Mitotic Arrest-associated Enhancement of O-Linked Glycosylation and Phosphorylation of Human Keratins 8 and 18*

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Arrest of the human colonic cell line HT29 at the G2/M phase of the cell cycle resulted in changes in keratin assembly that were coupled with a significant increase in the O-linked glycosylation and serine phosphorylation of keratin polypeptides 8 and 18 (K8/18). With mitotic arrest, enhanced keratin phosphorylation occurred preferentially on K8, whereas K18 showed a higher glycosylation level than K8. Removal of the arresting agent allowed cells to proceed through the cell cycle with a concomitant decrease in K8/18 glycosylation. In contrast, keratins isolated from S phase-enriched cells, obtained after synchronization with aphidicolin, did not show enhanced glycosylation. Tryptic peptide analysis of keratins in G2/M-arrested cells showed changes in the glycopeptide pattern of K8 and in the phosphopeptide patterns of K8 and K18. Labeling of K8/18 immunoprecipitates, isolated from G2/M-arrested cells, with [3H]galactose followed by β-elimination showed that K8/18 glycosylation consisted of single N-acetylglucosamine residues. Threonine was identified as the site of glycosylation after comparing acid hydrolysis products of β-eliminated and non-β-eliminated K8 and K18. Specific cleavage at tryptic phospho-residues indicated that K18 glycosylation and phosphorylation were restricted to the head and proximal rod domains, whereas K8 did not show the same restriction. Our results show a unique association of O-linked glycosylation and phosphorylation (Steinert and Idler, 1975). Recently, we showed that K8/18 have multiple O-linked glycosylation sites that consist of single N-acetylglucosamine (GlcNAc) residues (Chou et al., 1992). This type of glycosylation appears to be characteristic of several cytoplasmic and nuclear proteins (Torres and Hart, 1984; Hart et al., 1989a; Davis and Blobel, 1987; Haltiwanger et al., 1992). K8/18 glycosylation was also found to be a dynamic process with the biosynthesis and degradation rates faster than the corresponding rates for the protein backbone, suggestive of a functional significance for this modification (Chou et al., 1992). The amino acid(s) involved in the single O-GlcNAc modifications has been described for only a few proteins including the nuclear pore complex glycoproteins (Holt et al., 1987b), α-crystallins (Roquemore et al., 1992), and the serum response transcription factor (Reason et al., 1992). In all cases, serine appears to be the predominant involved O-linked amino acid. Since phosphorylation and O-linked glycosylation can theoretically involve identical Ser/Thr residues, it has been tempting to hypothesize that Ser/Thr O-linked glycosylation may function, at least in some settings, as a functional regulator of protein phosphorylation (Hart et al., 1992).

Keratin phosphorylation occurs primarily on serine residues (Steinert, 1988; Chou and Omary, 1991) with evidence for the involvement of several kinases (Yano et al., 1991), including cAMP-dependent protein kinase (Gilmartin et al., 1984) and protein kinase C (Omary et al., 1992). The function of keratin phosphorylation is unknown; however, there is evidence that hyperphosphorylation plays a role in the disassembly of IF proteins (Inagaki et al., 1987, 1988; Gonda et al., 1990; Chou et al., 1989) and may also play a role in signal transduction as shown after epidermal growth factor binding to rat hepatocytes (Baribault et al., 1989). Increased phosphorylation was also noted in keratins obtained from mitotic amnion cells as compared with keratins obtained from interphase cells (Celis et al., 1983).

In this study, we initially confirmed serine hyperphosphorylation of K8/18 in the human colonic epithelial cell line HT29 after colcemid-induced arrest in the G2/M phase of the cell cycle. We then examined keratin glycosylation during distinct human keratin polypeptides described. Keratins have been catalogued into acidic type I keratins (K9-K21, pl < 6) and basic/neutral type II keratins (K1-K8, pl > 6) (for reviews see Lazurides, 1982; Steinert and Roop, 1988; Klymkowsky et al. 1989; Skalli and Goldman, 1991). Single layered "simple" epithelial cells such as the intestine express at least one major type I keratin (K18) and one major type II keratin (K8) that exist in cells as obligate noncovalent heteropolymers (K8/18).

