Journal of Cell Science 107, 1833-1843 (1994)
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# Mitotic arrest with anti-microtubule agents or okadaic acid is associated with increased glycoprotein terminal GlcNAc's

**Chih-Fong Chou* and M. Bishr Omary†**

Palo Alto Veterans Administration Medical Center, and the Digestive Diseases Center, Stanford University School of Medicine, 3801 Miranda Avenue, 154J, Palo Alto, CA 94304, USA

*Present address: Institute of Molecular and Cell Biology, National University of Singapore, Singapore 0511
†Author for correspondence

## SUMMARY

**The two major intermediate filament glycoproteins in human simple epithelia are keratins 8 and 18 (K8/18). A dramatic increase in terminal N-acetylglucosamine (GlcNAc) residues in K8/18 was previously noted after arresting cells in $G_2$/M using anti-microtubule agents. Here we use in vitro galactosylation to show that increased terminal GlcNAc's is a general phenomenon that occurs in glycoproteins isolated from nuclear and plasma membrane fractions after cells are arrested in mitosis using colcemid, nocodazole, or okadaic acid. All three agents also resulted in a hyperphosphorylated form of K8 as determined by phosphatase treatment and tryptic phosphopeptide mapping. The altered glycosylation was found to be independent of microtubule disassembly, and was not directly related to the $G_2$/M phase of the cell cycle after aphidicolin synchronization. Staurosporine (1 μM) inhibited K8/18 phosphorylation in okadaic acid- or nocodazole-treated cells, and inhibited the increase in K8/18 glycosylation without inhibiting the increase in terminal GlcNAc's of membrane-associated glycoproteins. In contrast, brefeldin A resulted in a dramatic increase in terminal GlcNAc's of membrane-associated but not intermediate filament proteins. Golgi complex-related staining using anti-β-COP antibody showed significant fragmentation under conditions associated with altered membrane protein glycosylation. Our results suggest that Golgi disruption may be involved in the observed increase in terminal GlcNAc's of membrane but not intermediate filament glycoproteins. The mechanism of increased glycoprotein terminal GlcNAc's in association with mitotic arrest appears to be distinct for intermediate filaments and membrane-associated proteins, and in the case of intermediate filament proteins, phosphorylation may play an important role. Some of the effects of agents that induce mitotic arrest may be mediated by glycosylation changes.**

Key words: mitotic arrest, glycosylation, keratin phosphorylation, okadaic acid, colcemid, nocodazole

## INTRODUCTION

Keratins, the intermediate filaments (IFs) of epithelial cells, are a multigene family that consists of at least 21 catalogued proteins (keratins 1-21(K1-21); Moll et al., 1982, 1990; Quaroni et al., 1991). They exist in cells as obligate heteropolymers such that all keratin-expressing cells have at least one type II (K1-K8) and one type I (K9-21) keratin that are expressed in a tissue-specific manner. For example, 'simple' glandular-type epithelia express the K8/18 pair predominantly. The K8/18 pair undergo serine phosphorylation (Gilmartin et al., 1984; Tolle et al., 1987; Omary et al., 1992a) and serine/threonine glycosylation that consists of single O-linked *N*-acetylglucosamine (GlcNAc) residues (Chou et al., 1992). Mitotic arrest of cells in $G_2$/M, using the anti-microtubule (anti-MT) agents colcemid or nocodazole (NO), results in a dramatic increase in K8/18 phosphorylation and glycosylation although an anti-MT versus a cell cycle-specific effect could not be distinguished (Chou and Omary, 1993).

Our recent identification of an 85 kDa keratin-associated protein (KAP, termed KAP85) that contains high mannose and complex oligosaccharides and localizes to a plasma membrane-associated compartment (Chou et al., 1994), suggested to us that alterations in GlcNAc residues may be a generalized phenomenon that occurs in association with anti-MT agents and/or with mitotic arrest. This suggestion was based on the strong in vitro labeling of KAP85 using UDP[$^3$H]galactose and galactosyltransferase, after isolation of the protein from colcemid or NO-arrested $G_2$/M cells. This galactosylation labeling method attaches a [$^3$H]galactose to accessible terminal GlcNAc moieties.

The organizational changes that occur in Golgi network in the presence of anti-MT agents (reviewed in Thyberg and Moskalewski, 1985) may have an effect on protein glycosylation and/or sorting. For example, in the presence of colchicine, the incorporation of [$^3$H]fucose-labeled glycoproteins into rat small intestinal villus cells was markedly inhibited in the microvillus membrane and was increased in the basolateral

membrane (Quaroni et al., 1979). This was supported by studies in the polarized epithelial cell line Caco-2, which showed that NO drastically retarded the direct apical and basolateral-to-apical transcytosis without having a significant effect on basolateral sorting (Matter et al., 1990), and interfered with apical secretion in the same cell line (Eilers et al., 1989). Similarly, colchicine strongly inhibited the discharge of albumin into plasma in rats but increased its secretion into bile (Saucan and Palade, 1992). However, anti-MT agents do not alter the incorporation of galactose and sialic acid into serum proteins (Banerjee et al., 1976), and do not alter the movement of newly synthesized glycoproteins between the Golgi apparatus and the cell surface in non-polarized cells (Stults et al., 1989).

The use of anti-MT agents to arrest cells at prometaphase is a tool that is commonly used to study mitosis-related cellular events (for review see Rieder and Palazzo, 1992). Arrest of cells at $G_2$/M can also be achieved using okadaic acid (OA), a phosphatase type 1 and 2A inhibitor (Bialojan and Takai, 1988), as described for the two myeloid leukemia cell lines HL-60 and U937 (Ishida et al., 1992). The effect of OA was concentration-dependent such that high concentrations (500 nM range) arrested cells at $G_1$/S whereas low concentrations (2-8 nM) arrested cells at $G_2$/M. In this report, we used two independent methods of mitotic arrest, namely anti-MT agents and OA, to compare IF phosphorylation and glycosylation in mitotically arrested cells with $G_2$/M-enriched cells obtained by aphidicolin synchronization. We show that, in contrast with aphidicolin-synchronized $G_2$/M cells, mitotic arrest with anti-MT agents or OA results in an increase in the number of terminal GlcNAc's of K8/18 intermediate filaments and of several plasma membrane-associated proteins in a microtubule-independent fashion. Hence, significant glycosylation changes appear to be a biochemical hallmark of mitotic arrest, and may underlie some of the effects of mitotic arrest-inducing agents. Although K8/18 phosphorylation was increased in both mitotically arrested and aphidicolin-synchronized mitotically enriched cells, a distinct hyperphosphorylated form of K8 was seen only in the mitotically arrested cells. Staurosporine, a kinase inhibitor (Tamaoki et al., 1986), abrogated the formation of the hyperphosphorylated form of K8 in the presence of NO and also inhibited the increased K8/18 glycosylation without altering the increase in KAP85 in vitro galactosylation. This suggests that phosphorylation may play a role in the altered glycosylation of IF but not membrane proteins in association with mitotic arrest. Potential mechanisms involved in the mitotic arrest-associated increase in terminal GlcNAc's of membrane proteins and intermediate filaments are discussed.

