Mitotic Arrest with Nocodazole Induces Selective Changes in the Level of O-Linked N-Acetylgalcosamine and Accumulation of Incompletely Processed N-Glycans on Proteins from HT29 Cells*

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O-Linked N-acetylgalcosamine (O-GlcNAc) is a ubiquitous and abundant protein modification found on nuclear and cytoplasmic proteins. Several lines of evidence suggest that it is a highly dynamic modification and that the levels of this sugar on proteins may be regulated. Previous workers (Chou, C. F., and Omary, M. B. (1993) J. Biol. Chem. 268, 4465–4472) have shown that mitotic arrest with microtubule destabilizing agents such as nocodazole causes an increase in the O-GlcNAc levels on keratins in the human colon cancer cell line HT29. We have sought to determine whether this increase in glycosylation is a general (i.e. occurring on many proteins) or a limited (i.e. occurring only on the keratins) process. A general increase would suggest that the microtubule destabilizing agents were somehow affecting the enzymes responsible for addition and/or removal of O-GlcNAc. Our results suggest that the changes in O-GlcNAc induced by nocodazole are selective for the keratins. The levels of O-GlcNAc on other proteins, including the nuclear pore protein p62 and the transcription factor Sp1, are not significantly affected by this treatment. In agreement with these findings, nocodazole treatment caused no change in the activity of the enzymes responsible for addition or removal of O-GlcNAc as determined by direct enzyme assay; UDP-HexNAc, uridine diphospho-N-acetylglucosamine 2-epimerase (10); PNGase F, peptide N-glycosidase F; PAGE, polyacrylamide gel electrophoresis; RIPA, radioimmune precipitation assay; UDP-HexNac, uridine diphospho-N-acetylhexosamine.

bearing this modification have been identified to date, no example of a specific function for this modification on any of these proteins has yet been demonstrated. Nonetheless, these proteins share some common features that may suggest a role. Significantly, most of these proteins are also known to be phosphoproteins (1–3). Since both phosphate and GlcNAc modify the hydroxyl groups of serines and threonines, it has been proposed that the sugar may function by blocking sites of phosphorylation (4). Several recent reports demonstrating competition between glycosylation and phosphorylation on individual proteins have added support to this concept (5, 6). Modifying a protein with a neutral sugar such as O-GlcNAc could have significantly different effects on the protein compared with phosphorylation. Thus, O-GlcNAc modification of proteins may add an additional level of control to protein phosphorylation by constitutively blocking phosphorylation sites.

In addition to competing with phosphate, several lines of evidence suggest that O-GlcNAc may be a regulated modification much like phosphorylation. For example, in several cases, O-GlcNAc has been shown to turn over more rapidly than the protein it modifies (7, 8). This suggests that the sugar is dynamically added to and removed from proteins during their lifetimes. Enzymes capable of addition and removal of the sugar from proteins have been purified and characterized (9, 10). They exist as large multisubunit soluble proteins in the cytoplasm of cells. Thus, a system capable of regulated addition and removal of the sugar exists. In addition to simply turning over more rapidly than the protein, changes in the levels of glycosylation induced by various stimuli have been observed in two separate systems. Kearse and Hart (11) showed that mitotic activation of either isolated T lymphocytes or T-cell hybridomas resulted in rapid (<1 h) changes in the level of glycosylation of several nuclear and cytoplasmic proteins. Subsequently, Chou and Omary (12, 13) demonstrated that mitotic arrest of a human colon cancer cell line (HT29) with okadaic acid or microtubule destabilizing agents (such as nocodazole) resulted in an increase in glycosylation of keratins 8 and 18. In addition, Chou and Omary (13) observed increased glycosylation of proteins in a crude nuclear extract as well as on several known plasma membrane proteins. The increase in terminal GlcNAc residues of the plasma membrane proteins could be mimicked by brefeldin A, a Golgi disrupter, suggesting that these changes were on extracellular glycosidic, not O-GlcNAc. The brefeldin A treatment had no effect on the glycosylation of keratins 8 and 18. Nonetheless, the increase in terminal GlcNAc residues on proteins from the nuclear extracts of these cells suggested that nocodazole may cause a general increase in the levels of O-GlcNAc on proteins in these cells.