Several post-translational modifications have been described for keratins including phosphorylation (Gilmartin et al., 1984; Steinert, 1988; Chou and Omary, 1991), glycosylation (King and Housell, 1989; Chou et al., 1992), and acetylation (Steinert and Idler, 1975). Particularly, we showed that K8/18 have multiple O-linked glycosylation sites that consist of single N-acetylglucosamine (GlcNAc) residues (Chou et al., 1992). This type of glycosylation appears to be characteristic of several cytoplasmic and nuclear proteins (Torres and Hart, 1984; Hart et al., 1989a; Davis and Blobel, 1987; Haltiwanger et al., 1992). K8/18 glycosylation was also found to be a dynamic process with the biosynthesis and degradation rates faster than the corresponding rates for the protein backbone, suggestive of a functional significance for this modification (Chou et al., 1992). The amino acid(s) involved in the single O-GlcNAc modifications has been described for only a few proteins including the nuclear pore complex glycoproteins (Holt et al., 1987b), α-crystallins (Roquemore et al., 1992), and the serum response transcription factor (Reason et al., 1992). In all cases, serine appears to be the predominant involved O-linked amino acid. Since phosphorylation and O-linked glycosylation can theoretically involve identical Ser/Thr residues, it has been tempting to hypothesize that Ser/Thr O-linked glycosylation may function, at least in some settings, as a functional regulator of protein phosphorylation (Hart et al., 1992).

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**Keratins are a subgroup of cytoskeletal intermediate filament (IF) proteins that have a characteristic expression on most epithelial tissues (Moll et al., 1982, 1980; Quaroni et al., 1991). Keratins are the most complex of IF proteins with 21 distinct human keratin polypeptides described. Keratins have been catalogued into acidic type I keratins (K9-K21, pl < 6) and basic/neutral type II keratins (K1-K8, pl > 6) (for reviews see Lazurides, 1982; Steinert and Roop, 1988; Klymkowsky et al. 1989; Skalli and Goldman, 1991). Single layered "simple" epithelial cells such as the intestine express at least one major type I keratin (K18) and one major type II keratin (K8) that exist in cells as obligate noncovalent heteropolymers (K8/18).

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different stages of the cell cycle and showed that it increased in G2/M colcemid-arrested cells but not in aphidicolin-synchronized S phase cells. The glycosylation during G2/M was similar to that found in asynchronously growing cells in that it consisted of single O-GlcNAc residues, with the site of glycosylation identified as threonine. Increased glycosylation and phosphorylation of keratins occurred in parallel. However, K8 showed a preferential increase in phosphorylation, whereas K18 showed a higher level of glycosylation during G2/M arrest. In addition, the head and proximal rod domain of K18 were the primary regions of glycosylation and phosphorylation.

MATERIALS AND METHODS

Cell Culture and Synchronization—HT29 cells (American Type Culture Collection, Rockville, MD) growing at 30–60% confluence were plated in 35-mm tissue culture dishes (2 × 10⁶ cells in 1 ml of medium). Colcemid or nocodazole (Sigma) was used at 0.5 μg/ml. Adherent cells were removed by treatment for 10 min with phosphate-buffered saline (PBS) containing 0.5% trypsin and 1 mM EDTA for the cell cycle analysis or by scraping for the biochemical studies. Aphidicolin (Sigma, 5 μg/ml) was used to arrest cells at the G1/S boundary.

To obtain floater cells from log phase growing cells, confluent HT29 cells were split 1:4. After allowing cells to settle for 6 h, nonadherent cells were discarded followed by rinsing. Fresh pre-warmed medium (37°C) was added followed by 10% CO₂ incubation for 1 h and then shaking in a 37°C room for 1 h. Floater cells were then collected, placed over ice immediately (viability greater than 95%), and then used for immunoprecipitation or cell cycle analysis.

Reagents—Uridine diphosphate UDP-[4-5-3H]galactose (34.6 Ci/mmol), [3H]sodium borohydride (NaB₃H₄, 12.6 Ci/mmol), and orthophosphate (PO₄) were purchased from Du Pont-New England Nuclear. HT29 cells were split 1:4. After allowing cells to settle for 6 h, nonadherent cells were discarded followed by rinsing. Fresh pre-warmed medium (37°C) was added followed by 10% CO₂ incubation for 1 h and then shaking in a 37°C room for 1 h. Floater cells were then collected, placed over ice immediately (viability greater than 95%), and then used for immunoprecipitation or cell cycle analysis.

Cell Cycle Analysis and Immunofluorescence—Cells were fixed with 70% ethanol for at least 30 min and then treated with propidium iodide (20 μg/ml) containing RNase A (40 μg/ml) in PBS for 30 min, followed by cell cycle analysis using the FACScan (RFP program, Becton Dickinson). The homogeneity of individual cell populations was estimated by calculating the standard deviation (i.e. dispersion from the mean) and coefficient of variation (i.e. standard deviation divided by the mean) using the RFIT program. For indirect immunofluorescence, cells were transfected to collagen-coated slides using a Cytospin centrifuge (7,000 rpm, 6 min), fixed in acetone (−20°C, 2 s), washed then stained with anti-K8/K18 monoclonal antibody L2A1 in PBS (7.4) containing 10% human serum and 0.1% sodium azide. After 45 min (22°C), cells were washed and then incubated with Texas Red-conjugated goat anti-mouse antibody. Photomicrographs were taken using black and white Kodak p3200 film.