## MATERIALS AND METHODS

### Reagents and antibodies

Tissue culture cell lines were purchased from the American Type Culture Collection (Rockville, MD). Uridine diphosphate (UDP) [4-5-$^3$H]galactose (32.1Ci/mmol), carrier-free $H_3{}^{32}PO_4$, and ENTENSIFY™ autoradiography enhancer were purchased from DuPont-New England Nuclear (Wilmington, DE). Other reagents used were: bovine milk galactosyltransferase, aphidicolin, colcemid, nocodazole, staurosporine, goat anti-mouse IgG Sepharose conjugate, anti-β-tubulin clone TUB 2.1 antibody (Sigma); okadaic acid (L-C Services, Woburn, MA); brefeldin A (Epicentre Technologies, Madison, WI); potato acid phosphatase (Boehringer Mannheim, Indianapolis, IN). Monoclonal antibodies used were: L2A1, which recognizes human K8/18 (Chou and Omary, 1993); B3/25 and I4D4, which recognize the transferrin receptor (TrR) (Trowbridge and Omary, 1981) and the KS-1 antigen (Omary et al., 1992b), respectively. Rabbit anti-Golgi-associated β-COP protein was kindly provided by Dr S. Pfeffer.

### Cell culture, synchronization and mitotic arrest

The tissue culture cell lines HT29 (human colon) and HeLa (human cervix) were grown at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were used during asynchronous near-confluent growth (primarily $G_0/G_1$ cells, referred to as G cells) or were arrested at the $G_2$/M stage of the cell cycle (M cells) using nocodazole (NO) or colcemid (0.5 μg/ml) for 24 hours and 36 hours, respectively. Optimal enrichment of $G_2$/M-arrested cells was obtained when the anti-microtubule agents were added to cells at 30-50% confluency. Alternatively, cells were arrested at $G_2$/M using okadaic acid (6 nM, 48 hours). The percentage of $G_2$/M cells was determined by cell cycle analysis after 70% ethanol fixation then propidium iodide staining as described (Chou and Omary, 1993). Synchronization of cells at the $G_1$/S boundary was carried out by incubating near-confluent HT29 cells with aphidicolin (5 μg/ml, 24 hours), a DNA polymerase alpha inhibitor (Pedrali-Noy et al., 1980). After synchronization, cells from each dish were split 1:3 then incubated with nocodazole or staurosporine (1 μM) plus nocodazole, or chased with normal medium for different time points to enrich for cells at different stages of the cell cycle.

### Immunoprecipitation

Cells ($2\times10^6$) were solubilized (40 minutes, 4°C) in 300 μl of 1% NP40 (after in vivo $^{32}$P-labeling), or DNS (1% deoxycholate, 1% NP40, 0.1% SDS) (for in vitro $^3$H-galactosylation) in PBS. A 26G needle-syringe was used to shear the DNS-solubilized material. All solubilization solutions also contained 5 mM sodium pyrophosphate, 50 mM NaF and 0.1 μg/ml okadaic acid (phosphatase inhibitors); and 0.1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 10 μM pepstatin, 10 μM leupeptin and 25 μg/ml aprotinin. After centrifugation (16,000 ***g***, 5 minutes), 150 μl of solubilized lysate, 2 μl of monoclonal antibody (mAb) ascites (non-immune, L2A1, B3/25, or I4D4) were incubated (45 minutes, 4°C with rocking). Immune complexes were collected using Protein A conjugated to Sepharose that had been precoated with rabbit anti-mouse Ig. Alternatively, mAb L2A1 or I4D4 conjugated to agarose beads were used to directly immunopurify K8/18 and the KS-1 antigen, respectively. Immunoprecipitates were analyzed using SDS-PAGE (Laemmli, 1970) under non-reducing conditions, except for TrR immunoprecipitates, which were analyzed under reducing conditions due to the near overlap of the TrR and immunoglobulin used for immunoprecipitation under non-reducing conditions (not shown). $^3$H-containing gels were processed for fluorography using ENTENSIFY™ as recommended by the manufacturer.

### Peptide mapping

Tryptic peptide mapping of $^{32}$P-labeled K8 and hyperphosphorylated K8(HK8) was carried out as described (Chou et al., 1992). K8 and HK8 were isolated by cutting out the individual bands from preparative SDS-PAGE gels of K8/18 immunoprecipitates, obtained from $^{32}PO_4$-labeled $G_2$/M-arrested HT29 cells. Proteins were electroeluted from the cut Coomassie-stained bands (Amicon electrolocution apparatus, Davers, MA) followed by concentration, acetone precipitation then trypsinization.

### Immunofluorescence

HT29 cells were grown on 8-well chamber slides (Nunc, Roskilde,

Denmark) then aphidicolin, nocodazole plus aphidicolin, or nocodazole were added to different chambers for 48 hours. Cells were then rinsed with prewarmed PBS (37°C) then fixed by dipping in –20°C methanol (5 minutes). After rinsing, anti-microtubule antibody was added (30 minutes, 22°C) followed by washing then incubating with Texas Red-conjugated goat anti-mouse antibody. For Golgi staining, cells were fixed in 1% paraformaldehyde in PBS (15 minutes, 22°C), rinsed with PBS then blocked with 0.1 M glycine in PBS (5 minutes, 22°C). Cells were then permeabilized with 0.1% Triton X-100 (TX-100) (15 minutes, 22°C) then blocked with PBS containing 0.1% TX-100, 2% normal rabbit serum. Rabbit anti-βCOP antibody was used at 1:400 (30 minutes) followed by incubating with fluorescein-labeled goat anti-rabbit antibody. Photographs were taken using black and white Kodak T-MAX 400 film.

### Subcellular fractionation

Subcellular fractionation was carried out using differential centrifugation (Omary and Trowbridge, 1980). Cells were suspended in 10 mM HEPES, pH 7.3, 130 mM KC1, 5 mM EDTA, 5 mM NaC1 then homogenized by nitrogen cavitation (150 lbf/in$^2$, 5 minutes, 4°C), which disrupts >99% of cells while leaving the nuclei intact, as determined by trypan blue staining. Pellet fractions were collected sequentially as follows: nuclei (N) (450 ***g***, 15 minutes), mitochondria (4,000 ***g***, 15 minutes), plasma membrane (M) (20,000 ***g***; 30 minutes), and endoplasmic reticulum/Golgi fraction (E) (125,000 ***g***; 1 hour). The mitochondrial fraction was discarded since it gave results that were intermediate between the N and M fractions (not shown). Each pellet was solubilized using 1% NP40 in 10 mM HEPES, pH 7.3, followed by centrifugation (16,000 ***g***, 10 minutes), protein determination then $^3$H-galactosylation.

### Radioisotope labeling

Aphidicolin-synchronized HT29 cells (0, 5.5, 10 and 13.5 hours after release from the $G_1$/S block) were labeled for 30 minutes with $H_3{}^{32}PO_4$ (carrier-free, 125 μCi/ml) in phosphate-free medium supplemented with 5% dialyzed fetal calf serum. After immunoprecipitation using L2A1-agarose, non-reducing sample buffer was added followed by SDS-PAGE (10% acrylamide) and autoradiography. Identical cell lysates from aphidicolin-synchronized cells (or from other preparations) were immunoprecipitated with L2A1 (or B3/25, I4D4) antibody. Immunoprecipitates were galactosylated using UDP[$^3$H]galactose (0.6 μCi) and 25 mU galactosyltransferase in 15 μl of 100 mM sodium cacodylate (pH 6.5), 20 mM $MnCl_2$ for 1.5 hours at 37°C (Chou et al., 1992). Subcellular fractionated lysates were galactosylated using 10 μg of total protein and 1 μCi UDP[$^3$H]galactose, 30 mU galactosyltransferase, 2.5 mM 5′-AMP, 25 mM $MnCl_2$, 10 mM galactose in 10 μl of 10 mM HEPES, pH 7.3 (Holt and Hart, 1986), for 1 hour at 37°C. The reaction was stopped by adding 2× non-reducing sample buffer followed by SDS-PAGE and fluorography.

