O-GlcNAcylation Determines the Solubility, Filament Organization, and Stability of Keratins 8 and 18*

Received for publication, December 24, 2009, and in revised form, July 26, 2010. Published, JBC Papers in Press, August 21, 2010, DOI 10.1074/jbc.M109.098996

Budnar Srikanth 1, Milind M. Vaidya, and Rajiv D. Kalraiya 2

From the Advanced Centre for Treatment Research and Education in Cancer, Tata Memorial Centre, Kharghar, Navi Mumbai 410210, India

Keratins 8 and 18 (K8/18) are intermediate filament proteins expressed specifically in simple epithelial tissues. Dynamic equilibrium of these phosphoglycoproteins in the soluble and filament pool is an important determinant of their cellular functions, and it is known to be regulated by site-specific phosphorylation. However, little is known about the role of dynamic O-GlcNAcylation on this keratin pair. Here, by comparing immortalized (Chang) and transformed hepatocyte (HepG2) cell lines, we have demonstrated that O-GlcNAcylation of K8/18 exhibits a positive correlation with their solubility (Nonidet P-40 extractability). Heat stress, which increases K8/18 solubility, resulted in a simultaneous increase in O-GlcNAc on these proteins. Conversely, increasing O-GlcNAc levels were associated with a concurrent increase in their solubility. This was also associated with a notable decrease in total cellular levels of K8/18. Unaltered levels of transcripts and the reduced half-life of K8 and K18 indicated their decreased stability on increasing O-GlcNAcylation. On the contrary, the K18 glycosylation mutant (K18 S29A/S30A/S48A) was notably more stable than the wild type K18 in Chang cells. The K18-O-GlcNAc mutant accumulated as aggregates upon stable expression, which possibly altered endogenous filament architecture. These results strongly indicate the involvement of O-GlcNAc on K8/18 in regulating their solubility and stability, which may have a bearing on the functions of these keratins.

Intermediate filaments form the largest group of cytoskeletal proteins (1–4), and among them keratins constitute the most diverse family. At least 35–37 keratin (K) 5 polypeptides (K1 to K37) are known until now, which are predominantly expressed in human epithelial tissues. They are further classified as type I (K9 to K28) and type II (K1 to K8 and K71 to K80) (5). These exhibit coordinated expression in specific pairs of type I and type II keratins in various tissues in a development- and differentiation-dependent manner (2, 4, 6–8). This feature of keratin expression has been utilized in the development of tissue-specific markers and distinguishes different epithelial cell types. For example, basal epidermal keratinocytes express K5/14, suprabasal keratinocytes in the upper layer of skin express K1/10, and wound healing epithelial cells express K6/16 (5, 8, 9). Simple epithelial tissues like pancreas, liver, and intestinal lining express keratin pair K8/18 along with variable levels of K19, K20, and K7 (10). Keratins, like all other intermediate filament proteins, share a common structure consisting of a central coiled coil, α-helical conserved “rod” domain that is flanked by non-α-helical amino-terminal “head” and carboxyl-terminal “tail” domains (7, 11, 12).

The head and tail domains of keratins are relatively nonconserved and harbor sites for post-translational modifications like phosphorylation and glycosylation (13). Keratin pair 8/18 and its regulation by phosphorylation are the most widely studied. Several sites of serine phosphorylation have been identified in the head and tail domains of K8 (Ser-23/Ser-73/Ser-431) and K18 (Ser-33/Ser-52) (10, 14). Site-specific phosphorylation on K8/18 regulates their association with cellular proteins (1, 15), ubiquitination, turnover (16, 17), and most importantly their filament assembly and organization (1). It plays a major role in regulating the dynamic exchange of subunits between the soluble keratins present as a minor fraction and the polymerized keratin intermediate filaments (18–23). This dynamic reorganization of keratins is essential for progression of cells through mitosis (24) and in enabling them to resist various mechanical (20, 21, 25) and nonmechanical stresses (26). The hyperphosphorylation of K8/18 is known to correlate with progression of chronic liver disease in patients (27), suggesting that alterations in filament reorganization because of phosphorylation could affect their cellular functions.

K8/18 glycosylation occurs as addition of a single O-linked β-N-acetylglucosamine (O-GlcNAc) sugar moiety. Occurrence of O-GlcNAc was first described in 1984 in lymphocytes (28). Since then, this unique type of glycosylation is described on serine and threonine residues of various classes of nuclear and cytoplasmic proteins that include transcription factors, kinases, phosphatases, metabolic enzymes, proteasomal subunits, oncoproteins, tumor suppressors, and cytoskeletal proteins (29–31). Accumulating evidence indicates its role in diseases like neurodegeneration, diabetes, and cancer. Like phosphorylation, O-GlcNAc is a dynamic modification with its addition and...
removal regulated by two enzymes, O-GlcNAc transferase (32, 33) and O-GlcNAcase (34–36), respectively. Due to its dynamic nature, O-GlcNAc is known to regulate numerous functions like protein phosphorylation, subcellular localization, protein-protein interactions, gene transcription, and degradation (31).