Cell Labeling and Immunoprecipitation—HT29 cells were labeled with orthophosphate (125 μCi/ml, 2 h, 5 × 10⁶ cells/ml) in phosphate-free RPMI 1640 medium supplemented with 5% dialyzed fetal calf serum. Cells were then washed with PBS and solubilized (30 min) with 1% Nonidet P-40 in PBS containing 25 μg/ml aprotinin, 10 μM leupeptin, 10 μM pepstatin, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM sodium pyrophosphate, and 50 mM sodium fluoride. After spinning to remove nonsolubilized material, immunoprecipitation was done using L2A1-Sepharose (anti-K8/18-specific monoclonal antibody coupled to Sepharose (Chou et al., 1992). Relative levels of K8/18 phosphorylation and glycosylation were determined using densitometric scanning of the corresponding bands on the radiograph (LRB Ultrascan XL enhanced laser densitometer).

Glycosylation and Reductive Focusing—Glycosylation of K8/K18 immunoprecipitates using UDP-[3H]galactose and galactosyltransferase was carried out to completion for 2 h as described (Chou et al., 1992). For β-elimination, K8 and K18 were separated using preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by electroelution of the Coomassie-stained bands (Amicon electroelution apparatus, Danvers, MA) using the manufacturer's recommendations. Eluted samples were concentrated and then precipitated with 10 volumes of acetone (−20°C). Precipitated samples were then treated with 0.3 ml of 1 M NaBH₄ in 0.1 M NaOH (37°C, 18 h), after which NaPO₃Cl₂ (20 mM) was added for 1 additional h. Samples were then gradually neutralized with glacial acetic acid (18 μl) followed by acetone precipitation of the remaining protein. Amino acid composition of the β-eliminated and duplicate β-eliminated material was done using a Beckman 6300 amino acid analyzer and coupled to a Beckman 6300 amino acid analyzer. To determine the location of the 0-galactosylated K8/18, immunoprecipitates were processed for β-elimination followed by analysis of the released carbohydrates exactly as described (Chou et al., 1992).

Protein Amino Acid Analysis, Peptide Mapping, and Isoelectric Focusing—Phosphorylated amino acids were analyzed by two-dimensional gel electrophoresis after extraction of the phosphate-labeled K8 and K18 protein bands from the SDS-polyacrylamide gels and hydrolysis in vacuo with constant boiling HCl (Cooper et al., 1983). Tryptic peptide mapping of in vitro ³H-galactosylated and in vivo metabolically ³²PO₄-labeled K8 and K18 was carried out as described (Chou et al., 1992). Isoelectric focusing was done using a 4:1 mixed of Amorphline (pH 5–7) and Amorphline (pH 3–10) (O’Farrell, 1975) in a Bio-Rad minigel system.

Dimethyl Sulfoxide-HBr Cleavage of [³H]Galactosylated or [³H]Phosphorylated K8 and K18 at Tryptophan Peptide Bonds—Cleavage of tryptophan residues was carried out similar to what has been described (Savige and Fontana, 1977). Immunoprecipitates of K8/18 were obtained from nonlabeled colcemid-arrested HT29 cells or from cells labeled with ³²PO₄ in vivo. Nonlabeled K8/18 immunoprecipitates were galactosylated using UDP-[³H]galactose. K8 or K18 was individually isolated using preparative SDS-PAGE as described above. K8 or K18 (10–20 μg) was then incubated with a mixture containing 24 μl of acetic acid, 12 μl of HCl (12 M), 1 μl of dimethyl sulfoxide, and 1 μl of phenol. After 30 min (22°C), 4 μl of HBr and 1 μl of dimethyl sulfoxide were added (30 min, 22°C) followed by the addition of 0.5 ml of H₂O. After lyophilization, samples were analyzed using SDS-PAGE. Amino acid composition of individual fragments was determined by transferring to polyvinylidene membranes followed by analysis using a Beckman 6300 analyzer.

RESULTS

Colcemid Arrest of HT29 Cells Results in Increased K8/18 Glycosylation and Phosphorylation—We used the colchicine analog colcemid, which arrests cells in the G2/M phase of the cell cycle (Ashihara and Baserga, 1979), to study the effect of cell arrest on the O-linked glycosylation and phosphorylation of K8/18 in the colonic epithelial cell line HT29. Incubation of HT29 cells growing in log phase with colcemid resulted in a progressive increase in the percentage of G2/M-arrested cells with increasing colcemid treatment intervals (not shown). As shown in Fig. 1B, colcemid treatment of HT29 cells for 36 h arrested more than 90% of the cells in the G2/M phase of the cell cycle. Coupled with this arrest, the fine evenly distributed network of keratin filaments seen when cells were predominantly in G0/G1 (Fig. 1C) changed to disassembled aggregates of cytoplasmic and perinuclear dots (Fig. 1D). A similar mitosis-associated reorganization of keratins has been observed in a variety of cell types (Lam et al., 1982; Franke et al., 1982).