We have sought to determine whether the increase in glycosylation of the keratins in HT29 cells induced by nocodazole is a selective or a general event. If there is an induction of an
O-GlcNAc-transferase or an inhibition of an O-GlcNAcase by a stimulus such as nocardazole, one would expect to see changes in the level of glycosylation of several proteins. This appears to be what was observed by Kearse and Hart (11) in mitogenically activated T lymphocytes. We have examined the glycosylation of whole cell extracts as well as several specific proteins (keratins, a nuclear pore protein, and the transcription factor Sp1) in nocardazole-treated HT29 cells. Our data suggest that the change in glycosylation of the keratins is a selective event. We saw no major change in O-GlcNAc levels on the other proteins we examined. In agreement with Chou and Omary (12, 13), we saw a generalized increase in terminal GlcNAc residues on numerous proteins upon nocardazole treatment. We have demonstrated that this increase is largely due to an accumulation of incompletely processed N-glycans in nocardazole-treated cells.

EXPERIMENTAL PROCEDURES

Materials—Nocardazole, bovine milk galactosyltransferase, protein G-agarose, and ovalbumin were from Sigma. Galactosyltransferase was autogalactosylated prior to use as described (14). Peptide N-glycosidase F (PNGase F) was purified from culture filtrate of Flavobacterium menengetispticum as described (15). Anti-keratin monoclonal antibody (L2A1) acitc fluid was generously provided by Dr. Bishr Omary (Stanford University). (16). Anti-nuclear pore monoclonal antibody (414) acites fluid was obtained from Babco (Berkeley, CA), and an isotype-matched control IgG (ascites fluid) was obtained from Sigma (mineral oil plasmacytoma 21). Rabbit anti-human Sp1 polyclonal antibody (PEP2) and competing peptide were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The human colon cancer cell line HT29 was obtained from the American Type Culture Collection (Rockville, MD). Dubecco's modified Eagle's medium, fetal bovine serum, penicillin/streptomycin, and trypsin/EDTA were obtained from Life Technologies, Inc. (Grand Island, NY). The human colon cancer cell line HT29 was obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin/streptomycin, and trypsin/EDTA were obtained from Life Technologies, Inc. (West Grove, PA). The human colon cancer cell line HT29 was obtained from the American Type Culture Collection (Rockville, MD).

Cell Culture and Nocodazole Treatments—HT29 cells were maintained in a humidified incubator with 5% CO2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin/streptomycin, and trypsin/EDTA. The cells were washed three times with cold Tris-buffered saline, and frozen at −80 °C until use.

Galactosyltransferase Labeling of Whole Cell Extracts—All galactosyltransferase labelings were performed as modifications of described procedures (14). Cell pellets prepared as described above were extracted with hot 1% SDS (0.5 ml/107 cells) for 5 min at 100 °C. Chromatin was sheared by repeated passage of the extracts through a 25-gauge needle. Insoluble material was removed by centrifugation at 10,000 × g for 10 min at 4 °C. Equivalent amounts of protein (100 μg) of the extracts were labeled with galactosyltransferase and UDP-[6-3H]galactose as described (14). Insoluble material was removed by centrifugation at 10,000 × g for 10 min at 4 °C. All immunoprecipitations were performed on the protein fraction as described above. The labeling was stopped by the addition of 25 μl EDTA, and the keratins were immunoprecipitated from the labeling mixture using monoclonal antibody L2A1 and protein G-agarose as described (18).

Galactosyltransferase Labeling of Nuclear Pore Proteins and Sp1—For galactosyltransferase labeling of nuclear pore proteins and Sp1, cells were extracted with RIPA buffer (50 mM Tris, pH 8.0, 0.15 M NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40) containing Pic 1 and Pic 2 for 30 min on ice. Aggregates were broken by passage of the lysates through a 25-gauge needle several times. Insoluble material was removed by centrifugation at 10,000 × g for 10 min at 4 °C. All immunoprecipitations were performed on the protein fraction as described above. The labeling was stopped by the addition of 25 μl EDTA, and the keratins were immunoprecipitated from the labeling mixture using monoclonal antibody L2A1 and protein G-agarose as described (18).