Three sites of O-GlcNAcylation have been mapped in the head domain of K18 at Ser-29, Ser-30, and Ser-48 (37). The sites on O-GlcNAcylation on K8 are not yet identified; however, peptide mapping indicates the presence of multiple O-GlcNAc sites (38). The stoichiometry of O-GlcNAc on K8/18 has been identified to be 1.5–2 (38). O-GlcNAc on K8/18 cycles rapidly and is known to increase upon G2/M arrest (39, 40). No reciprocal relationship has been demonstrated between K8/18 O-GlcNAcylation and phosphorylation under in vitro conditions. However, in vitro studies by peptide analysis indicate the reciprocal relationship of these two modifications at K18 Ser-48 and Ser-52 (41). Transient transfection of mutant K18 with all three O-GlcNAcylation sites mutated (Ser-29, -30, and -48 to Ala) exhibited no obvious change in the filament architecture as seen by immunostaining (37).

In this study, we demonstrate a positive correlation between O-GlcNAc levels on K8/18 and their solubility. Increased O-GlcNAcylation caused increased solubility accompanied by their simultaneous degradation by proteasomes. On the contrary, absence of O-GlcNAc in the K18 glycosylation mutant (S29A/S30A/S48A) imparted stability. Stable expression of the mutant caused altered filament architecture, its accumulation with all three GlcNAc sites mutated (Ser-29, -30, and -48 to Ala) exhibited no obvious change in the filament architecture as seen by immunostaining (37).

**EXPERIMENTAL PROCEDURES**

**Reagents**—Chang (human hepatocyte) and HepG2 (human hepatoma) cell lines were obtained from National Centre for Cell Science, Pune, India. Cell culture reagents were obtained from Invitrogen. Anti-human keratin 8 (clone M20), keratin 18 (clone CY-90), and anti-ubiquitin (clone 6C1) monoclonal antibodies were purchased from Sigma. Monoclonal antibodies to phosphoserine/threonine (O-Phos) (clone 22a) and O-GlcNAc (clone RL-2) were obtained from BD Biosciences and Affinity BioReagents, respectively. Anti-mouse Alexa Fluor 568 was obtained from Molecular Probes, Invitrogen; Vectashield mounting medium was from Vector Laboratories, and anti-mouse HRP was from Sigma. Protein G-Sepharose, PVDF membrane, and the ECL kit were purchased from Amersham Biosciences. Cultureware was obtained from Nunclon. Pfu polymerase and restriction enzymes were from Fermentas International Inc., Canada. GlcNAc was purchased from Sigma. All other chemicals were purchased locally and were of analytical grade.

**Cell Culture and Drug Treatment**—Chang (human hepatocyte cell line) and HepG2 (human hepatoma cell line) were routinely cultured and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.03% glutamine, 10 units/ml penicillin G-sodium, 10 μg/ml streptomycin sulfate, 25 μg/ml amphotericin B, and 10% FBS at 37 °C and 5% CO2. For heat stress, cells were grown at 42 °C for various time points. For different drug/inhibitor treatments, PUGNAc, GlcNAc, and MG-132 were added at concentrations of 100 μM, 20 mM, and 25 μM, respectively, for various time points. Cycloheximide was used to compare the half-life of K8/18 by growing cells in its presence at a concentration of 100 μM for different time points. In experiments involving two different drug treatments, cells were simultaneously treated with both the drugs for various time points.

**Protein Isolation, Immunoblotting, and Immunoprecipitation Analysis**—Total cell lysates were obtained by solubilizing Chang or HepG2 cells in cell lysis buffer (62.5 mM Tris and 2% SDS, pH 6.8). Keratin-enriched cytoskeletal preparations were made according to procedures published previously (17, 42). In brief, harvested cells were treated for 15 min at 4 °C with PBS (150 mM NaCl, 10 mM phosphate, pH 7.4) containing 1% Nonidet P-40, 5 mM EDTA, and a protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin, and 25 μg/ml aprotinin) followed by centrifugation (16,000 x g, 20 min). The supernatant was collected as the Nonidet P-40-soluble keratin fraction (soluble fraction). The resulting insoluble pellet was homogenized in phosphate-buffered saline containing 2% Empigen and incubated for 45 min at 4 °C, followed by centrifugation (16,000 x g, 20 min). The supernatant was collected as the Nonidet P-40-insoluble keratin fraction (filament fraction). Proteins (40 μg for total cell lysate, 80 μg for Nonidet P-40 soluble fraction, and 15 μg of filament fraction) were mixed with Laemmli sample buffer, boiled for 5 min, and resolved on 10% SDS-PAGE (43), transferred to PVDF membranes, and probed with one of the following antibodies: anti-K8, anti-K18, anti-O-GlcNAc, or anti-phosphoserine/threonine monoclonal antibodies. Membranes were washed three times with Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) containing 0.1% Tween for 30 min, then incubated with anti-mouse secondary antibody coupled to horseradish peroxidase, and visualized using enhanced chemiluminescence (Amersham Biosciences). After Western blotting, the membrane was stained with Coomassie Brilliant Blue R-250, which served as a control for equal loading.

For immunoprecipitation studies, soluble or filament fractions were incubated with anti-K18 antibody or anti-ubiquitin antibody for 1 h at room temperature. The immune complexes were incubated with protein G-Sepharose beads overnight at 4 °C. The beads were washed three times with RIPA buffer (20 mM HEPES, 140 mM NaCl, 5 mM EDTA, and 0.4% Nonidet P-40, pH 7.4). The immune complexes were solubilized in cell lysis buffer, resolved on 10% SDS-PAGE, blotted, and probed as described above. The amount of K8/18 in the immunoprecipitates of samples to be compared is normalized as close as possible so that the differences in the modifications can be easily evaluated.