We examined the phosphorylation of K8/18, after various treatment intervals with colcemid, by metabolic labeling of cells with ³²PO₄ for 2 h. K8/18 glycosylation was examined by galactosylating K8/18 immunoprecipitates using UDP-[³H]galactose and galactosyltransferase, which donates galactose to terminal O-linked GlcNAc residues. This galactosylation technique detects only accessible GlcNAc residues and may therefore underestimate the total glycosylation. For unclear reasons, the labeling of K8 with [³H]GlcnH₂O interfered with the ability of colcemid to induce G2/M cell arrest (not shown). As shown in Fig. 2, there was a gradual increase in K8/18 glycosylation and phosphorylation with increasing colcemid incubation times, as the percent of G2/M cells increased. After 36 h of colcemid treatment, there was a 10-fold (K8) and 5-fold (K18) increase in keratin phosphorylation. The faint Coomassie-stained band migrating slightly slower than K8 represents a hyperphosphorylated form of K8 (Fig.)
We asked if the increased glycosylation of K8/18 associated with G2/M arrest was reversible after removal of the arresting agent. Colcemid-arrested cells, however, could not be readily unblocked from their arrest even after incubating for more than 72 h in the absence of colcemid (not shown). We instead used nocodazole (Zieve et al., 1980), which arrested cells in a manner similar to colcemid, and resulted in a similar increase in K8/18 glycosylation after arrest (Fig. 3, lane b). Removal of nocodazole slowly returned K8/18 glycosylation to baseline levels commensurate with a decrease in the percent of cells in G2/M (Fig. 3, lane c). Of note, the increase in K8/18 glycosylation associated with colcemid/nocodazole G2/M arrest does not occur in all cell lines tested. For example, HeLa (human cervix) and PtK1 (marsupial kidney) do not show increased K8/18 glycosylation, whereas SK-CO-1 (human colon) does (not shown).

Glycosylation of K8/18 Isolated from S- and G2/M-enriched Cells After Aphidicolin Arrest or from G2/M-enriched Cells without Drugs—We asked if changes in K8/18 glycosylation occur in the S phase of the cell cycle and in G2/M cells obtained without the use of an antimicrotubule agent. An enriched population of G2/S-blocked cells was obtained by incubating with aphidicolin, a DNA polymerase α inhibitor (Pedrali-Noy et al., 1980). After washing off the aphidicolin and incubating with fresh medium, a substantial population of S phase cells can be obtained, which, on further incubation, proceed into G2/M and then G0/G1 (Fig. 4A). No difference in glycosylation was noted between the S-enriched cells, cells blocked at G0/S, or cells growing asynchronously (i.e. primarily G2/G1). Furthermore, G2/M cells obtained using the aphidicolin synchronization method also showed baseline G0/G1-like glycosylation (Fig. 4A). Multiple time points taken at 0.5-h intervals on both ends of the G0/G1 peak also showed baseline glycosylation (not shown).

A near base-line level of K8/18 glycosylation (not shown) was also noted in G2/M-enriched floater cells obtained after mechanical agitation of log phase growing HT29 cells without any drug treatment (Fig. 4B, histogram c). The lack of increased K8/18 glycosylation in G2/M-enriched cells obtained from floater cells or from aphidicolin-synchronized cells suggests that the increased glycosylation with colcemid/nocodazole mitotic arrest may be secondary to a microtubule disassembly-related effect rather than a mitosis-associated phenomenon. However, this remains unclear since nocodazole/
were used for immunoprecipitation followed by UDP-[3H]galactosylation. At the indicated time points after release, duplicate cell preparations enriched were released from their G1/S block by culturing in normal medium. Under "Materials and Methods." Histograms were obtained from: the G2/M peak. Homogeneity of the G2/M peak is expressed in terms of variation of variation.

then released cells (12-h time point shown in Fig. 4B). To this end, we consistently observe a more significantly depending on the method of isolation.