Carbohydrate Analysis—PNGase F digests of galactosyltransferase-labeled protein fractions were performed as described (19). The released glycans were separated by cellulose column chromatography and analyzed by SDS-PAGE and fluorography.

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as described by Chou and Omary (12), nocodazole induces a decrease in the level of the protein. We also saw an increase in the labeling of a protein that coprecipitates with the keratins. This protein is likely an N-glycosylated protein called KAP85 (13). Thus, as described by Chou and Omary (12), nocodazole induces a dramatic increase in the level of glycosylation of keratin 18.

**Nocodazole Induces a Significant Increase in Terminal GlcNAc Residues on Numerous Proteins in HT29 Cells**—To determine whether the effect of nocodazole on the keratins was a general or a selective event, we examined the glycosylation of proteins from crude lysates of HT29 cells. Lysates of HT29 cells were galactosyltransferase-labeled exactly as described above except that no immunoprecipitation was performed. A dramatic increase in the labeling of numerous protein species occurred between the 8- and 24-h time points (Fig. 2A), coincident with the increase in the glycosylation of keratin 18 shown in Fig. 1. During this same period, no significant changes were seen in the levels of the proteins themselves (Fig. 2B). These results demonstrate that mitotic arrest with nocodazole induces a large increase in terminal GlcNAc residues on numerous proteins from HT29 cells. Interestingly, the labeling of at least one protein decreased upon nocodazole treatment (Fig. 2A, asterisk). We believe that this decrease is due to a decrease in the solubility of this protein under the extraction conditions used (phosphate-buffered saline and 0.5% Nonidet P-40). The decrease is no longer observed when cell extracts are made with either RIPA buffer or 1% SDS (see Fig. 4). For this reason, subsequent lysates were all made with either RIPA buffer or 1% SDS.

**Increase in Terminal GlcNAc Residues Is Due to N-Glycans and Not O-GlcNAc**—Chou and Omary (13) had demonstrated that nocodazole could induce an increase in the level of terminal GlcNAc residues on cytokeratins, proteins in nuclear extracts, and extracellular proteins. Since the changes in GlcNAc on extracellular proteins were likely to result from changes in N-glycans, we sought to determine whether the increase in terminal GlcNAc residues shown in Fig. 2 was due to changes in O-linked and/or N-linked GlcNAc. We used the enzyme PNGase F to differentiate between GlcNAc linked to proteins through N-glycans or other means. In untreated cells, essentially all of the radiolabel was resistant to PNGase F digestion (Fig. 3A, compare 0 h — PNGase F with 0 h + PNGase F), implying that the majority of the GlcNAc residues are O-linked. In contrast, the nocodazole-treated samples showed significant sensitivity to the PNGase F digestion (Fig. 3A,
compare 32 h - PNGase F with 32 h + PNGase F). In fact, the increase in galactose incorporation caused by the nocodazole treatment could be completely accounted for by the increase in PNGase F-sensitive (i.e. N-linked) material. Thus, nocodazole appeared to induce an increase in terminal GlcNAc residues on N-glycans in these cells, but it caused no significant changes in the PNGase F-resistant (i.e. O-linked) material.

To confirm that the labeled sugars remaining on the proteins after PNGase F digestion were in the form of O-GlcNAc, the PNGase F-resistant material from both the control and nocodazole-treated samples was subjected to alkali-induced \(\beta\)-elimination. This caused the majority (>90%) of the radioactivity to be released from the protein fraction (Fig. 3B), indicating that the PNGase F-resistant radioactivity was O-linked to the protein. To determine the structure of the material released by the \(\beta\)-elimination, the samples were subjected to size (Fig. 3C) and high pressure anion-exchange chromatography analyses. These analyses demonstrated that the released sugars were essentially all in the form of [\(\text{H}^3\)]galactose-\(\beta\)1,3-\(\text{N}\)-acetylglucosaminitol (1), \(\text{H}^3\)galactose-\(\beta\)1,3-\(\text{N}\)-acetylgalactosaminitol (2), and \(\text{H}^3\)galactose-\(\beta\)1,4-\(\text{N}\)-acetylgalcosaminitol (3).