### Brefeldin A and potato acid phosphatase treatments

Brefeldin A (BFA; 5 μg/ml) was added to non-synchronized cells for 15 minutes, 30 minutes, 1 hour and 2 hours. Triplicates of the two-hour BFA-treated cells were washed and chased for 30 minutes, 1 hour and 2 hours. Immunoprecipitates of K8/18 and the TrR were then obtained. Densitometric scanning (LKB Ultrascan enhanced laser densitometer) was used to measure the relative amount of galactosylated KAP85 and TrR from the radiographs, or the amount of K8/18 protein from the Coomassie-stained gels prior to fluorography. For TrR immunoprecipitation, equal amounts of protein lysates (300 μg) were used from BFA-treated cells. Protein determination was done using the Bradford method.

Dephosphorylation, using potato acid phosphatase (PAP), was carried out using K8/18 immunoprecipitates obtained from NO or OA $G_2$/M-arrested HT29 cells. The reaction mixture included PAP (3 μg) in 10 μl of 40 mM PIPES (pH 6.0) and 1 mM DTT (22°C). After 1 hour, immunoprecipitates were washed then analyzed by SDS-PAGE.

## RESULTS

### Analysis of terminal GlcNAc's in mitotically enriched cells

Our recent results showed that K8/18 intermediate filaments and the membrane-associated glycoprotein KAP85 undergo a marked increase in their terminal GlcNAc's in association with mitotic arrest, induced using the anti-microtubule agents nocodazole (NO) or colcemid (Chou and Omary, 1993; Chou et al., 1994). We analyzed the level of terminal GlcNAc's in proteins present in several subcellular compartments, isolated from G and M HT29 cells, to determine how generalized were terminal GlcNAc alternations associated with mitotic arrest. This was carried out using galactosyltransferase, which in the presence of UDP[$^3$H]-galactose transfers a [$^3$H]galactose to accessible terminal GlcNAc residues. As shown in Fig. 1A, a crude separation of subcellular fractions into soluble (S), nuclear (N), plasma membrane (M), and endoplasmic reticulum/Golgi (E) fractions showed that these four fractions had similar protein profiles when comparing G and M cells. However, as expected, different protein profiles were noted when comparing the four different compartments (Fig. 1A). The $^3$H-galactosylated profile of the proteins from the four compartments obtained from G and M cells showed that there is a generalized increase in terminal GlcNAc's in the nuclear and membrane proteins obtained from nocodazole-arrested M cells (Fig. 1B). Arrest of HT29 cells in $G_2$/M using colcemid also showed a similar pattern (not shown). In contrast with $G_2$/M-arrested cells, if $G_2$/M-enriched cells were obtained using aphidicolin synchronization, no differences in the level of terminal GlcNAc were noted in membrane proteins isolated from these cells or from G cells (Fig. 1B, compare lanes G and A10 that are bracketed under M). Similarly, comparison of the S, E and N compartments isolated from $G_2$/M cells obtained using aphidicolin synchronization with G cells did not show any differences in the level of terminal GlcNAc's (not shown).

We also examined the terminal GlcNAc's of several proteins including K8/18, intermediate filament-associated protein KAP85, and the two cell surface proteins TrR and KS-1 antigen (Perez and Walker, 1989; Szala et al., 1990; Omary et al., 1992b). As shown in Fig. 1C-F, the density of terminal GlcNAc's increased signficantly, as determined by $^3$H-galactosylation of accessible GlcNAc's, in all of these proteins in mitotically arrested cells but not in mitotically enriched cells obtained after aphidicolin synchronization. Similar results were also obtained for K8/18, KAP85 and the membrane and nuclear fractions isolated from $G_2$/M-arrested HeLa cells (not shown). The increase in terminal GlcNAc's is not due to differences in the expression level of the protein as indicated by the Coomassie staining of K8/18 (Fig. 1C) and in finding that KS-1 and TrR levels in permeabilized and nonpermeabilized G and M cells are similar (as determined by flourescence-activated cell sorter analysis; not shown). Of note, arrest of cells in $G_2$/M using nocodazole (Fig. 1) or colcemid (not shown) results in a hyperphosphorylated form of K8 (HK8) that can be seen by Coomassie staining (see also results shown in Fig. 4), and faintly seen by galactosylation (Fig. 1D).

The increased terminal GlcNAc's of proteins in colcemid/NO-arrested $G_2$/M cells may be related to an anti-microtubule effect, a nonmicrotubule-mediated drug effect or

other causes such as the associated Golgi dispersion that occurs in association with mitotic arrest. We tested the first two possibilities by comparing the galactosylation of K8/18 and KAP85 in aphidicolin-arrested cells, with aphidicolin-arrested $G_1$/S cells treated with NO for 24 hours in the presence of aphidicolin. The treatment of cells with aphidicolin results in cell arrest at the $G_1$/S boundary as determined by cell cycle analysis (Fig. 2C). As shown in Fig. 2B, an increase in terminal GlcNAc's was observed only in nocodazole-treated cells, whereas nocodazole addition to $G_1$/S-arrested cells did not alter the GlcNAc level of KAP85 and K8/18 despite disassembly of the microtubules (Fig. 2D: compare panels AN and N, with G and A). Therefore, the increase in terminal GlcNAc's, in cells arrested at $G_2$/M using anti-microtubule agents, does not appear to be simply due to microtubule disruption or a nonspecific drug effect.

### $G_2$/M-enriched cells obtained by aphidicolin synchronization have increased K8/18 phosphorylation without any change in glycoslyation

The lack of altered glycosylation of K8/18 and several membrane proteins in $G_2$/M-enriched cells obtained by aphidicolin synchronization (in contrast with $G_2$/M nocodazole-arrested cells; Fig. 1) led us to evaluate phosphorylation changes in aphidicolin-synchronized $G_2$/M cells. We previously showed that K8/18 phosphorylation increased dramatically in nocodazole or colcemid $G_2$/M-arrested cells (Chou and Omary, 1993), with similar findings in keratins from mitotic HeLa cells obtained by mechanical detachment (Bravo et al., 1982; Celis, et al., 1983), and from maturing *Xenopus* oocytes (Klymkowsky et al., 1991). As shown in Fig. 3, aphidicolin synchronization blocked 88% of the cells at the $G_1$/S boundary,

