM PUGNAc (Fig. 6B, [32P]PO4). The Sp1 from PUGNAc-treated cells showed approximately a 30% reduction (when normalized to protein levels based on Western blots) in the level of phosphorylation relative to the untreated cells. Analysis of the O-GlcNAc level on Sp1 isolated from a parallel set of cells treated with or without PUGNAc showed approximately a 4-fold increase in the level of O-GlcNAc on the protein (Fig. 6B, [3H]Galactose). Western blots with anti-Sp1 antibodies on extracts of the same cells demonstrated that the level of Sp1 did not change significantly with PUGNAc treatment (Fig. 6B, Western blot). These results indicate that although PUGNAc does not affect the overall phosphorylation state of the cell, it does cause an increase in O-GlcNAc and a decrease in phosphate on Sp1. Thus, O-GlcNAc and phosphate appear to compete with each other for at least some modification sites on Sp1.

DISCUSSION

Our main goal in the studies described here was to determine whether inhibitors could be used to modulate O-GlcNAc levels on proteins in cells. We have examined the feasibility of using a known in vitro inhibitor of O-GlcNAcase, PUGNAc, on cultured mammalian cells in vitro. Our data demonstrate that we can in fact increase the level of O-GlcNAc on numerous proteins in a variety of cell types using this inhibitor. The increase in O-GlcNAc appears to be due to a specific inhibition of the O-GlcNAcase, since PUGNAc had no effect on the other components of the O-GlcNAc glycosylation machinery (O-GlcNAc transferase or UDP-GlcNAc). The increase in O-GlcNAc is the result of a true increase in the sugar to protein ratio and not just accumulation of O-GlcNAc-modified proteins, since we saw no increase in the amount of Sp1 protein in PUGNAc-treated cells. In addition, PUGNAc was non-toxic, indicating that at the levels tested it did not have negative effects on cell growth. These results indicate that PUGNAc can be used to inhibit the

![Fig. 2. PUGNAc induces an increase in O-GlcNAc levels in HT29 cells.](http://www.jbc.org/)

Galactosyltransferase-labeled samples from untreated and treated (0 and 40 μM PUGNAc; 24 h, as in Fig. 1A) HT29 cells were analyzed to determine how the [3H]galactose was linked to the protein. A, galactosyltransferase-labeled protein fractions were subjected to PNGase F digestion as described under "Experimental Procedures." The amount of terminal GlcNAc released from (N-glycans) or remaining with (O-glycans) the protein after digestion was quantified. Average values from duplicate samples are shown. The error bars represent the range observed from the duplicate samples. B, PNGase F-resistant material (from A) was subjected to β-elimination to release O-glycans as described under "Experimental Procedures." The released and resistant fractions were separated by gel filtration and quantified. The data are presented as percent of total PNGase F-resistant GlcNAc. Average values from duplicate samples are shown. C, the O-glycans released during the β-elimination (from B) were size-fractionated on a Superdex peptide column as described under "Experimental Procedures" (closed circles, 40 μM PUGNAc; open circles, no PUGNAc). The migration positions of hydrolyzed dextran standards (triangles) are indicated. D, the disaccharides from C were separated by high pH anion exchange chromatography as described under "Experimental Procedures" (closed circles, 40 μM PUGNAc; open circles, no PUGNAc). The migration positions of the following standards are indicated: β-galactose-β1,3-β-N-acetylgalactosaminitol (1), β-galactose-β1,5-β-N-acetylgalactosaminitol (2), and β-galactose-β1,4-β-N-acetylgalactosaminitol (3).
O-GlcNAcase in vivo, resulting in a general increase in O-GlcNAc levels on numerous proteins.