To examine whether we could see changes in the level of O-GlcNAc on proteins in response to nocodazole treatment, we examined the pattern of O-GlcNAc-modified proteins from control and nocodazole-treated cells by SDS-PAGE and fluorography after PNGase F treatment (Fig. 4A). As shown above, a large increase in PNGase F-sensitive terminal GlcNAc residues was induced by the nocodazole treatment. In contrast, the pattern of the PNGase F-resistant species (which represent the O-GlcNAc-labeled species as demonstrated above) did not change dramatically upon nocodazole treatment. Some minor changes could be seen on the gel, but the overall pattern was unchanged. Likewise, the overall total protein pattern was largely unchanged by nocodazole treatment (Fig. 4B). Thus, it appears that nocodazole does not induce major changes in the levels of glycosylation on the more abundant O-GlcNAc-bearing proteins (i.e. those that can be detected in a crude cell lysate) in HT29 cells. We can conclude from these data that global changes in the pattern of O-GlcNAc-modified proteins like those observed by Kearse and Hart (11) in mitogenically acti-
Selective Changes in O-GlcNAc

Nocodazole Treatment Has Little or No Effect on the Glycosylation of the Nuclear Pore Protein p62 or the Transcription Factor Sp1.—To more carefully examine changes in the glycosylation of specific O-GlcNAc-modified proteins other than the keratins, we performed galactosyltransferase labelings of the nuclear pore protein p62 and the transcription factor Sp1 from control and nocodazole-treated cells. Both of these proteins are known to be modified with O-GlcNAc (24–26). The nuclear pore proteins are among the most abundant and heavily O-GlcNAc-modified proteins in mammalian cells (24). Thus, they serve as a good marker of the level of O-GlcNAc from control and nocodazole-treated HT29 cells. The immunoprecipitates were then labeled with galactosyltransferase to detect O-GlcNAc. As can be seen in Fig. 5A, p62 labeled quite well using this technique. The presence or absence of nocodazole had very little effect on the level of its glycosylation. The levels of the p62 protein were monitored by Western blot analysis with monoclonal antibody 414 (Fig. 5B). If anything, this analysis showed that nocodazole induces a slight increase in the amount of the p62 protein. Thus, there does not appear to be an increase in the sugar/protein ratio on p62. We also examined the glycosylation of the transcription factor Sp1 using the same procedure. Sp1 exists as two major molecular mass species in cells (95 and 105 kDa), both of which have been shown to be glycosylated (26). Both species were present in untreated HT29 cells (Fig. 5D), and both appeared to be glycosylated (Fig. 5C). Nocodazole treatment caused an accumulation of the 105-kDa form (Fig. 5, C and D), but the overall level of glycosylation on the protein did not increase. Taken together, these data suggest that the changes seen in glycosylation of the keratins are selective in that similar changes do not occur on all O-GlcNAc-modified proteins in the cell.