Fig. 4. Analysis of K8/18 glycosylation in S- and G2/M-enriched HT29 cells. Panel A, cells were grown in serum-free medium for 40 h and then switched to serum-containing medium to which aphidicolin (5 μg/ml) was added for 24 h. After washing, cells were released from their G2/S block by culturing in normal medium. At the indicated time points after release, duplicate cell preparations were used for immunoprecipitation followed by UDP-[3H]galactosylation or for cell cycle analysis. Panel B, propidium iodide staining and cell cycle analysis and statistics were carried out as described under "Materials and Methods." Histograms were obtained from: a, cells arrested with nocodazole for 24 h; b, aphidicolin-synchronized then released cells (12-h time point shown in panel A above); c, floater cells obtained from log phase growing cells using mechanical agitation as described under "Materials and Methods." The bar corresponds to the G2/M peak. Homogeneity of the G2/M peak is expressed in terms of the standard deviation of the mean (S.D.) and percent coefficient of variation (% CV).

colcemid arrest provides a much sharper G2/M peak (i.e. more homogeneous) than observed in the G2/M peak obtained after aphidicolin synchronization or from floater cells without drugs (Fig. 4B). To this end, we consistently observe a much more homogeneous G2/M population when using colcemid/nocodazole compared with non-antimitcrotubule modalities. This suggests that the "G2/M" populations used can vary significantly depending on the method of isolation.

Tryptic Peptide Mapping and Isoelectric Focusing Analysis of Glycosylated and Phosphorylated K8/18 Species—Using isoelectric focusing, we asked if the phosphorylated and glycosylated K8/18 species had identical pI values, which indicates that both modifications occur on the same molecules. The major K8/18 phosphorylated species do not correspond to the major Coomassie-stained species in asynchronously growing cells (Fig. 5a). Colcemid treatment generated a second major Coomassie-stained K8 species which corresponded to one of two major phosphorylated spots with minimal change in the K18 profile (Fig. 5b). In contrast to the phosphorylated species, the major glycosylated species corresponded to the major Coomassie-stained K8 and K18 species in asynchronously growing and colcemid-treated cells (Fig. 5, c and d).

We also examined the effect of G2/M cell arrest on the phosphorylated and glycosylated tryptic peptide pattern of K8 and K18. As shown in Fig. 6, although the overall phosphorylation of K8 and K18 increased in association with colcemid treatment, there were several distinct patterns of change. For example, several K8 phosphopeptides showed a relative decrease (arrow) or an increase in labeling intensity (arrowheads). For K18 phosphopeptides, some became newly phosphorylated after G2/M arrest (arrowheads), and others were increased (dotted arrow). Similarly, two newly glycosylated peptides can be seen for K8 (arrowas), whereas K18 showed a uniform increase in peptide glycosylation.

Characterization of K8/18 Glycosylation and Phosphorylation Sites during Mitosis—Phosphoamino acid analysis of K8 and K18, obtained from asynchronously growing cells or G2/M-arrested cells, was carried out. As shown in Fig. 7, serine was the only phosphorylated amino acid in K8 and K18.

The in vitro galactosylation of K8/18 shown in Fig. 2 indicates that these keratins contain terminal GlcNAcs. We recently showed that β-elimination of in vitro galactosylated K8/18, isolated from asynchronously growing cells, generated the disaccharide N-acetyllactosaminitol. This indicated that K8/18 in G2/G0 cells contain single O-GlcNAc residues. The isolated glycosylated species had identical PI values, which indicates that both modifications occur on the same molecules.

For K18 phosphopeptides, some became newly glycosylated whereas K18 glycosylated residue since β-elimination of K8 and K18 using [3H]NaBH4 generated only a single unidentified labeled product (Chou et al., 1992). Similarly, as shown in Fig. 8, the increased glycosylation in K8/18 during G2/M arrest can be accounted for by single O-linked GlcNAc.

We determined the site of K8/18 glycosylation using NaBH4/NaOH β-elimination. This reaction converts O-glycosylated serine and threonine residues to alamine and α-aminobutyrate, respectively. If [3H]NaBH4 is used, labeled amino acid products are generated as was done for the identification of serine as the O-linked site in nuclear pore proteins (Holt et al., 1987b). The addition of palladium(I1) cation as a catalyst dramatically enhances threonine to α-aminobutyrate conversion without affecting the generation of alanine from serine (Tanaka and Pigman, 1965). Treatment of K8/18 with [3H]NaBH4 (which is available only in the mM range of specific activity) resulted, after acid hydrolysis, in a single species which did not comigrate on TLC with alanine or α-aminobutyrate (not shown). This suggested that reduction of the diene amino acid derivative was incomplete, which was confirmed by carrying out β-elimination using I M unlabeled NaBH4 followed by HCl hydrolysis and amino acid analysis. As shown in Fig. 9, c and d, both K8 and K18 generated α-aminobutyrate (indicated by an arrow) after reductive β-elimination, consistent with threonine being a site for K8/18 O-linked glycosylation. The mol of α-aminobutyrate generated were 2.0 and 1.0 mol of K8 and K18, respectively. This is likely an underestimate of the number of glycosylated threonine residues since the recovery of amino acids in the β-eliminated samples was low (compare a and b with c and d, respectively, Fig. 9). Since quantitative and complete recovery of hydrolyzed amino acids are not feasible using this technique, it is possible that K8/18 serine residues may also be O-glycosylated. However, it is unlikely that serine is a major glycosylated residue since β-elimination of K8 and K18 using [3H]NaBH4 generated only a single unidentified labeled product, which upon addition of I M NaBH4, generated α-aminobutyrate (not shown).