In addition to demonstrating that PUGNAc can be used to increase O-GlcNAc levels on proteins in cells, we have used the inhibitor to test the hypothesis that protein modifications by O-GlcNAc and phosphate are reciprocally related to one another. PUGNAc had no effect on the overall phosphorylation pattern in HT29 cells, indicating that the major phosphoproteins in these cells are not affected by changes in O-GlcNAc levels, perhaps because many of these proteins are not modified with O-GlcNAc. These results also suggest that PUGNAc does not have any global detrimental effect on the protein phosphorylation machinery. In contrast, when we looked specifically at a protein known to be modified with both O-GlcNAc and phosphate (Sp1 (5, 23)), we observed that the increase in O-GlcNAc caused by PUGNAc was accompanied by a decrease in phosphorylation. The decrease in phosphorylation following the PUGNAc treatment was smaller than the corresponding increase in O-GlcNAc levels, indicating that some phosphorylation sites on Sp1 may be unaffected by changes in O-GlcNAc levels. It is likely that there is not complete overlap between the glycosylation and phosphorylation sites. Neither the sites

FIG. 3. PUGNAc causes an increase in O-GlcNAc levels on numerous proteins in HT29 cells. A, galactosyltransferase-labeled samples from HT29 cells treated with increasing concentrations of PUGNAc (as in Fig. 1A) were treated with PNGase F and separated by SDS-PAGE as described under “Experimental Procedures.” The Coomassie Blue stain of the gel is shown on the right, and the fluorograph of the same gel is shown on the left. Molecular masses (in kilodaltons) of standard proteins are shown between the panels. B, selected bands from A (labeled with lowercase letters a–e in A) were quantified by densitometry and plotted versus PUGNAc concentration. Relative intensity (arbitrary units) is shown.

FIG. 4. PUGNAc inhibits O-GlcNAcase but has no effect on O-GlcNAc transferase or UDP-GlcNAc levels in HT29 cells. A, the O-GlcNAcase (closed circles) and O-GlcNAc transferase (open circles) in lysates of HT29 cells were assayed as described under “Experimental Procedures” in the presence of increasing concentrations of PUGNAc. Data are presented as the percent of maximal activity (activity in the absence of PUGNAc). B, UDP-GlcNAc levels were determined in perchloric acid extracts of HT29 cells treated with 0, 20, or 40 μM PUGNAc for 24 h as described under “Experimental Procedures.” For both A and B, each data point represents the average of duplicate samples. The error bars represent the range in the duplicate samples.
of glycosylation nor phosphorylation on Sp1 are known, so we cannot conclusively state that O-GlcNAc and phosphate compete for identical sites, although at least three Class I O-GlcNAc sites (1) are discernible in the sequence (24). These sites could also be modified by proline-directed or mitogen-activated protein kinases. Nonetheless, these results suggest that glycosylation and phosphorylation are in a reciprocal relationship on this particular protein. To our knowledge, this is the first demonstration of altering the level of phosphorylation by increasing the level of O-GlcNAc on a protein in vivo, and it serves as an important step toward understanding the role of O-GlcNAc on nuclear and cytoplasmic proteins in cellular function.

Although the majority of the PUGNAc-induced increase in terminal GlcNAc moieties in cells was due to O-GlcNAc, a small increase in terminal GlcNAc on N-glycans also occurred. Since PUGNAc is a known inhibitor of lysosomal hexosaminidases (25), this is most likely the result of inhibition of lysosomal hexosaminidases and accumulation of N-glycans bearing terminal GlcNAc moieties. Alternatively, PUGNAc could be indirectly affecting the biosynthesis of N-glycans by some unknown mechanism. The fact that PUGNAc had little or no effect on UDP-GlcNAc pools suggests that the effect is not being mediated through alterations in the nucleotide sugar pools in the cell.

The inhibition of the O-GlcNAcase in HT29 cells resulted in an increase in O-GlcNAc levels on essentially all O-GlcNAc-modified proteins (see Fig. 3). This suggests that the O-GlcNAcase which has been purified and characterized (17) is the enzyme responsible for the removal of O-GlcNAc from most proteins in these cells. Alternatively, if other O-GlcNAcase
enzymes exist, PUGNAc may have similar inhibitory effects on these activities as well. Interestingly, the extent of PUGNAc-induced changes on different proteins varied somewhat. The increase in O-GlcNAc on Sp1 was greater than that seen on the proteins analyzed in Fig. 3, suggesting that the rate of O-GlcNAc turnover may differ from protein to protein. Similar increases in O-GlcNAc levels were seen in HT29, NIH-3T3 cells, and CV-1 cells, although much smaller changes were seen in HeLa cells. The reason for differences in the effect of PUGNAc on the HeLa cells is not clear. It may be the result of lower rates of O-GlcNAc turnover in these cells. If the cells express low levels of O-GlcNAcase then will they not be greatly affected by PUGNAc. Tissue searches of the O-GlcNAcase enzyme activity demonstrated variability from tissue to tissue (17), suggesting that the expression levels may vary. Alternatively, PUGNAc may be efficiently excluded from HeLa cells, preventing significant intracellular accumulation of the inhibitor.