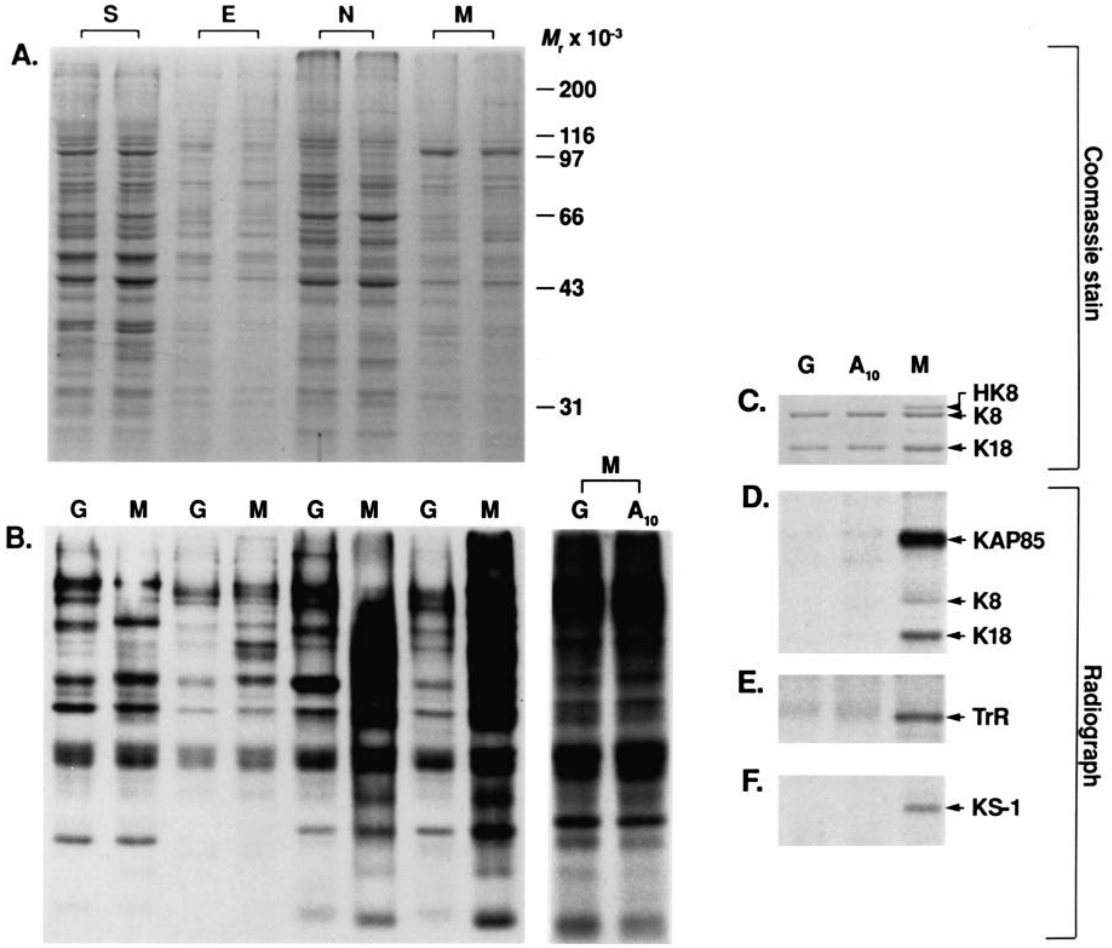



**Fig. 1.** An increase in terminal GlcNAc's occurs in mitotic cells obtained using antimicrotubule agents but not using aphidicolin synchronization. (A and B) Asynchronous (primarily $G_0/G_1$) or nocodazole $G_2$/M-arrested HT29 cells were disrupted by nitrogen cavitation followed by subcellular fractionation as described in Materials and Methods. (B) Lanes marked G ($G_0/G_1$ cells) or M ($G_2$/M cells) represent the radiograph of A. From each subcellular fraction (S, E, N, and M correspond to soluble cytosolic, endoplasmic reticulum/Golgi, nuclear and plasma membrane enriched, respectively) 10 μg of protein was galactosylated using UDP[$^3$H]galactose followed by SDS-PAGE as described in Materials and Methods. Similarly, cells enriched in $G_2$/M, which were obtained using aphidicolin synchronization followed by washing off the aphidicolin and chasing for 10 hours ($A_{10}$) or G cells were fractionated followed by galactosylation of the plasma membrane fraction (B). The last two lanes of B (bracketed under M) were from a different representative experiment (4 day exposure) as compared with the other lanes in B (2 day exposure). (C-F) G, M or $A_{10}$ cells were solubilized in DNS followed by immunoprecipitation of K8/18, TrR or KS-1.) (D) The radiograph of C.

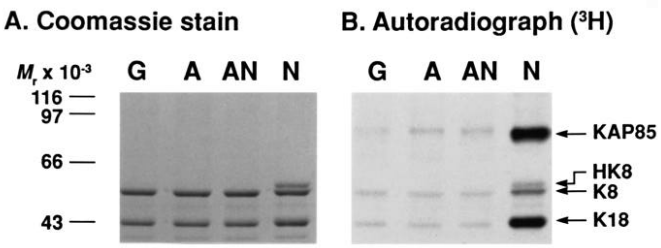
which, after washing off the aphidicolin and chasing, yielded 82% of cells in S phase after 5.5 hours, and 66% of cells in $G_2$/M after 10 hours. Under all conditions examined, K8/18 glycosylation was unchanged whereas phosphorylation of K8 and K18 began to increase during the S phase, peaking during $G_2$/M and decreasing as cells pass through $G_2$/M (Fig. 3). Although a discrete hyperphosphorylated K8 (HK8) species was noted with colcemid/NO arrest, we were unable to obtain a similar species using aphidicolin synchronization (Fig. 3) or after examining mitotically enriched cells obtained by mechanical shaking of HT29 cells (not shown).

### Arrest of cells in $G_2$/M using OA or NO alters the glycosylation of K8/18 and cell surface receptors, and generates a distinct hyperphosphorylated K8 species

We asked if a modulator, other than anti-MT agents, which arrests cells in $G_2$/M will also alter cellular glycosylation. For this we used OA (6 nM), which arrested 85-90% of cells in $G_2$/M after a 48 hour treatment (histogram is similar to NO-arrested cells; not shown). High concentrations of OA (500 nM range) arrested HT29 cells at $G_1$/S as reported for U937 and HL60 cells (not shown). Arrest of cells at $G_2$/M using OA resulted in a 60 kDa HK8 species, similar to what was obtained with NO (Fig. 4A). In addition, as noted for NO or colcemid $G_2$/M arrest, OA $G_2$/M arrest also resulted in increased terminal GlcNAc density of K8/18, KAP85, KS-1 and TrR although the altered glycosylation of the cell surface receptors was not as dramatic as that of K8/18 (Fig. 4A,B). Both K8 and HK8 become hyperglycosylated to similar proportions after NO or OA arrest as seen in Fig. 2B (lane N) or on a lighter exposure of Fig. 4A (not shown). Subcellular fractionation of OA-arrested HT29 cells also showed increased terminal GlcNAc's of the membrane fraction as compared with asynchronous cells (not shown), although the effect was not as pronounced as with NO arrest.

Evidence that HK8 is indeed a stable hyperphosphorylated form of K8 was obtained by treatment of K8/18 immunoprecipitates, obtained from OA or NO $G_2$/M-arrested cells with potato acid phosphatase, which resulted in dephosphorylation of HK8 to form K8 (Fig. 4C). Furthermore, tryptic phosphopeptide mapping of individually purified K8 and HK8 bands showed that they have identical phospho-peptides except for an additional phosphorylated peptide in HK8 indicated by the arrow (Fig. 4D). Therefore, arrest of cells in $G_2$/M using NO or OA results in a specific hyperphosphorylated form of K8, and an increase in the terminal GlcNAc's of IF and membrane-associated proteins.

We examined the role that phosphorylation may play in the observed increase in terminal GlcNAc's of K8/18 cytoplasmic IF proteins and KAP85 membrane-associated protein. As shown in Fig. 5 (lane c), treatment of aphidicolin-synchronized cells with NO for 12 hours resulted in generation of hyperphosphorylated K8 coupled with hyperglycosylation of K8/18 and increased terminal GlcNAc's of KAP85. However, coincubation of the aphidicolin-synchronized cells with NO and staurosporine (Fig. 5, lane d) abolished most of the effects observed in the presence of NO alone, including mitotic arrest (not shown), except for the increase in terminal GlcNAc's of KAP85. As a control, the galactosylation of K8/18 and KAP85 from asynchronous HT29 cells (Fig. 5, lane a) and asynchronous cells treated with NO alone for 12 hours (Fig. 5, lane b) are shown. The galactosylation level of K8/18 and KAP85 (Fig. 5) and cell cycle analysis (not shown) indicate that treatment of asynchronous cells with NO for 12 hours results only in partial $G_2$/M arrest as compared with the complete $G_2$/M arrest obtained by treating aphidicolin-synchronized cells with NO for 12 hours. Hence, aphidicolin synchronization followed by the addition of NO provides a better homogenous cell population with a shorter duration of NO treatment. This is reflected by the inhibition of both membrane and K8/18 altered glycosylation if NO and staurosporine are added concurrently (not shown). Therefore, the results shown in Fig. 5 suggest that phosphorylation plays a role in mitotic arrest-associated increase in terminal GlcNAc's of K8/18, but not in the disruption of oligosaccharide processing of membrane glycoproteins.