**Enrichment and Analysis of O-GlcNAcylated Keratins by Wheat Germ Agglutinin (WGA)**—O-GlcNAcylated proteins from the soluble fraction were precipitated by WGA-Sepharose beads. Amounts of keratins in the soluble fraction of Chang and HepG2 cells were matched as close as possible by analyzing different amount of the same on SDS-PAGE. Equal volumes of these soluble fractions containing matched amounts of keratins were precleared with Sepharose beads for 1 h at room temperature. The precleared fractions were then incubated with WGA-Sepharose overnight at 4 °C, followed by centrifugation (2000 rpm, 5 min). The supernatant was collected as unbound
fraction. The WGA beads were washed three times with buffer containing 10 mM Tris, 150 mM NaCl, and 0.4% Nonidet P-40, pH 7.4. The WGA-bound proteins were eluted in equal volumes of cell lysis buffer and then prepared for immunoblotting as described above.

Reverse Transcription-PCR—Total RNA was isolated from Chang cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was assessed by spectrophotometry at 260 nm, and the quality was confirmed by resolving 2 μg of total RNA from each preparation on 1% agarose gel. The first strand cDNAs were synthesized by逆转录酶 First Strand cDNA synthesis kit (New England Biolabs) using random primers. The primers used for PCR amplifications were as follows: K18 forward primer 5'-GACA-CACAATCAGACGTGC-3' and reverse primer 5'-G GCC- TTGTTAGGCCTTACTTCC-3', which correspond to nucleotides 640–661 and 747–726, respectively, of K18 (GenBankTM accession number NM_002273.3); 18 S rRNA forward primer 5'-GACCGCGGTTCTATTTTGGC-3' and reverse primer 5'-TTGGCCGCGTGTTAAGATTCA-3', which correspond to nucleotides 874–895 and 981–960, respectively, of K8 (GenBankTM accession number NM_002273.3); 18 S rRNA forward primer 5'-CATAGAAGGTACCTTTCCG-3' and reverse primer 5'-GTGGTCCATGTTGCTTCAG-3', which correspond to nucleotides 418–439 and 518–497, respectively, of 18 S rRNA (GenBankTM accession number NR_003286); YFP cDNA forward primer 5'-TGACCCTGAAGTTCCATCTGCAC-3' and reverse primer 5'-AAGCTGTGTGCTGAAAAGTTAC-3', which correspond to nucleotides 128–149 and 251–230, respectively, of YFP (GenBankTM accession number GQ221700). The PCR was performed in a gradient thermocycler, DNAEngine (Bio-Rad). Four microliters of the first strand product was used as a template in each 25-μl PCR. The reaction parameters consisted of one cycle of 95 °C for 5 min, followed by cycles of 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and a single 15 min cycle at 72 °C for extension. The reactions were performed for various (20, 25, 30) and 30 cycles of PCR amplification. The RT-PCR products were electrophoresed on a 2% agarose gel using 50- and 100-bp DNA ladders as a standard to determine the molecular size. Levels of 18 S rRNA served as control for equal loading.

DNA Cloning—The K18-GM cDNA from the vector construct was amplified by PCR using high fidelity polymerase Pfu. The 5' forward (5’-TGTGATTCTTTCAGCTACCTGCTCC-3') and 3' reverse (5’-TTGTTACCCCACTGTCATTACGT-3') primers incorporated an EcoRI site and KpnI site, respectively, so that they are in-frame with the YFP coding sequence of the mammalian expression vector pEGFP N1 (Clontech). These primers were used to amplify the K18-GM cDNA. The PCR amplicon and the vector pEGFP N1 were sequentially restriction-digested with EcoRI and KpnI. The digested amplicon was ligated into the linearized vector and transformed into a DH5α strain of competent Escherichia coli cells, and the positive clone was confirmed by restriction digestion and sequencing.

cDNA Transfection and Selection of Stable Cell Lines—Transfection of Chang cells with YFP-tagged cDNAs of either K18-WT or K18-GM was performed using calcium phosphate precipitation as described previously (44). Stable cell line of Chang cells expressing YFP-K18 either WT or GM was selected using G418 antibiotic (USB Corp.) followed by sorting cells on a fluorescence-activated cell sorter FACSAria (BD Biosciences).

Immunostaining and Imaging—Chang cells stably or transiently expressing YFP-tagged K18-WT or GM were fixed in chilled methanol (–20 °C for 5 min). Antibody staining for indirect immunofluorescence was carried out as described elsewhere (45). Briefly, cells were incubated with 0.4% Triton X-100 in PBS, pH 7.4, for 5 min followed by a 10-min incubation in PBS. The coverslips were overlaid with 5% BSA for 15 min, followed by incubation for 1 h with anti-K8 or -K18 antibody. The coverslips were subsequently incubated with PBS for 30 min with three changes. This was followed by incubation with antimouse Alexa Fluor 568 for 1 h with subsequent incubation with PBS for 30 min with three changes. Following this, the nucleus was stained with DAPI for 1 min, and the coverslips were incubated for 15 min in PBS with three changes. All the incubations were at room temperature. The coverslips were mounted onto microscope slides using Vectashield mounting medium. Cells were examined in a Zeiss LSM510 META confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). Images were obtained using a 63× PlanApochromat phase contrast objective (NA: 1.4) and processed using LSM510 imaging software, version 4.2.