Distribution of K8 and K18 Glycosylation and Phosphorylation within the Protein Backbone—Both K8 and K18 contain a large number of Ser/Thr residues scattered throughout the entire molecules (59/21 for K8 and 37/30 for K18; Kulesh and Oshima, 1988; Yamamoto et al., 1990). Using selective cleavage at tryptophan residues (Savige and Fontana, 1977), we asked if K8/18 phosphorylation and glycosylation localized to a particular domain (i.e. head, rod, or tail domains of the
Fig. 5. Two-dimensional gel analysis of K8/18 labeled metabolically with $^{32}$PO$_4^-$ or in vitro with $[^3]$Hgalactose. The larger panels show radiographs of the inset panels which in turn show the Coomassie stains. Immunoprecipitates of K8/18 were obtained from cells with or without colcemid treatment (0.5 μg/ml, 36 h). Metabolic labeling with $^{32}$PO$_4^-$ and in vitro $[^3]$Hgalactosylation of isolated immunoprecipitates were carried out as described in Fig. 2. Labeled immunoprecipitates were analyzed using isoelectric focusing and then SDS-PAGE. Basic peptides are to the left. Panels a and b, circled peptides (inset) correspond to dotted circles in the radiograph where minimal phosphorylation was observed. Note that Coomassie-stained spot 2 (inset and panels b and d) has become a major spot after colcemid treatment. Panels c and d, arrows correspond to K8/18 species that represent identical major glycosylated and Coomassie-stained species. The unmarked faint spot in radiograph d located between K8 and K18 likely represent degradation products of spots 1 and 2 of K8.

Fig. 6. Tryptic peptide maps of K8/18 labeled metabolically with $^{32}$PO$_4^-$ or in vitro with $[^3]$Hgalactose. Labeled K8/18 immunoprecipitates were obtained as described in the legend from asynchronous growing or colcemid arrested cells. K8 and K18 were purified using preparative gels and then electroeluted, followed by acetone precipitation and tryptic peptide mapping as described under "Materials and Methods." Electrophoresis is shown in the horizontal axis, and chromatography is in the vertical axis. For $^{32}$PO$_4^-$ labeled maps, 2,000 cpm were spotted at the origin marked by a small x. For $[^3]$Hgalactosylated keratin, 10,000 cpm were spotted. The solid arrowhead (K8, $^{32}$PO$_4^-$ labeled) indicates an increase in the labeling of a K8 peptide after colcemid treatment; solid arrowheads (K18, $^{32}$PO$_4^-$) indicate newly labeled peptides; arrows (K8, $^{32}$PO$_4^-$) indicate relatively decreased labeled peptides; dotted arrows (K18, $^{32}$PO$_4^-$ labeled) indicate a relative increase in labeled peptides; arrows (K8, $[^3]$H) indicates new glycosylated peptides. Of note, tryptic peptide maps of galactosylated $^{32}$PO$_4^-$ labeled (using unlabeled galactose) and nongalactosylated $^{32}$PO$_4^-$ labeled K8 and K18 were similar (not shown).

keratin molecule; for review see Steinert and Roop, 1988). Cleavage at tryptophan residues of K18 showed that most of the phosphorylation and glycosylation occurred in fragments 1 and 4 (Fig. 10). Since fragments 2, 3, and 5 were not phosphorylated or glycosylated, it can be concluded that fragment 1 contains most of the Ser/Thr modifications (Fig. 10). This fragment corresponds to the N-terminal head and proximal rod region of K18. Confirmation of the fragment assignment, aside from approximate $M_r$, was also done using the amino acid composition of fragments 2 and 5 (not shown). In contrast, K8 phosphorylation and glycosylation were not restricted to the N-terminal region because fragments 1 and 2 of K8 contained $^3$H-sugar and $^{32}$PO$_4^-$ labeled sites (Fig. 10).