Despite the fact that removal of O-GlcNAc from proteins was inhibited, the growth rate of all of the cells examined was unaffected by continued exposure to PUGNAc. This suggests that turnover of O-GlcNAc is not essential for cell growth. Effective inhibitors of the O-GlcNAc transferase will need to be developed before it can be determined how the lack of O-GlcNAc on proteins affects growth. It is somewhat surprising that there is no growth effect, especially in light of the finding that changes in O-GlcNAc also affect phosphorylation of proteins like Sp1. The results suggest that the presence of O-GlcNAc does not inhibit growth but may stimulate growth. Since several lines of O-GlcNAc modification resemble mitogen-activated protein kinase sites, it is possible that the presence of O-GlcNAc or phosphate on a protein at these sites may have a similar growth stimulatory effect. Thus, instead of competition at individual sites, there may be sharing of sites by different systems that are responding to different sets of upstream signals, both resulting in cell growth. Further work comparing the effects of O-GlcNAc to the effects of phosphate on a protein’s function when the modifications occur at the same site needs to be done before such conclusions can be drawn.

Our results on the lack of growth effects are somewhat in conflict with a preliminary report by Snow and co-workers (2, 26). They demonstrated that expression of galactosyltransferase in the cytoplasm of Chinese hamster ovary cells resulted in rapid cell death. The galactosyltransferase put a galactose “cap” on O-GlcNAc moieties in the cytoplasm. They concluded that the galactose prevented turnover of O-GlcNAc, and thus, turnover of O-GlcNAc must be essential for cell viability (26). In view of our data, an alternative interpretation may be that unmodified GlcNAc moieties are essential for cell viability, possibly to allow interaction with specific GlcNAc receptors. Other workers (27) have shown the existence of GlcNAc-binding proteins in nuclear and cytoplasmic compartments. Treatment with PUGNAc causes an accumulation of terminal GlcNAcs and may have no effect on the binding of these GlcNAc-binding proteins. Capping with galactose would “hide” the O-GlcNAcs and prevent these essential interactions resulting in cell death. Alternatively, the galactose capping could cause detrimental conformational changes or protein-protein interactions that result in cell death.

The use of PUGNAc to selectively alter the level of glycosylation of proteins in cells is a valuable new tool for the study of the role of O-GlcNAc on individual proteins. There is a great deal of interest in identifying specific inhibitors of the O-GlcNAc transferase, which will be extremely useful for lowering O-GlcNAc levels in cells for similar types of studies. In combination, these types of inhibitors will be invaluable for assessing the role of O-GlcNAc on individual proteins. Others have shown that O-GlcNAc levels can be reduced by lowering UDP-GlcNAc levels in cells (28). The treatments required to lower UDP-GlcNAc levels are somewhat drastic (either glucose starvation or treatment with nonspecific amidotransferase inhibitors such as 6-diazo-5-oxonorleucine). These treatments will affect all forms of glycosylation containing GlcNAc or GalNAc (N-glycans, mucin-type O-glycans, glycosylphosphatidylinositol anchors, glycosaminoglycans, glycosphingolipids, and O-GlcNAc) and must be interpreted with caution. Nonetheless, intriguing results have been generated with this approach.

Recently, it was shown that the transcriptional activation of certain genes (28) as well as the degradation rate of Sp1 (29) could be dramatically affected by reduced O-GlcNAc levels. As inhibitors such as PUGNAc are used more widely, further insights into the function of O-GlcNAc modification of proteins will be possible.