Densitometric Quantitation and Statistical Analysis—Densitometric quantitation of scanned images was done by ImageJ 1.43 (National Institutes of Health). Band intensities were normalized to respective loading controls. Statistical analysis was performed using GraphPad Prism 4. Comparisons were done by unpaired Student's t test, and one-way analysis of variance was used to analyze the data. p < 0.01 (**) and p < 0.001 (*** values were considered significant.

RESULTS

O-GlcNAcylation of K8 and K18 Correlates Positively with Their Solubility—Malignant transformation reportedly results in increased activity of the enzyme O-GlcNAcase leading to decreased O-GlcNAcylation on cellular proteins (46). This decrease in O-GlcNAcylation on cellular proteins is seen even in highly malignant HepG2 (hepatoma) cell line as compared with the immortalized Chang cells (hepatocyte) (supplemental Fig. 1D). These cell lines were compared to assess the effect of altered O-GlcNAcylation of K8/18 on their solubility/filament forming properties. Comparison of Western-blotted keratin immunoprecipitates from filament fractions (Nonidet P-40 insoluble) showed that keratins from HepG2 cells are indeed notably less glycosylated as compared with those from Chang cells (0.47-fold for both K8 and K18) (Fig. 1A). Decreased glycosylation was also accompanied by decreased phosphorylation on these proteins (0.41- and 0.39-fold for K8 and K18, respectively) (Fig. 1A).

The extent of O-GlcNAcylation on soluble keratins was assessed by the extent of their binding to WGA (a lectin that exhibits specific affinity for GlcNAc)-Sepharose. Although the amount of K8/18 in the soluble fractions of Chang and HepG2 cells used for WGA affinity was closely matched (Fig. 1B), keratins from HepG2 cells exhibited decreased binding to WGA.
O-GlcNAc Regulates Keratin Solubility and Stability

FIGURE 1. O-GlcNAc on K8 and K18 correlates with their solubility. A–D, Western-blotted proteins were probed with antibodies to O-GlcNAc, O-Phos, K8, and K18 as indicated. A, immunoprecipitates (IP) of K8/18 from filament fraction of Chang and HepG2 cells. B, WGA-bound and -unbound eluates and soluble fractions of Chang and HepG2 cells containing matching amounts of K8/18 (matched as described under “Experimental Procedures”). Soluble fraction served as control for equal loading on WGA-Sepharose. C, equal amounts of proteins in the total cell lysates (40 μg), soluble (80 μg), and filament fractions (15 μg) from Chang and HepG2 cells. The membrane stained with Coomassie Brilliant Blue serves as control for equal loading (C). D, immunoprecipitates from soluble and filament fractions of Chang cells.

FIGURE 2. Changes in K8/18 solubility coincide with changes in their O-GlcNAcylation and vice versa. A–D, Western-blotted proteins probed with antibodies to O-GlcNAc, O-Phos, K8, and K18 as indicated. A and B, HepG2 cells subjected to heat stress (42 °C for 8 h). A, equal amounts of proteins from soluble fraction of control (Con) (37 °C) and heat stressed (HS) cells. B, immunoprecipitated (IP) K8/18 from filament fraction of control and heat-stressed cells. C and D, HepG2 cells treated with GlcNAc (20 mM for 0.5 and 1 h). C, immunoprecipitated K8/18 from filament fraction of untreated and GlcNAc-treated cells. D, equal amounts of proteins from soluble fraction of untreated and GlcNAc-treated cells. A and D, membrane stained with Coomassie Brilliant Blue serves as control for equal loading.

(0.61- and 0.65-fold for K8 and K18, respectively). This suggests that K8 and K18, even from the soluble fraction, are less O-GlcNAcylated in HepG2 cells (Fig. 1B).

To see if the extent of O-GlcNAc on keratins has any effect on their solubility (extractability by Nonidet P-40), total, soluble, and insoluble filament fractions from the two cell lines were compared. Results showed that although HepG2 cells exhibit higher levels of K8 and K18 (2.28- and 1.54-fold for K8 and K18, respectively), very little remains in the soluble fraction (0.57- and 0.71-fold for K8 and K18, respectively), and most of it appears to be assembled as filaments (2.18- and 2.58-fold for K8 and K18, respectively). In contrast, higher amount of keratins in Chang cells remained in the soluble fraction (Fig. 1C). Thus, keratins in HepG2 cells exhibit decreased O-GlcNAcylation and also decreased solubility. The above observations indicate a positive correlation between K8/18 O-GlcNAcylation and their solubility properties.

Phosphorylation, a known regulator of keratin solubility, exhibits a gradient between soluble and filament keratins (14). If O-GlcNAc also has a role in such regulation, it would also exhibit such a gradient. Comparison of immunoprecipitated K8 and K18 showed that keratins in the soluble fraction are not only considerably more phosphorylated but are also more O-GlcNAcylated as compared with those in the filament fraction (Fig. 1D). This suggests that alterations in K8/18 O-GlcNAcylation possibly lead to simultaneous changes in their partitioning between soluble and filament fractions.