C. Cell cycle analysis

| | | G | A | AN | N |
|---|---|---|---|---|---|
| | aphidicolin: | – | + | + | – |
| | nocodazole: | – | – | + | + |
| Cell cycle analysis | % G0/G1 | 79 | 85 | 90 | 3 |
| | % S | 14 | 10 | 7 | 5 |
| | % G2/M | 7 | 5 | 3 | 92 |

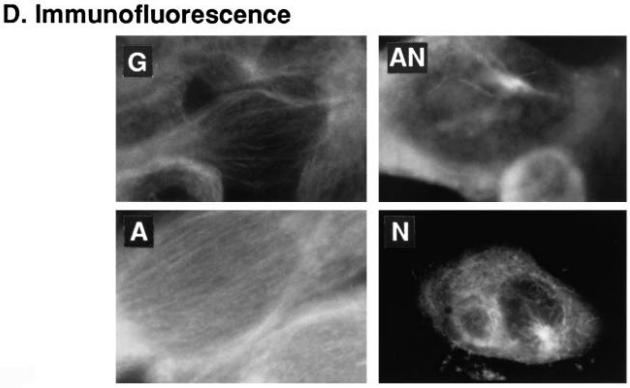
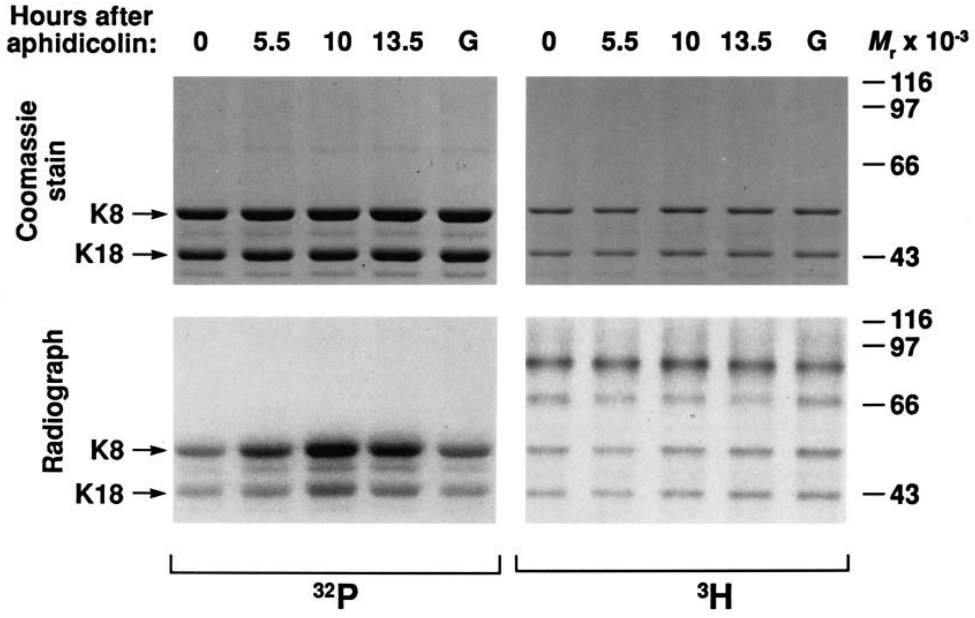



**Fig. 2.** Microtubule disassembly per se does not result in increased K8/18 glycosylation. Four preparations of HT29 cells were used: asynchronous (lanes G), synchronized at $G_1$/S for 48 hours using aphidicolin (lanes A), synchronized with aphidicolin for 24 hours followed by the addition of nocodazole for 24 hours in the presence of aphidicolin (AN) or treated with nocodazole for 24 hours (N). Triplicates of each preparation were used for immunoprecipitation and $^3$H-galactosylation of K8/18 (A and B), cell cycle analysis (C) and immunofluorescence using anti-tubulin antibody (D) as described in Materials and Methods.




| | Hours after aphidicolin: | 0 | 5.5 | 10 | 13.5 | G |
|---|---|---|---|---|---|---|
| Cell cycle analysis | % G0/G1 | 88 | 8 | 7 | 37 | 74 |
| | % S | 10 | 82 | 27 | 24 | 21 |
| | % G2/M | 2 | 10 | 66 | 39 | 6 |

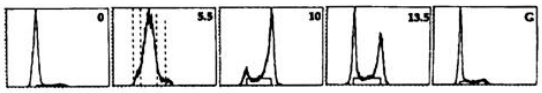


**Fig. 3.** Aphidicolin synchronization increases K8/18 phosphorylation but does not alter K8/18 glycosylation: HT29 cells were synchronized in $G_1$/S using aphidicolin followed by washing, then incubating without aphidicolin for the indicated time points. Triplicates of each time point were then processed for cell cycle analysis, or labeled with $^{32}PO_4$ for 30 minutes, or incubated in phosphate-free medium for 30 minutes. $^{32}PO_4$-labeled and nonlabeled cells were then processed for immunoprecipitation using anti-K8/18 mAb, followed by galactosylation of the nonlabeled immunoprecipitates as described in Materials and Methods. The lower panel shows percentage of cells at $G_0/G_1$, S, and $G_2$/M as calculated from the inset histograms.

### Effect of BFA on IF and membrane protein terminal GlcNAc's

We used BFA to ask what happens to the level of terminal GlcNAc's of IF and membrane glycoproteins. BFA, a fungal microcyclic lactone (Harri et al., 1963), is an agent that is widely used to study protein and membrane traffic (see Klausner et al., 1992, for review). Its effects on protein glycosylation and secretion appear to be mediated by fusion of Golgi stacks with the endoplasmic reticulum. In the case of N-linked glycosylation and processing in the presence of BFA, initial oligosaccacharide trimming appears to be intact but further processing including the addition of terminal galactose and outer branch fucose residues is significantly affected (Sampath et al., 1992). This was clearly demonstrated in N-linked glycoproteins of bovine pulmonary artery and endothelial cells, where 65% of the N-linked chains terminated with β-GlcNAc residues in the presence of BFA (Sampath et al., 1992).

Incubation of HT29 cells with BFA resulted in a time-dependent and significant increase in the level of terminal GlcNAc's of the TrR and KAP85 membrane proteins (Fig. 6A,B). In addition, a generalized increase in terminal GlcNAc's was noted in proteins isolated from the plasma membrane fraction of BFA-treated cell (Fig. 6C). In contrast, no change was noted in the level of terminal GlcNAc's of K8/18 (Fig. 6A), as would be expected for a cytoplasmically directed glycosylation of the single O-linked GlcNAc type.