Nocodazole Treatment Has No Effect on the O-GlcNAc Glycosylation Machinery.—In addition to examining the level of O-GlcNAc on proteins in nocodazole-treated cells, we assayed for changes in the activity levels of the enzymes responsible for the addition (O-GlcNAc-transferase) or removal (O-GlcNAcase) of O-GlcNAc. Changes in the activity of these enzymes would be indicative of a general effect of nocodazole on glycosylation. We have modified the standard assay for both of these enzymes so that they can be detected in crude extracts of ~10^7 cells (see “Experimental Procedures”). Using these modified assays on extracts of HT29 cells treated for increased times with nocodazole, we saw no significant changes in activity that correlate with the increase in glycosylation seen on the keratins (Fig. 6, A and B). In addition, since UDP-GlcNAc is a substrate for O-GlcNAc-transferase and could be limiting, we examined the levels of UDP-GlcNAc over the course of nocodazole treatment (Fig. 6C). The technique used for analysis of UDP-GlcNAc was not capable of resolving UDP-GlcNAc from UDP-GalNAc (21). Thus, the data are shown as the total UDP-HexNAc levels. The ratio of UDP-GlcNAc to UDP-GalNAc in a cell is determined by an epimerase capable of interconverting the two, and a relatively constant ratio of ~2:1 (UDP-GlcNAc/UDP-GalNAc) is found in most cells (see “Experimental Procedures”). A dramatic increase in UDP-HexNAc levels was observed during the latter time points of the nocodazole treatment, but these changes occurred much later than the observed increase in O-GlcNAc levels on the keratins. Thus, we could detect no gross changes in the glycosylation machinery responsible for the addition and/or removal of O-GlcNAc from proteins. These data are consistent with the observations discussed above and suggest that the change in glycosylation of the keratins induced by nocodazole is a selective phenomenon.

**DISCUSSION**

The modulation of post-translational modifications such as phosphate groups on proteins is well documented. The level of phosphate on proteins is controlled by the relative activity of the enzymes responsible for addition (kinases) and removal (phosphatases) of the phosphate. One implication of such systems is that stimuli that activate or inhibit these enzymes can cause changes in modification of numerous target proteins. For example, stimulation of cAMP-dependent protein kinase with cAMP results in phosphorylation of numerous target proteins. Thus, if nocodazole were a modulator of a system controlling O-GlcNAc levels, one would predict that the levels of glycosylation on numerous proteins would change. Since nocodazole is a microtubule-stabilizing agent that arrests the cell at the G2/M interface of the cell cycle, such an effect could be associated with cell cycle-induced changes. The goal of this report was to test this prediction and to determine whether such a system is operating in HT29 cells with nocodazole as a stimulator.

In this report, we have shown that nocodazole causes a marked increase in the level of O-GlcNAc on keratin 18, as was originally reported by Chou and Omary (12). In contrast, analysis of O-GlcNAc levels on other proteins in the nocodazole-treated cells showed few, if any, changes. Consistent with this
observation is the fact that we saw no changes in the glycosylation machinery (O-GlcNAc-transferase, O-GlcNAc'ase, and UDP-GlcNAc levels) induced by nocodazole that would explain the increase in glycosylation of keratin 18. Thus, nocodazole appears to induce an increase in glycosylation of keratin 18 selectively. This implies that the glycosylation machinery is not activated by nocodazole treatment and that the increase in glycosylation of keratin 18 must result from a change in the keratins themselves.

Since nocodazole causes a reorganization of the cytoskeletal components, including the intermediate filaments (12), the simplest explanation for the change in glycosylation of the keratins is that it is related to this reorganization. Omary’s group (12, 13) has done extensive work showing that agents that cause reorganization of intermediate filaments in HT29 cells result in dramatic increases in both the glycosylation and phosphorylation of the keratins. In addition to the changes induced by inhibitors that arrest the cell cycle at the G₂/M interface (okadaic acid and microtubule-destabilizing agents such as nocodazole and colcemid) (12, 13), Omary and co-workers (27) have shown that heat shock causes a rapid increase in the glycosylation and phosphorylation of keratins 8 and 18. These results suggest that the changes in phosphorylation and/or glycosylation are related to the reorganization. Mutation of a major phosphorylation site in keratin 18 does not alter intermediate filament assembly, but it does interfere with filament reorganization of keratins 8 and 18 induced by G₂/M arrest in transfected cells (29). Thus, phosphorylation appears to play an active role in filament reorganization in HT29 cells. A similar role for the glycosylation of the keratins is not yet clear. Mutation of the major glycosylation sites of keratin 18 had no effects on filament assembly in transfected cells (29), although the effects of the mutation on reorganization induced by G₂/M arrest have not been examined. Recent studies have demonstrated that mutation of a conserved arginine in keratin 18 is sufficient to induce a significant reorganization of intermediate filament structures. The glycosylation and phosphorylation of this mutated keratin 18 are significantly increased compared with the wild-type protein (30). The increase in glycosylation observed on this keratin 18 bearing a point mutation strongly suggests that the change in glycosylation is a result of intermediate filament reorganization, and not a cause. Such a conclusion is consistent with our findings that the increase in glycosylation caused by nocodazole is apparently selective for the keratins. Further work will be needed to conclusively determine the role glycosylation plays in intermediate filament reorganization. Interestingly, not all treatments that cause reorganization of intermediate filaments have the same effects. Rotavirus infection of HT29 cells, which causes intermediate filament reorganization, results in an increase in phosphorylation of keratins 8 and 18, but no change in their glycosylation (27). Thus, the signals that result in changes in glycosylation or phosphorylation of the keratins appear to be distinct.