**DISCUSSION**

The primary findings of our study are: (i) the unique association of enhanced O-linked glycosylation of keratins with mitotic arrest in HT29 cells, thereby providing evidence for the functional significance of this modification; (ii) identification of threonine as the predominant K8/18 glycosylation site; (iii) K8/18 phosphorylation and glycosylation occur for the most part on different molecules with the N-terminal third of K18 containing most of the modifications. The stage of the cell cycle that K8/18 glycosylation increased was at the G2/M boundary, whereas G0/G1, G1/S, and S cells showed basal levels of K8/18 glycosylation. Both colcemid- and no-
Phosphorylated on serine residues. were analyzed using SDS-PAGE followed by electroelution of the colcemid-induced arrest were labeled metabolically with 32P/O4 for 2 h followed by immunoprecipitation of K8/18. Phosphorylated species were analyzed using SDS-PAGE followed by electroelution of the indicated band and phosphoamino acid analysis using two-dimensional electrophoresis as described under "Materials and Methods." PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

**FIG. 7.** K8/18 from asynchronous or G2/M-arrested cells are phosphorylated on serine residues. HT29 cells (with or without colcemid-induced arrest) were labeled metabolically with 32P/O4 for 2 h followed by immunoprecipitation of K8/18. Phosphorylated species were analyzed using SDS-PAGE followed by electroelution of individual K8 and K18. β-Elimination and TLC were carried out as described under "Materials and Methods." After TLC, the cellulose plate was cut into strips and counted. The plot shows strip counts versus migration distance on the TLC plate. Migration of the indicated standards is shown.

colcemid-arrested cells showed a similar increase in K8/18 glycosylation. In the case of nocodazole, the mitotic arrest and increased glycosylation were reversible upon washing off the nocodazole, concomitant with continued growth of the cells and progression through the cell cycle. Our working model is that increased keratin glycosylation occurs in some but not all cells and relates to a specific perimitotic stage of the cell cycle. However, since we were unable to observe increased K8/18 glycosylation in G2/M-enriched cells using nonmicrotubule-disrupting methods, we cannot exclude a microtubule effect for the observed glycosylation enhancement (see also below). Altered glycosylation does not depend on simple roundness of cells. For example, in colcemid/nocodazole-treated cells (36 h), the ratio of round floater to adherent cells varies depending on the confluence level when the drug is added. However, in all cases, both adherent and floater HT29 cells show nearly similar percent of G2/M cells as well as similar increased levels of glycosylation (not shown). In addition, treatment of asynchronous cells with trypsin, which results in single rounded cells, does not affect K8/18 glycosylation (not shown).

The precise association of increased K8/18 glycosylation with keratin aggregation, an antimicrotubule effect, or specific cell cycle events remains to be determined. The microtubule effects of colcemid/nocodazole are also associated with intermediate filament reorganization (Fig. 1) and, in addition to mitotic arrest, may have resulted in the observed increase in K8/18 glycosylation in a mitosis-independent manner. However, keratin reorganization alone is not necessarily related to increased glycosylation. For example, disruption of keratins using cold hypotonic solution treatment of interphase cells (Tolle et al., 1987) does not affect the glycosylation level of K8/18 (not shown). Our inability to reproduce the increase in K8/18 glycosylation using aphidicin-synchronized or log phase floater G2/M-enriched cells suggests that increased K8/18 glycosylation may be more related to an antimicrotubule phenomenon than a mitosis event. However, the G2/M-enriched cells obtained as floaters from log phase growing cells or aphidicolin synchronization are not as homogeneous in their cell cycle stage as colcemid/nocodazole-arrested cells (Fig. 4B). This lack of homogeneity may account for the observed basal level of K8/18 glycosylation in G2/M-enriched cells obtained without the use of mitosis-arresting agents.

Well defined roles for the single O-GlcNAC modification remain to be elucidated. To date, several cytoplasmic and nuclear proteins with this modification have been described, and the list is growing (for reviews see Hart et al., 1989a; Haltiwanger et al., 1992). One hypothesis has been that single O-GlcnAcNs play a role in the assembly and/or organization of multiprotein complexes (Roquemore et al., 1992). This is based on finding this modification in proteins involved in multimeric structures including nuclear pore proteins (Davis and Blobel, 1987; Holt et al., 1987b; Starr and Hanover, 1990), erythrocyte band 4.1 (Holt et al., 1987a), SP-1 (Jackson and Tjian, 1988), and serum response transcription factor (Reason et al., 1992). The presence of single O-GlcNAC in keratins (K8 and 18 [Chou et al., 1992]; K13 (King and Hounsell, 1989)) also falls in line with this hypothesis. For example, glycosylation may be involved in blocking or enhancing specific sites of proposed head-to-tail interactions involved in keratin filament assembly (Lu and Lane, 1990). Alternatively, glycosylation may alter keratin solubility or effect interaction with other intermediate filament-associated proteins. With regard to keratin solubility, we find that the soluble and insoluble K8/18 fractions have a similar level of glycosylation and a similar tryptic glycopeptide pattern. This suggests that K8/18 glycosylation does not play a direct role in keratin solubility.