### Analysis of the Golgi apparatus under different treatment and synchronization conditions

Altered membrane protein glycosylation was noted when cells were treated with BFA (Fig. 6), or arrested at $G_2$/M using anti-MT agents or OA (Figs 1 and 4). No alteration of membrane protein glycosylation was noted in cells arrested at $G_1$/S with aphidicolin then treated with NO (Fig. 2, lane AN) or aphidicolin-synchronized then $G_2$/M-enriched cells (Fig. 3, 10 hour chase). Since a common alteration among BFA, OA and anti-MT agents is significant Golgi disruption, we examined Golgi-associated staining using anti-βCOP antibody (Duden et al., 1991). As shown in Fig. 7, asynchronous G cells show a curvilinear/reticular staining pattern (A) that dramatically fragments

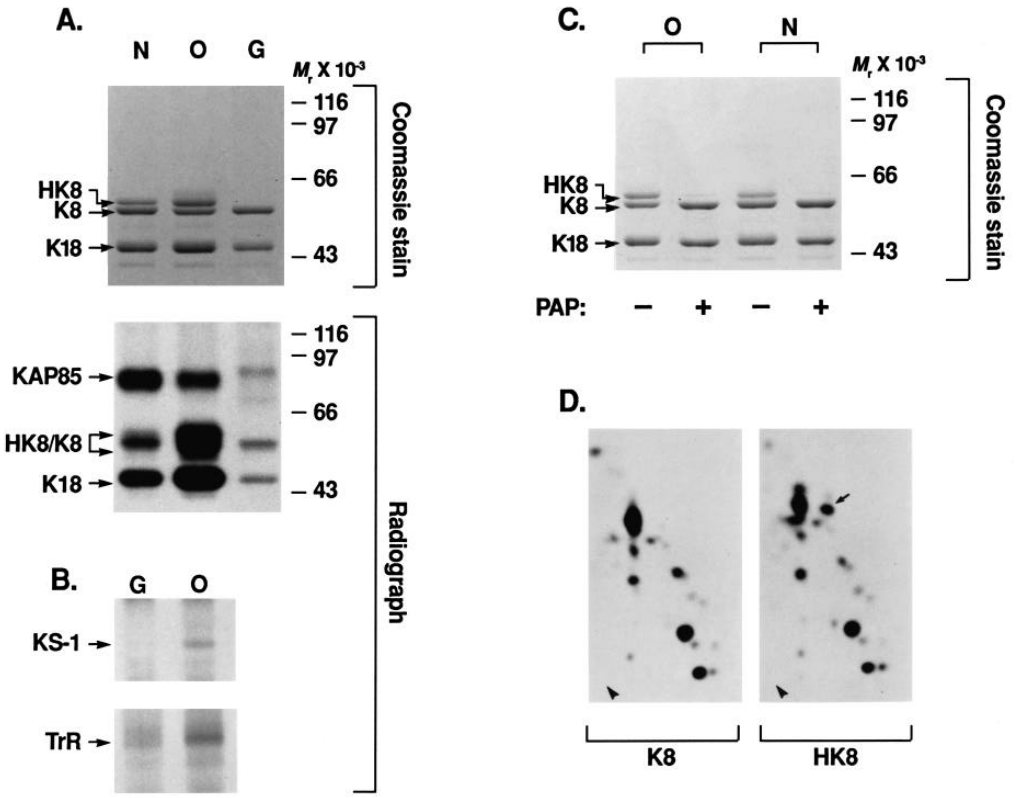



**Fig. 4.** OA, similar to NO, arrests cells in $G_2$/M and alters the glycosylation of K8/18 and several membrane proteins and generates a distinct hyperphosphorylated K8 species. Asynchronous HT29 cells (G) were arrested at $G_2$/M using nocodazole (N) or okadaic acid (O) as described in Materials and Methods. (A) Immunoprecipitates of K8/18 were galactosylated using UDP[$^3$H]galactose. (B) $^3$H-galactosylated immunoprecipitates of the KS-1 antigen and TrR. (C) Immunoprecipitates of K8/18 were obtained from O or N cells followed by treatment with potato acid phosphatase (PAP) as described in Materials and Methods. (D) HT29 cells were arrested in $G_2$/M using nocodazole followed by labeling with [$^{32}$P]orthophosphate for 3 hours. The individual K8 or hyperphosphorylated K8 bands were then isolated using preparative gels, followed by tryptic peptide mapping. Arrowheads indicate origin where samples were loaded, and arrow shows a peptide that was phosphorylated only in HK8.

in OA-arrested cells (B). Similar staining to that noted in B was obtained with BFA-treated (2 hours) or NO-arrested HT29 cells (not shown). In contrast, the Golgi staining in cells treated with NO and aphidicolin (C) or $G_2$/M-enriched cells after aphidicolin synchronization (D) showed smaller aggregates and less fragmentation than seen in B.

## DISCUSSION

The major findings of our study are: (i) arresting cells in mitosis using anti-MT agents or OA results in generalized alteration in the terminal GlcNAc density of intermediate filaments and membrane glycoproteins. (ii) This observed phenomenon is not simply related to MT disruption and does not appear to be a specific mitosis-associated event. In the case of membrane proteins it may be related to the persistent Golgi disruption that occurs in mitotic arrest. (iii) Phosphorylation appears to play a role in the mitotic arrest-associated increase in IF glycosylation, but not in the alteration of membrane protein glycosylation. Our results raise the possibility that some of the effects of anti-MT agents may be mediated by cellular protein glycosylation changes.

The observed increase in terminal GlcNAc density occurred after arresting cells using anti-MT agents or OA in a microtubule-independent fashion, since disruption of MT in aphidicolin-arrested $G_1$/S cells for the same duration used to arrest asynchronous cells at $G_2$/M did not alter GlcNAc levels (Fig. 2). The most likely explanation for the observed increase in terminal GlcNAc's of membrane proteins is that Golgi disruption, which occurs in association with anti-MT agents (Thyberg and Moskalewski, 1985) and OA (Lucocq et al., 1991; Thyberg and Moskalewski, 1992), prevents the maturation of synthesized oligosaccharides thereby resulting in an

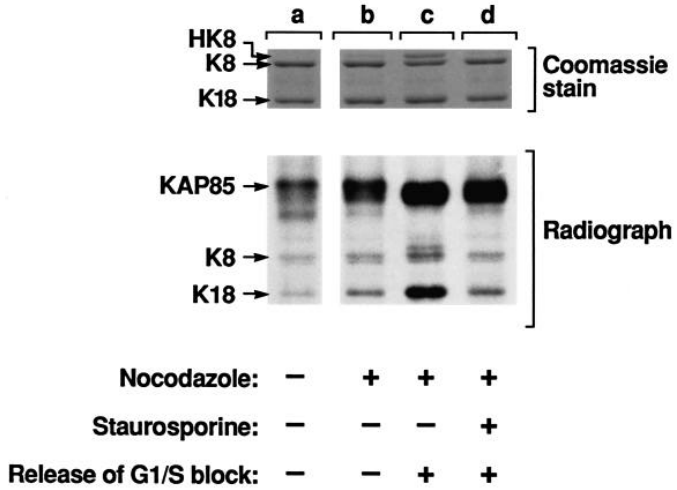



**Fig. 5.** Staurosporine preferentially blocks the alteration in K8/18 but not membrane protein glycosylation. Immunoprecipitates of K8/18 (which also co-immunoprecipitate KAP85) were obtained from HT29 cells that were grown using four conditions: asynchronously (a); in the presence of NO (0.5μg/ml) for 12 hours (b); blocked at $G_1$/S using aphidicolin followed by washing off the aphidicolin and chasing for 8 hours, then incubating with NO alone (0.5 μg/ml) (c), or NO and staurosporine (1 μM) for 12 hours (d). Cells subjected to the four conditions were solubilized using DNS followed by immunoprecipitation of K8/18, then galactosylation as described in Materials and Methods.

accumulation of terminal GlcNAc's. This explains the increased GlcNAc's of plasma membrane proteins and is supported by the data obtained using BFA (Fig. 6), which also increases oligosaccharide termination with GlcNAc (Sampath et al., 1992). Another supportive correlation is the Golgi-related staining observed in Fig. 7. This staining showed dramatic fragmentation when cells were arrested with NO or OA or treated with BFA (conditions associated with altered membrane protein GlcNAc's), but less aggregation and punctate patterns when cells were treated with NO and aphidicolin or $G_2$/M-enriched after aphidicolin synchronization. Alternatively, the mitotic arrest-associated alteration in glycosylation of a number of cellular proteins may be related to an accumulating effect of a drug metabolite. However, this is unlikely since different drugs (colcemid, NO, OA), all of which result in Golgi dispersion and mitotic arrest, altered protein glycosylation.