Alterations in Keratin Solubility Result in Simultaneous Changes in Their O-GlcNAcylation and Vice Versa—If the above observations are correct, then altering keratin solubility should change their O-GlcNAcylation status. Heat stress is known to increase keratin solubility (47). HepG2 cells were subjected to heat stress to investigate if alteration in keratin 8/18 solubility would be accompanied by changes in their O-GlcNAc status. As expected, heat stress resulted in an increase in the amount of soluble K8 and K18 in these cells (1.59- and 1.33-fold, respectively) (Fig. 2A). This coincided with increased O-GlcNAcylation and also phosphorylation on K8/18 (2.14- and 1.75-fold for O-GlcNAcylated and 1.05- and 1.33-fold for phosphorylation, on K8 and K18, respectively) (Fig. 2B). The increase in O-GlcNAcylation on K8 and K18 during heat stress is more pronounced than changes in phosphorylation (Fig. 2B). To determine whether increased O-GlcNAcylation of K8/18 would lead to their increased solubility, HepG2 cells were incubated with GlcNAc. GlcNAc treatment of HepG2 cells caused increased O-GlcNAc levels on several cellular proteins (supplemental Fig. 1A), including that on K8 and K18 at 30 min (1.57- and 1.35-fold, respectively) (Fig. 2C). This coincided with increased extractability of K8 and K18 in the soluble fraction at 30 min (1.54- and 1.45-fold respectively) (Fig. 2D). These results imply a definite role of O-GlcNAcylation in regulating K8/18 solubility.

PUGNAc Treatment Results in Increased Solubility but Decreased Total Levels of K8 and K18—Chang cells subjected to heat stress or GlcNAc treatment exhibited little change either in the levels of soluble K8 and K18 or in their O-GlcNAcylated (data not shown). This could possibly be due to higher basal glycosylation on these proteins. To confirm this, O-GlcNAcylated in Chang cells was altered using PUGNAc, a potent inhibitor of the deglycosylating enzyme O-GlcNAcase. Treatment of Chang cells with PUGNAc led to a notable
increase in O-GlcNAc levels on cellular proteins (supplemental Fig. 1C) and on K8/18 in the soluble (data not shown) and filament fraction (1.6- and 1.2-fold at 12 and 24 h, respectively for K8; 1.6- and 1.8-fold at 12 and 24 h, respectively, for K18) (Fig. 3A) in a time-dependent manner. As expected, this correlated with an increase in K8 and K18 in the soluble fraction (1.66-, 1.78-, and 1.89-fold at 6, 12, and 24 h for K8; 1.8-, 2.22-, and 2.33-fold at 6, 12, and 24 h for K18) (Fig. 3B). However, this also coincided with a notable decrease in the total levels of K8 and K18 (Fig. 3, C, E, and F). HepG2 cells treated with PUGNAc exhibited a similar increase in O-GlcNAc on cellular proteins (supplemental Fig. 1E) and on K8/18 in both soluble and filament fractions (data not shown). This was associated with a concomitant decrease in K8/18 levels in a time-dependent manner (0.74-, 0.53-, and 0.45-fold at 6, 12, and 24 h for K8; 0.88-, 0.76-, and 0.65-fold at 6, 12, and 24 h for K18) (Fig. 3D). This decrease in keratin levels could be either due to reduced transcription or reduced stability of these proteins.

O-GlcNAcylation of K8/18 Determines Their Degradation by Proteasomes—The levels of mRNA and half-life of K8/18 were assessed to investigate the reason for their decreased levels upon PUGNAc treatment. The mRNA levels of K8 and K18 were assessed upon PUGNAc treatment by semi-quantitative reverse transcriptase PCR performed in the linear range of amplification (data not shown). The results showed that PUGNAc treatment had no effect on the abundance of K8 and K18 mRNA transcripts (Fig. 4A) in Chang cells, suggesting that decreased levels could be due to the decreased stability of these proteins. To test for altered half-life of K8/18, Chang cells were treated with cycloheximide, an inhibitor of protein synthesis, for various time periods up to 16 h either in the presence or absence of PUGNAc. PUGNAc treatment caused a notable decrease in the half-life of K8/18 (Fig. 4, B–D), suggesting that O-GlcNAc on K8/18 regulates their degradation by proteasomes. To confirm this, Chang cells were treated with MG-132, a potent inhibitor of proteasomes, in the presence or absence of PUGNAc. As expected, blocking proteasomes with MG-132 caused stabilization of ubiquitin-conjugated cellular proteins (Fig. 4E). Interestingly, the extent of ubiquitin conjugation of proteins due to MG-132 was notably more during PUGNAc treatment (Fig. 4E). To verify whether O-GlcNAcylaton on K8/18 determines their ubiquitination, ubiquitin-conjugated proteins were immunoprecipitated from the soluble fraction of Chang cells treated either with MG-132 or with both PUGNAc and MG-132. A substantial increase in the levels of ubiquitin-conjugated keratin 8 and 18 seen as higher molecular weight bands upon PUGNAc treatment further corroborates the finding that O-GlcNAcylation on K8/18 determines their targeting and subsequent degradation by proteasomes (Fig. 4F). These results suggest that increased O-GlcNAcylation of K8/18 upon PUGNAc treatment causes an increase in the levels of K8/18 in the soluble fraction, which is possibly targeted for degradation by proteasomes.