The most dramatic change in glycosylation induced by nocodazole is the increase in terminal GlcNAc residues on N-glycans. Other workers have shown that short-term treatments with nocodazole (e.g., 1–3 h) have no effect on the movement or glycosylation of proteins through the Golgi apparatus (31). Microtubules are believed to be involved in retrograde transport of proteins from the Golgi apparatus to the endoplasmic reticulum (32), and nocodazole has been used to inhibit this transport. The effects of nocodazole on retrograde transport are also seen after fairly short treatments (<3 h). We see no effect on glycosylation until after 8 h (see Fig. 2A). Thus, the effects we see are apparently not related to the short-term effects of microtubule disassembly, but are more likely related to arrest of the cells at the G₂/M interface. Most vesicular traffic in cells, including endoplasmic reticulum to Golgi apparatus and intra-Golgi traffic, has been shown to be interrupted during mitosis (33). The Golgi apparatus fragments, assisting in even distribution of the Golgi apparatus between the two daughter cells. Thus, the accumulation of the terminal GlcNAc residues on N-glycans that we see is most likely a result of the mitosis-induced interruption of medial to trans-Golgi transport. Since galactosyltransferase is a trans-Golgi enzyme (34), lack of medial to trans-Golgi movement would result in an accumulation of unmodified GlcNAc residues. The accumulation we see may suggest that there is a slight time delay between interruption of intra-Golgi transport (medial to trans) and that of endoplasmic...
mic reticulum to Golgi transport. The mechanism of Golgi fragmentation, as well as the rationale for why transport stops at mitosis, is not well understood (33). Several workers have suggested that Golgi vesiculation may result from the normal process of vesicular budding from Golgi stacks coupled with an inhibition of vesicle fusion. Thus, vesicles form but are unable to fuse with the next stack. The fact that terminal GlcNAc moieties on N-glycans increase under these conditions may serve as a useful assay to follow this process and allow for a better understanding of the underlying mechanisms.

The results presented in this paper suggest several important factors that must be kept in mind when analyzing changes in the levels of O-GlcNAc on proteins. Galactosyltransferase is still the best method available for analyzing O-GlcNAc levels, and PNGase F digests must be performed on each labeling to assess the contribution of N-glycans to the final pattern. If we had analyzed only the galactosyltransferase-labeled samples from the untreated cells with PNGase F, we would have concluded that >90% of the galactose incorporated was on O-GlcNAc and that PNGase F controls were unnecessary in this cell type. In contrast, the nocodazole treatment caused a dramatic change in galactose labeling of N-glycans. In addition, this study underscores the importance of following protein levels as well as sugar levels before conclusions about changes in glycosylation of any protein can be made. Initial studies with Nonidet P-40 lysates of HT29 cells showed an apparent decrease in glycosylation of some proteins (see Fig. 2). We believe that the decrease has to do with changes in the solubility of proteins induced by the nocodazole treatment of the cells, and not with true changes in the level of glycosylation. Once the change in the levels of the proteins was abrogated by a more efficient extraction technique (e.g. RIPA buffer or 1% SDS), the changes in the glycosylation level disappeared. Thus, in future studies on the levels of O-GlcNAc on proteins, it will be essential to control for labeling of N-glycans with PNGase F and to closely follow the level of the proteins being examined as well as the level of the sugar on them.

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