The effect of mitotic arrest induced by colcemid, NO, or OA on membrane protein glycosylation in this study is different to the lack of effect of colcemid on protein glycosylation and vectoral transport from ER to Golgi to cell surface that was described in Chinese hamster ovary cells (Stults et al., 1989). However, the study by Stults et al. focused on the anti-MT effect of colcemid, and the maximum incubation of cells with colcemid (10 μM) was 3 hours, which in HT29 and HeLa cells does not result in any significant accumulation of cells in $G_2$/M (not shown). Although the concentration of colcemid we used was 1.35 μM (0.5 μg/ml), incubation of HT29 cells with 10 μM of colcemid for 3 hours does not result in any significant alteration in GlcNAc levels (not shown).

In contrast to membrane-associated proteins, several lines of evidence indicate that the mitotic arrest-associated increase in terminal GlcNAc of IF is independent of direct Golgi function. First the single O-GlcNAc modification is a unique form of glycosylation that occurs normally in the cytoplasm (Hart et al., 1989), and appears to be independent of BFA-induced Golgi fusion with the endoplasmic reticulum (Fig. 6). The O-GlcNAc transferase that mediates this glycosylation, a two subunit phosphoprotein (Haltiwanger et al., 1992a), was recently purified from rat liver cytosol (Haltiwanger et al., 1992b). Second, phosphorylation appears to play a role in the GlcNAc level of IF but not membrane proteins. To that end, staurosporine blocked the increase in K8/18 GlcNAc's without affecting the increase in membrane-associated KAP85 after NO-induced mitotic arrest (Fig. 5). The effect of staurosporine on K8/18 glycosylation was coupled with lack of generation of the hyperphosphorylated K8. It is not known if the activity of the O-GlcNAc transferase phosphoprotein (Haltiwanger et al.,

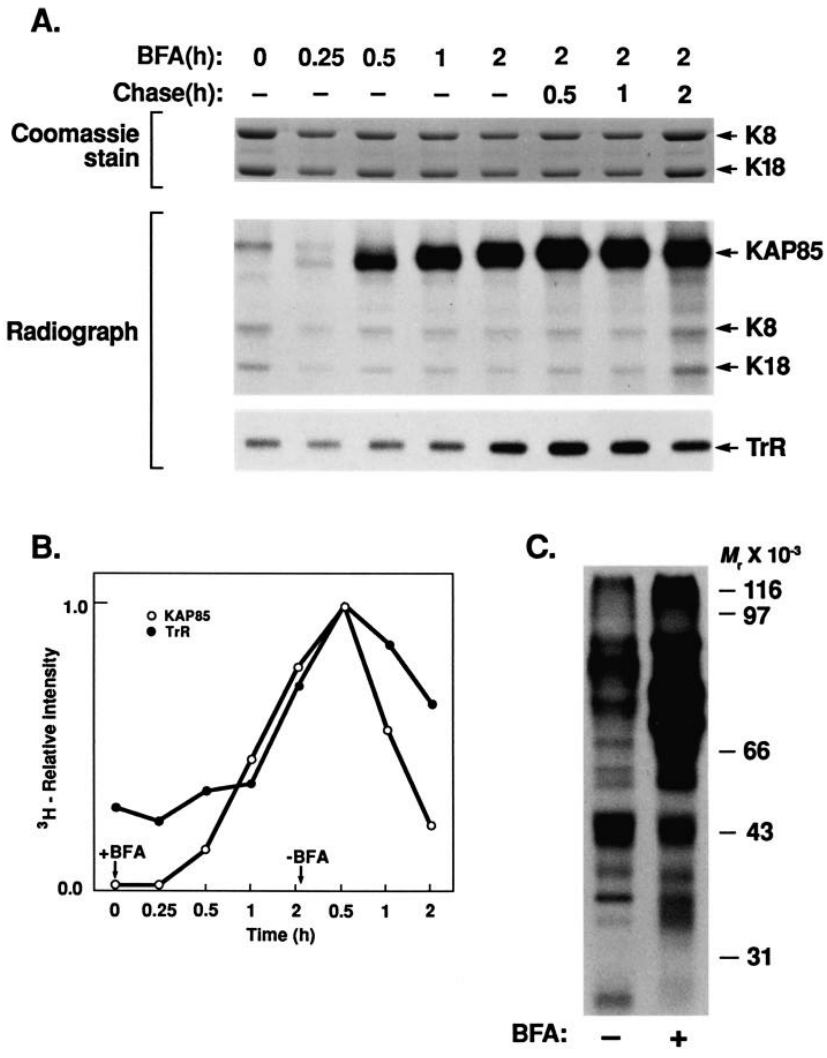



**Fig. 6.** Golgi disruption by BFA modulates membrane but not K8/18 terminal GlcNAc levels: (A) Asynchronous HT29 cells were treated with BFA for 0, 0.25, 0.5, 1 and 2 hours followed by immediate harvesting of cells or chasing in the absence of BFA for the indicated times. Immunoprecipitates were then prepared using anti-K8/18 and anti-TrR mAb. (B) Densitometry scanning of the TrR and of KAP85 $^3$H-galactosylated species, corrected for the amount of K8/18 present based on densitometry scanning of the Coomassie-stained K8/18 bands shown in A. The peak intensity (0.5 hours chase) was defined as having a relative intensity of 1. (C) HT29 cells with and without BFA treatment (2 hours) were disrupted followed by subcellular fractionation and $^3$H-galactosylation of the plasma membrane fraction as shown in Fig. 1, then fluorography.

1992b) is regulated by phosphorylation. However, it is attractive to speculate that the hyperphosphorylated state of cells generated by OA or anti-MT agents could potentially activate the O-GlcNAc transferase by hyperphosphorylation of the enzyme. Alternatively, a cytosolic *N*-acetylglucosaminidase (Haltiwanger et al., 1992a) may be inactivated with a resultant net increase in the O-GlcNAc level of K8/18.

The concurrent increase in phosphorylation and glycosylation of K8/18 during mitotic arrest may be interrelated. Interestingly, the phosphorylation and glycosylation of K8 and K18 molecules is mutually exclusive such that K8 (or K18) molecules that have the single O-GlcNAc are not phosphorylated and vice versa (Chou and Omary, 1993). A similar finding was described for RNA polymerase II (Kelly et al., 1993) and the eukaryotic peptide chain initiation factor 2-associated p67 polypeptide (Datta et al., 1989), which have the single O-GlcNAc modification. However, hyperphosphorylation of K8/18 per se, as noted in aphidicolin-synchronized $G_2$/M cells, does not alter K8/18 glycosylation (Fig. 3). Furthermore, HK8 and K8 appear to be similarly galactosylated (e.g. see Fig. 5). Identification of specific phosphorylation and glycosylation sites coupled with transfection experiments should allow a better understanding of the relationship between these two modifications.