The small number of tryptophan residues in K8/18 has allowed us to take advantage of cleavage at these sites to define better the region(s) of glycosylation and phosphorylation. In the case of K18, most of the glycosylation and phosphorylation occurs in the N-terminal domain spanning the first 122 amino acids (Fig. 10). This domain has eight three-nine potential glycosylation sites (Kulesh and Oshima, 1988) with four major labeled glycosylated tryptic peptides for the entire K18 species (Fig. 6). The same domain also has 18 serine potential phosphorylation sites, with ~10 labeled phosphopeptides (Fig. 6). In the case of K8, glycosylation and phosphorylation are not restricted to the N-terminal domain.

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**Fig. 9.** Thrreonine is a site of K8/18 O-linked glycosylation. HT29 cells were grown in the presence of colcemid for 36 h followed by isolation of K8/18 immunoprecipitates. K8 and K18 were purified using preparative gels. Duplicate samples were analyzed directly by acid hydrolysis (6 M HCl, 110 °C, 24 h) or were treated with 1 M NaBH₄, 0.1 M NaOH (37 °C, 19 h) in the presence of 20 mM Na₃PdCl₄ for the last hour of incubation and then subjected to acid hydrolysis. The plots show the amino acid elution profile with time. The α-aminobutyrate standard eluted at 24.59 min, which corresponds to the position indicated by the arrow (panels c and d). For the elution profiles corresponding to non-β-eliminated K8 and K18, arrowheads indicate the retention time of the α-aminobutyrate standard (panels a and b).

**Fig. 10.** Cleavage of [³H]glycosylated and [³²P]phosphorylated K8 and K18 at tryptophan residues. Panel A, schematic showing the location of tryptophan residues in K8/18 and the predicted Mr of the partial or complete cleavage products. Panel B, [³H]galactosylated or [³²P]labeled K8 and K18 were individually isolated then treated with HCl/dimethyl sulfoxide/HBr as described under “Materials and Methods.” Numbered arrows represent the K8 and K18 fragments shown in panel A. The fluorograph shown in lanes c and d was obtained by exposing the Coomassie-stained gel (12% acrylamide) shown in lanes e and f (15% acrylamide, not shown) is similar to that shown in lanes a and b. Lanes g and h show electroeluted but uncleaved K8 and K18.
phorylation in intact cells using staurosporine (Chou and colleagues, 1989) and lamin (Liesch et al., 1991), and in keratins from HeLa cells (Celis et al., 1983; Tolle et al., 1987), ME-180 cells (Gillmartin et al., 1984), and the Xenopus oocyte (Klymkowsky et al., 1991). All reported studies indicate that type II keratin of the keratin heterodimer (e.g. K8) is the predominant species that is hyperphosphorylated. Our results extend these findings by showing that specific serine-containing peptides are phosphorylated during mitotic arrest. Furthermore, it appears that most of the glycosylation and phosphorylation of K8 and K18 after G2/M arrest showed differing tryptic peptide patterns of enhancement for K8 and K18. For example, K18 exhibited a uniform increase in glycosylation of the four major labeled tryptic forms of cell activation such as stimulation of murine T-cells and Hart, 1991). The increase in K8/18 glycosylation associated with mitotic arrest occurs for the most part on the head or tail domains (for review see Skalli and Goldman, 1991). For example, analysis of asynchronous cells showed that vimentin is phosphorylated primarily in the head domain, whereas desmin is phosphorylated in the head and tail domains (Evans, 1988). During mitosis, phosphorylation increases in both IF proteins primarily in the head domain (Evans, 1988).

Several studies showed mitosis-associated increased phosphorylation of non-keratin IF including vimentin (Chou et al., 1989) and lamin (Liesch et al., 1991), and in keratins from HeLa cells (Celis et al., 1983; Tolle et al., 1987), ME-180 cells (Gillmartin et al., 1984), and the Xenopus oocyte (Klymkowsky et al., 1991). All reported studies indicate that type II keratin of the keratin heterodimer (e.g. K8) is the predominant species that is hyperphosphorylated. Our results extend these findings by showing that specific serine-containing peptides are phosphorylated during mitotic arrest. Furthermore, it appears that most of the glycosylation and phosphorylation of K8 and K18 after G2/M arrest showed differing tryptic peptide patterns of enhancement for K8 and K18. For example, K18 exhibited a uniform increase in glycosylation of the four major labeled tryptic forms of cell activation such as stimulation of murine T-cells and Hart, 1991). The increase in K8/18 glycosylation associated with mitotic arrest occurs for the most part on the head or tail domains (for review see Skalli and Goldman, 1991). For example, analysis of asynchronous cells showed that vimentin is phosphorylated primarily in the head domain, whereas desmin is phosphorylated in the head and tail domains (Evans, 1988). During mitosis, phosphorylation increases in both IF proteins primarily in the head domain (Evans, 1988).

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