K18 O-GlcNAc Mutant Is More Stable than Wild Type—To confirm the role of O-GlcNAcylation in regulating keratin stability, we transfected Chang cells with cDNA constructs of yellow fluorescent protein (YFP)-tagged K18, either WT or O-GlcNAc Mutant (GM) (Ser-29, -30, -48 to Ala). Semi-quantitative RT-PCR was performed in the linear range of amplification to assess the levels of mRNA transcripts produced for the same amount of K18-WT and K18-GM cDNA transfected. As seen in Fig. 5A, the levels of K18-YFP transcripts of WT and GM are similar for equal amounts of respective cDNA transfected. We then analyzed the levels of YFP-K18 after transfecting various amounts of K18-WT and GM cDNA in Chang cells. As expected, the level of K18-GM was notably more as compared with K18-WT, at all the different amounts of cDNA transfected. We then analyzed the levels of YFP-K18 after transfecting various amounts of K18-WT and GM cDNA in Chang cells. As expected, the level of K18-GM was notably more as compared with K18-WT, at all the different amounts of cDNA transfected. We then analyzed the levels of YFP-K18 after transfecting various amounts of K18-WT and GM cDNA in Chang cells. As expected, the level of K18-GM was notably more as compared with K18-WT, at all the different amounts of cDNA transfected.
transfected (Fig. 5, B and C). Furthermore, upon prolonged exposure, we observed higher molecular weight bands of K18-GM at approximate multiples of 8 kDa (Fig. 5D), which possibly are the ubiquitin conjugates of K18. The above observations confirm that glycosylation indeed regulates stability, as loss of glycosylation resulted in increased stability of K18-GM. To verify this, these transfected cells were treated with cycloheximide, and the half-life of K18-WT and GM was assessed. As expected, K18-GM exhibited an increased half-life as compared with K18-WT (0.93- and 0.8-fold at 12 and 18 h for K18-WT; 1.02- and 0.99-fold at 12 and 18 h for K18-GM) (Fig. 5E). To finally confirm the above results, Chang cells transfected with K18-WT and GM were treated with PUGNAc for various times. PUGNAc treatment caused a time-dependent decrease in K18-WT levels (Fig. 5, F and G) as seen earlier for endogenous K8/18. On the contrary, K18-GM levels remained unaltered during PUGNAc treatment (Fig. 5, F and G), indicating that lack of O-GlcNAc on K18 is responsible for its stabilization. These observations highlight the role of O-GlcNAcylation in regulating K18 stability.

Overexpression of K18-GM Results in Cell Death, Probably Because of Altered Filament Architecture and Keratin Aggregate Formation—To understand the role of keratin O-GlcNAcylation in regulating their solubility, Chang cells were transiently transfected with YFP-tagged K18-WT and K18-GM cDNA constructs. As reported earlier, no significant alterations were observed in the filament formation of YFP-tagged K18-WT and K18-GM or its endogenous partner K8 as assessed by immunostaining and confocal imaging (supplemental Fig. 2, A–H). However, most cells expressing K18-GM exhibited altered cell shape and rounding (supplemental Fig. 3, A–F) suggesting decreased cell viability or increased cell death.

Because transient transfection did not yield satisfactory answers, we tried developing Chang cells which stably overexpress YFP-tagged K18-WT and K18-GM. Chang cells exhibit good stable expression of K18-WT; however, most of the cells overexpressing K18-GM did not survive; only cells showing very little expression survived, and even these cells exhibited progressive cell death over time and over passages and never grew to confluency. The K18-GM expressed high levels of the mutant protein, and the highly vacuolar structures suggest an unhealthy state of these cells. Stable expression of K18-WT in Chang cells showed normal filament architecture and cell morphology (supplemental Fig. 4, A–H).

To assess keratin filament characteristics, K8 and K18 were immunostained in these stable cell lines. Most of the K18-WT-expressing cells showed normal endogenous K8 filament architecture characterized by intense network of fine filaments spanning the cytoplasm (Fig. 6A). However, cells expressing even low levels of K18-GM exhibited abnormal filament architecture characterized by diffused staining that cannot be distinguished as filaments (Fig. 6C). Similar observations were noted upon immunostaining K18 in these cells (supplemental Fig. 4, A–D). Exogenous YFP-tagged K18-WT and GM in these cells can be seen in Fig. 6, B and D, as bright dense filaments and diffused patterns, respectively. The normal and
diffused filament patterns in these cell lines were scored after immunostaining with K8 (n = 393 for K18-WT and n = 630 for K18-GM) and K18 (n = 318 for K18-WT and n = 366 for K18-GM). As seen in Fig. 6E, 95 ± 2.78% of cells expressing K18-WT and only 28 ± 7.6% of cells expressing K18-GM exhibit normal K8 filaments. Similarly, the percent of cells exhibiting normal K18 filaments are 92 ± 3.23 and 26 ± 9.9 in K18-WT- and K18-GM-expressing cells, respectively (Fig. 6D).

These results indicate that O-GlcNAc on K18 plays an important role in determining the filament organization and architecture in a cell.

Interestingly, the exogenous K18-GM in most of these cells form intracellular aggregates (Fig. 6, F–J, and supplemental Fig. 5, A–E) characterized by the absence of endogenous K8 staining (Fig. 6, H and I, and supplemental Fig. 5, C and E). This indicates that absence of O-GlcNAc on K18 prevents its degradation causing accumulation and finally leading to the formation of intracellular aggregates.