The increased glycosylation of K8/18 in association with mitotic arrest was not observed in $G_2$/M-enriched cells obtained after aphidicolin synchronization. It is possible that a mitosis-specific alteration in keratin glycosylation may be extremely transient and could have been missed by our aphidicolin synchronization. However, if altered glycosylation does indeed occur peri-mitotically, it would have to be more transient than the increased phosphorylation noted with aphidicolin synchronization.

Anti-MT agents are widely used in studying mitosis-associated events in hundreds of experimental systems. Their effects, either directly or indirectly, apear to be pleotropic and presumably are reflected by binding to MT (Olmstead and Borisy, 1973), binding to smooth ER membranes (Riordan and Alon, 1977), dispersion of the Golgi apparatus (Thyberg and Moskalewski, 1985), alteration of location and morphology of lysosomes (Matteoni and Kreis, 1987). Although drugs such as colchicine, colcemid and NO are typically referred to as anti-MT agents, they can also have profound effects on IF with formation of juxtanuclear aggregates in a number of cell lines (Goldman, 1971; Franke et al., 1982). The finding of this report also indicates that anti-MT agents, independent of a direct MT effect but in the setting of mitotic arrest, significantly alter the glycosylation of a number of cellular proteins. The altered glycosylation of a number of cellular proteins in association with colcemid raises the possibility, which remains to be tested, that

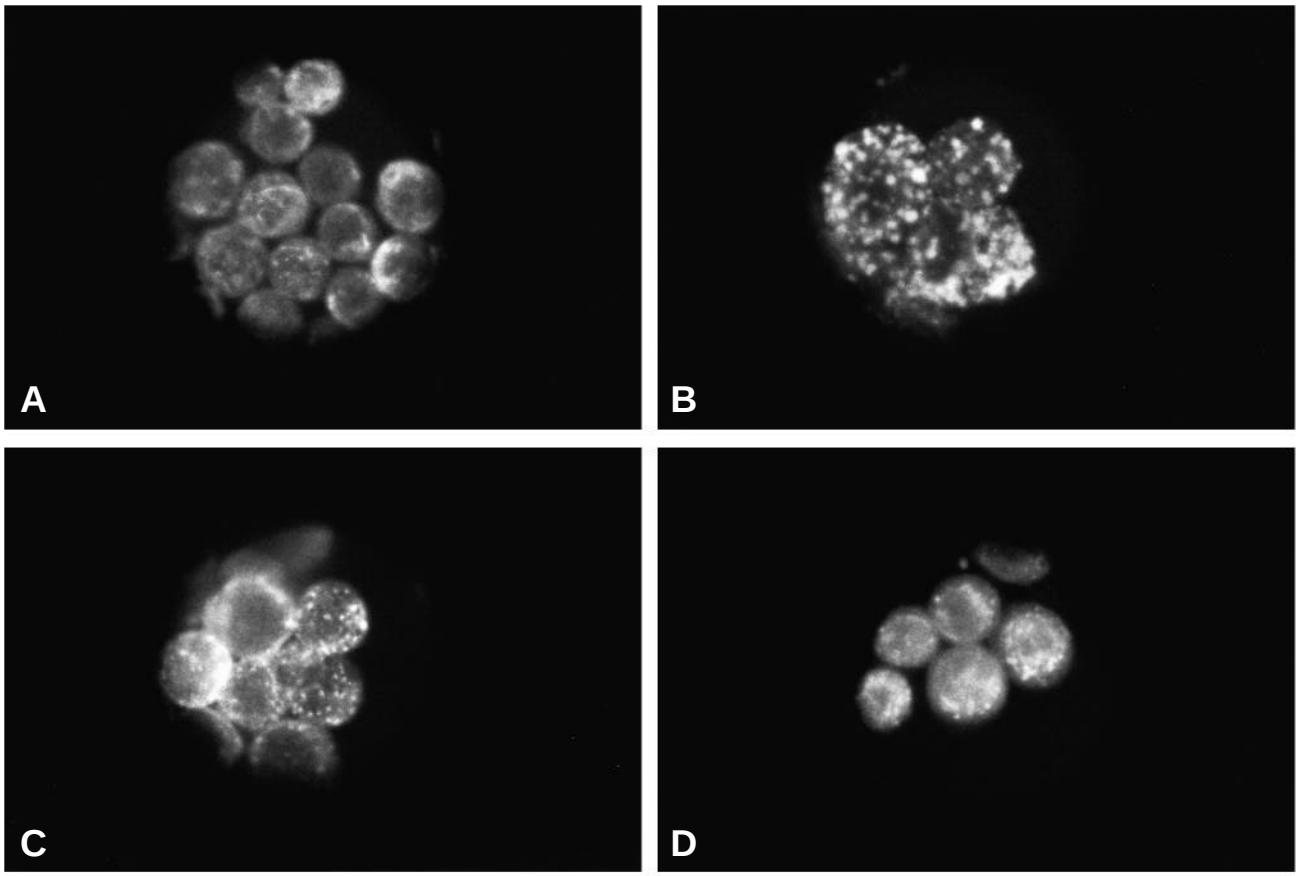



**Fig. 7.** Golgi-related staining using anti-βCOP antibody: (A) Asynchronous HT29 cells. (B) HT29 cells arrested at $G_2$/M using 6 nM okadaic acid for 48 hours. (C) HT29 cells were treated with aphidicolin for 24 hours, then aphidicolin plus NO for 24 hours as in Fig. 2. (D) Cells were synchronized with aphidicolin then enriched for $G_2$/M cells by collecting after a 10-hour chase (similar to the 10 hour chase in Fig. 3). Cells were then fixed and stained with anti-βCOP antibody as described in Materials and Methods.

some of the side effects noted in patients taking colchicine may be related to changes in glycosylation. Colchicine has been used since 1763 to treat gouty arthritis (Hartung, 1954). It is also used in other diseases including familial mediteranian fever (Dinarello et al., 1974), and liver cirrhosis (Kershenobich et al., 1988). Although generally well tolerated, its most frequent side effect is diarrhea, which is generally managed by decreasing the dose (Wallace and Singer, 1988). The mechanism of diarrhea in patients taking colchicine is unknown but is felt to be related to its anti-MT effect (Malawista and Bensch, 1967). A finding of altered glycosylation of intestinal apical membrane proteins would support the hypothesis, based on our data, that such altered glycosylation may play a pathogenic role in colchicine-induced diarrhea. Interestingly, small intestinal biopsies from patients taking colchicine showed that the enzyme activities of sucrase, maltase and lactase were significantly decreased as compared with normal controls (Race et al., 1970). Furthermore, a recent study showed that differences in the glycosylation of lactase reflect different enzyme activities (Naim and Lentze, 1992). An examination of cell surface glycoproteins isolated from small intestinal biopsies of patients taking colchicine as compared with control patients not on colchicine could further test our hypothesis.

We are grateful to Theresa L. Hooper and Olivia A. Hernandez for preparing the manuscript and to Dr Suzanne Pfeffer for providing the anti-βCOP antibody and optimum staining conditions. This work was supported by a Veteran Administration Merit Award, National Institute on Alcohol Abuse and Alcoholism grant AA09478-01, the PEW Scholars Program (M.B.O.), and Digestive Diseases Center grant DK38707.

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(Received 4 January 1994 - Accepted, in revised form, 31 March 1994)