Acknowledgments—We thank Dr. James Trimmer, Scott Busby, and Daniel Moloney for critical reading of the manuscript and helpful discussions. We also thank Oxford Glycosciences for their generous provision of the PUGNAc, which made this study possible, and Dr. Martin Freundlich (Department of Biochemistry and Cell Biology, SUNY, Stony Brook) for the use of the densitometer. Peptide synthesis was developed before it can be determined how the lack of O-GlcNAc production affects growth. It is somewhat surprising that there is no growth effect, especially in light of the finding that changes in O-GlcNAc also affect phosphorylation of proteins like Sp1. The results suggest that the presence of O-GlcNAc does not inhibit growth but may stimulate growth. Since several lines of O-GlcNAc modification resemble mitogen-activated protein kinase sites, it is possible that the presence of O-GlcNAc or phosphate on a protein at these sites may have a similar growth stimulatory effect. Thus, instead of competition at individual sites, there may be sharing of sites by different systems that are responding to different sets of upstream signals, both resulting in cell growth. Further work comparing the effects of O-GlcNAc to the effects of phosphate on a protein’s function when the modifications occur at the same site needs to be done before such conclusions can be drawn.

Our results on the lack of growth effects are somewhat in conflict with a preliminary report by Snow and co-workers (2, 26). They demonstrated that expression of galactosyltransferase in the cytoplasm of Chinese hamster ovary cells resulted in rapid cell death. The galactosyltransferase put a galactose “cap” on O-GlcNAc moieties in the cytoplasm. They concluded that the galactose prevented turnover of O-GlcNAc, and thus, turnover of O-GlcNAc must be essential for cell viability (26). In view of our data, an alternative interpretation may be that unmodified GlcNAc moieties are essential for cell viability, possibly to allow interaction with specific GlcNAc receptors. Other workers (27) have shown the existence of GlcNAc-binding proteins in nuclear and cytoplasmic compartments. Treatment with PUGNAc causes an accumulation of terminal GlcNAcs and may have no effect on the binding of these GlcNAc-binding proteins. Capping with galactose would “hide” the O-GlcNAcs and prevent these essential interactions resulting in cell death. Alternatively, the galactose capping could cause detrimental conformational changes or protein-protein interactions that result in cell death.

The use of PUGNAc to selectively alter the level of glycosylation of proteins in cells is a valuable new tool for the study of the role of O-GlcNAc on individual proteins. There is a great deal of interest in identifying specific inhibitors of the O-GlcNAc transferase, which will be extremely useful for lowering O-GlcNAc levels in cells for similar types of studies. In combination, these types of inhibitors will be invaluable for assessing the role of O-GlcNAc on individual proteins. Others have shown that O-GlcNAc levels can be reduced by lowering UDP-GlcNAc levels in cells (28). The treatments required to lower UDP-GlcNAc levels are somewhat drastic (either glucose starvation or treatment with nonspecific amidotransferase inhibitors such as 6-diazo-5-oxonorleucine). These treatments will affect all forms of glycosylation containing GlcNAc or GalNAc (N-glycans, mucin-type O-glycans, glycosylphosphatidylinositol anchors, glycosaminoglycans, glycosphingolipids, and O-GlcNAc) and must be interpreted with caution. Nonetheless, intriguing results have been generated with this approach.

Recently, it was shown that the transcriptional activation of certain genes (28) as well as the degradation rate of Sp1 (29) could be dramatically affected by reduced O-GlcNAc levels. As inhibitors such as PUGNAc are used more widely, further insights into the function of O-GlcNAc modification of proteins will be possible.
Modulation of O-LinkedN-Acetylglucosamine Levels on Nuclear and Cytoplasmic Proteins in Vivo Using the PeptideO-GlcNAc-β-N-acetylglucosaminidase InhibitorO-(2-Acetamido-2-deoxy-dglucopyranosylidene)amino-N-phenylcarbamate

Robert S. Haltiwanger, Kathleen Grove and Glenn A. Philipsberg

J. Biol. Chem. 1998, 273:3611-3617.
doi: 10.1074/jbc.273.6.3611

Access the most updated version of this article at http://www.jbc.org/content/273/6/3611

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 15 of which can be accessed free at http://www.jbc.org/content/273/6/3611.full.html#ref-list-1