**DISCUSSION**

Malignant transformation appears to be associated with increased activity of O-GlcNAcase, thereby leading to decreased O-GlcNAc on cellular proteins (46). By comparing the hepatoma (HepG2) cell line with the immortalized hepatocytes (Chang), we demonstrate that this decrease is evident even in the transformed hepatoma cell line (supplemental Fig. 1D). This decrease is apparent even on proteins like K8 and K18 (Fig. 1, A and B), which have been shown to be O-GlcNAcylated (38). This lowered O-GlcNAc on K8 and K18 in HepG2 cells appeared to affect their solubility (Fig. 1C), a property known to regulate their filament organization and hence their cellular functions (20, 21, 25, 26). Solubility and filament organization have been vividly demonstrated to be regulated by phosphorylation that exhibits a clear gradient between the polymerized filament and soluble keratin subunits (14, 18–23). Interestingly, a similar gradient was observed for O-GlcNAcylation as

**FIGURE 5. Absence of O-GlcNAc on K18 makes it more stable.** A, analysis of YFP transcripts by semi-quantitative RT-PCR in Chang cells transiently transfected with 2.5 µg of YFP-tagged cDNA constructs of either K18-WT or K18-GM (S29A/S30A/S48A). Transcripts of 18 S rRNA serve as control for equal loading. B, D–F, Western-blotted proteins probed with antibody to K18. B and D, equal amounts of proteins from total cell lysate of Chang cells transiently transfected with different amounts of either WT or GM, YFP-K18 cDNA. C, graph representing protein levels of K18-WT and K18-GM, normalized to respective mRNA levels after transfecting equal amount of cDNA in Chang cells by quantitating images of three independent experiments. Bars represent mean ± S.E. E, equal amounts of proteins from total cell lysate of untreated and cycloheximide (CHX) (100 µM for 12 and 18 h)-treated Chang cells transfected either with K18-WT or GM cDNA.

F, equal amount of proteins from total cell lysate of Chang cells transfected with either K18-WT or GM cDNA and treated with PUGNAc for indicated times. G, graph representing changes in the protein levels of K18-WT and K18-GM upon PUGNAc treatment by quantitating scanned images of three independent experiments, including F. Bars represent mean ± S.E. The membrane stained with Coomassie Brilliant Blue serves as control for equal loading (B, D–F).
well, wherein K8/18 in the soluble fraction showed both higher glycosylation and phosphorylation than in the filament fraction in Chang cells (Fig. 1D). These observations imply a possible correlation between keratin solubility and their O-GlcNAcylation and envisage that alterations in either keratin solubility or O-GlcNAcylation would result in simultaneous changes in the other.

Heat stress and N-acetylglucosamine treatments are known to increase keratin solubility and cellular O-GlcNAc, respectively (47, 48). HepG2 cells under both these conditions exhibited a simultaneous increase in K8/18 solubility and O-GlcNAc (Fig. 2). However, these conditions had a minimal effect on Chang cells in altering either K8 or K18 solubility or their O-GlcNAcylation (data not shown). Interestingly, treatment with PUGNAc, the potent inhibitor of O-GlcNAcase, in Chang cells exhibited a notable increase in O-GlcNAc levels on keratins and several cellular proteins (Fig. 3A and supplemental Fig. 4C), with a simultaneous increase in the levels of K8 and K18 in the soluble fraction (Fig. 3B).

Taken together, these findings suggest that like phosphorylation, O-GlcNAc on K8 and K18 may also regulate their abundance in the soluble fraction and thus their filament organization. Our experiments with K18-GM also support the above findings. We observed that transient expression of YFP-tagged K18-GM in Chang cells caused their incorporation into the pre-existing endogenous filament network and resulted in filament architecture similar to that of K18-WT (supplemental Fig. 2). Similar observations have been reported earlier using baby hamster kidney cells (37).

Surprisingly, stable clones of Chang cells expressing even low levels of K18-GM exhibited altered endogenous K8/18 filament architecture, observed as diffused staining that cannot be distinguished as normal filaments as seen in K18-WT-expressing cells (Fig. 6, C and D, and supplemental Fig. 4, C and D). Together, these observations indicate that the absence of O-GlcNAc does not affect initial filament assembly under in vivo conditions. However, during subsequent cell divisions, the K18-GM might exhibit aberrant filament reorganization and remodeling, in turn affecting the endogenous filament architecture. The above hypothesis seems true as most cells expressing high levels of K18-GM exhibit abnormal filament architecture and cell morphology (supplemental Fig. 4, G and H). They also show cell death over a period of time, and cells expressing very low levels of the mutant eventually get selected in culture. Similar observations have also been reported for the K18 phosphorylation mutant (S33A) (15). Interestingly, experiments with PUGNAc also suggested an apparent role of O-GlcNAc on K8 and K18 in regulating their stability. PUGNAc treatment caused a gradual decrease in the total levels of K8 and K18 both in Chang and HepG2 cells (Fig. 3, C–F) without affecting their mRNA levels (Fig. 4A). A notable decrease in the half-life of K8 and K18 (Fig. 4, B–D) and an increase in the ubiquitination of this protein pair upon blocking proteasomes in the presence of PUGNAc indicated that the decreased stability of K8/18 is due to their rapid degradation via proteasomes (Fig. 4F). Interestingly, blocking proteasomes along with PUGNAc treatment caused a prominent increase in ubiquitin conjugation on cellular proteins (Fig. 4E). This correlation indicates that protein ubiquitination and subsequent degradation on the majority of cellular proteins is regulated by their O-GlcNAc. O-GlcNAc-dependent ubiquitination of cellular proteins has been reported recently (49). Together, these observations indicate that increased O-GlcNAc on K8/18 causes their disassembly and targets them to degradation.

Expression of K18-WT and K18-GM in Chang cells further strengthens the above paradigm. For similar levels of transcripts, the levels of K18-GM were much higher as compared with that of K18-WT (Fig. 5, A–C) indicating the increased stability of K18-GM. Experiments using cycloheximide (Fig. 5E) and PUGNAc (Fig. 5, F and G) confirmed that K18-GM is more stable as compared with K18-WT, which possibly accumulated as ubiquitin-conjugated aggregates (Fig. 5D). The
results (Fig. 5, E–G) also reaffirm that O-GlcNAcylation indeed regulates stability and degradation of K8 and K18.

Abnormal increase in the stability of many proteins is associated with their accumulation as intracellular aggregates (50). K8 and K18 also accumulate as intracellular aggregates under many experimental and disease conditions. Hyperphosphorylated ubiquitin-conjugated K8/18 are the major constituents of Mallory bodies, the inclusion bodies in hepatocytes (51, 52). The occurrence and formation of keratin aggregates is linked either to their phosphorylation status or to the cellular levels of O-GlcNAc prevents its degradation. This might be similar to accumulation of the microtubule-associated protein Tau in neurons, wherein reduced O-GlcNAcylation leads to its accumulation as aggregates due to reciprocal increase in its phosphorylation (54).

Accumulation of ubiquitin-conjugated keratin aggregates is also seen in many types of cancers (51–53, 55, 56). Cancer cells also show decreased O-GlcNAcylation on several cellular proteins because of increased activity of O-GlcNasease (46). It would thus be important to investigate whether reduced O-GlcNAc on K8 and K18 is the cause of their accumulation as aggregates in cancer cells. It would be equally important to characterize the O-GlcNAc site(s) on K18 that regulate its filament organization and stability and to confirm whether O-GlcNAcylation and phosphorylation on K8/18 function independently or are dependent on each other. These aspects are currently being investigated.

Acknowledgments—We thank Dr. M. Bishr Omary, University of Michigan Medical School, for the keratin 18 O-GlcNac mutant (S29A/S30A/S48A) cDNA construct. We also thank Dr. Rudolf E. Leube and Dr. Reinhard Windoffer, Johannes Gutenberg University, Germany, for the YFP-tagged keratin 18 wild type construct and the National Centre for Cell Science, Pune, India, for the human hepatocyte and hepatoma cell lines. We acknowledge the help extended by Vaishali Kailaje and Tanuja Dighe for confocal imaging; Shymal Talen, E., O’Neill, R. A., Hart, G. W., and Omary, M. B. (2006) Exp. Cell Res. 270, 8752–8758

Haltiwanger, R. S., and Philipsberg, G. A. (1997) J. Biol. Chem. 272, 8752–8758

Chou, C. F., and Omary, M. B. (2004) Methods Cell. Biol. 78, 297–319

Sawson, C., Pidala, J., and Potter, R. (2001) Biochim. Biophys. Acta 1537, 147–157

Liao, J., Lowthert, L. A., and Omary, M. B. (1996) J. Biol. Chem. 271, 345–357

Spector, D. L., Goldman, R. D., and Leinwand, L. A. (1998) Cells: A Laboratory Manual, pp. 86.2–86.3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Flitney, E. W., and Goldman, R. D. (2004) Methods Cell. Biol. 78, 297–319

Slawson, C., Pidala, J., and Potter, R. (2001) Biochim. Biophys. Acta 1537, 147–157

Liao, J., Lowthert, L. A., and Omary, M. B. (1998) Exp. Cell Res. 219, 348–357

Yki-Järvinen, H., Virkamäki, A., Daniels, M. C., McClain, D., and Gottschalk, W. K. (1998) Metabolism 47, 449–455

Guinez, C., Mir, A. M., Dehennaut, V., Cacan, R., Harduin-Lepeers, A.,
Michalski, J. C., and Lefebvre, T. (2008) *FASEB J.* 22, 2901–2911
50. Goldberg, A. L. (2003) *Nature* 426, 895–899
51. Zatloukal, K., French, S. W., Stumptner, C., Strnad, P., Harada, M., Toivola, D. M., Cadrin, M., and Omary, M. B. (2007) *Exp. Cell Res.* 313, 2033–2049
52. Strnad, P., Zatloukal, K., Stumptner, C., Kulaksiz, H., and Denk, H. (2008) *Biochim. Biophys. Acta* 1782, 764–774
53. Omary, M. B., Ku, N. O., Strnad, P., and Hanada, S. (2009) *J. Clin. Invest.* 119, 1794–1805
54. Liu, F., Iqbal, K., Grundke-Iqbal, I., Hart, G. W., and Gong, C. X. (2004) *Proc. Natl. Acad. Sci. U.S.A.* 101, 10804–10809
55. Iwaya, K., and Mukai, K. (2005) *Semin. Cancer Biol.* 15, 309–318
56. Denk, H., Stumptner, C., Fuchsbichler, A., Müller, T., Farr, G., Müller, W., Terracciano, L., and Zatloukal, K. (2006) *J. Pathol.* 208, 653–661