Stress, Apoptosis, and Mitosis Induce Phosphorylation of Human Keratin 8 at Ser-73 in Tissues and Cultured Cells*

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Simple epithelia express keratins 8 (K8) and 18 (K18) as their major intermediate filament proteins. We previously showed that several types of cell stress such as heat and virus infection result in a distinct hyperphosphorylated form of K8 (termed HK8). To better characterize K8/18 phosphorylation, we generated monoclonal antibodies by immunizing mice with hyperphosphorylated keratins that were purified from colonic cultured human HT29 cells pretreated with okadaic acid. One antibody specifically recognized HK8, and the epitope was identified as $^{32}$ LPSP which corresponds to K8 phosphorylation at Ser-73. Generation of HK8 occurs in mitotic HT29 cells, basal crypt mitotic cells in normal mouse intestine, and in regenerating mouse hepatocytes after partial hepatectomy. Prominent levels of HK8 were also generated in HT29 cells that were induced to undergo apoptosis using anisomycin or etoposide. In addition, mouse hepatotoxicity that is induced by chronic feeding with griseofulvin resulted in HK8 formation in the liver. Our results demonstrate that a “reverse immunological” approach, coupled with enhancing in vivo phosphorylation using phosphatase inhibitors, can result in the identification of physiologic phosphorylation states. As such, K8 Ser-73 phosphorylation generates a distinct HK8 species under a variety of in vivo conditions including mitosis, apoptosis, and cell stress. The low steady state levels of HK8 during mitosis, in contrast to stress and apoptosis, suggest that accumulation of HK8 may represent a physiologic stress marker for simple epithelia.

Epithelial cells express the keratin subfamily of intermediate filament (IF) proteins in a cell-type preferential manner (1). More than 20 unique keratin gene products (K1–K20) are characterized and classified into type I (K9–K20) and type II (K1–K8) IFs. This classification has biologic relevance since epithelial cells express in a tissue preferential manner at least one type I and one type II keratins that form noncovalent obligate heteropolymers in a soluable tetrameric form or a more complex filamentous cytoskeletal form (2, 3). For example, glandular simple type epithelia express K5 and K18 (K5/18) with variable levels of K19 and K20, and stratified epithelia express K5/14 basally and K1/10 suprabasally. Although the function of IF proteins, including keratins, remains poorly understood, their importance in human disease is accumulating. For example, mutations in epidermal type keratins (e.g. K5/14, K1/10) result in several blistering and scaling skin diseases (4–6). More recently, a mutation in K18 that is associated with an in vitro assembly defect was identified in a patient with chronic liver disease of “unknown etiology” (7), thereby suggesting a predisposition or cause of the patient’s cryptogenic cirrhosis (8).

Although IF protein functions remain poorly understood, characterization of the dynamic and regulatory modification of phosphorylation is beginning to provide important functional clues (9, 10). A functional and regulatory role for IF protein phosphorylation is supported by the location of this modification within the N-terminal “head” and C-terminal “tail” domains of IF proteins which are the domains that impart most of the structural heterogeneity and hence tissue-specific expression of these proteins. In the case of K8/18, phosphorylation regulates filament reorganization in vivo, enhances keratin solubility, plays a role in dictating the localization of K8/18 with specific cellular compartments, regulates the association with the 14-3-3 protein family, and is associated with a variety of physiologic stresses (10). With regard to cell stress, several stress modalities have been associated with hyperphosphorylation of K8/18 including heat stress and virus infection (11), and mitotic arrest (which can be considered a form of stress) of several cultured cell lines (12). Also, drug-induced hepatotoxic stress, induced in mice by feeding with a griseofulvin-supplemented diet, resulted in dramatic K8/18 hyperphosphorylation (13). Of note, stress-induced K8 hyperphosphorylation is associated with generation of a distinct K8 species, termed HK8 (11, 12), that migrates slightly slower than K8 after one-dimensional gel analysis. To date, two in vivo K8 phosphorylation sites have been described (14). One site, Ser-431, is located in the tail domain of K8 and is phosphorylated after stimulation of cells by epidermal growth factor and is likely to be phosphorylated by mitogen-activated protein kinase (MAPK). The second site, Ser-23, is located in the head domain of K8 and is a conserved site in all type II keratins. This latter site is also phosphorylated in K6 and hence is likely to be a conserved phosphorylation site that serves a common function in all type II keratins. Other in vitro phosphorylation sites of K8 that were phosphorylated by purified cAMP-dependent protein kinase have also been described (15).

In this report we used a “reverse immunological” approach, which entailed utilizing several existing strategies, to identify physiologic K8/18 phosphorylation sites. This approach in-
volves generating monoclonal antibodies that selectively recognize phosphorylated K8 or K18, by immunizing mice with keratins that were purified from cultured human cells that were treated with okadaic acid to generate hyperphosphorylated K8/18. One such characterized antibody (LJ4) selectively recognized HK8, and the epitope of the antibody was identified as Ser(P)-73 of K8. The antibody was then used to demonstrate that formation of HK8 occurs in association with several physiologic events including cell stress, apoptosis, and mitosis. The biologic implications of these findings are discussed.

MATERIALS AND METHODS

Cell Culture and Animal Studies—HT29 (human colon) and baby hamster kidney cultured cells and the cDNA for human K8 were obtained from the American Type Culture Collection (Rockville, MD). Transgenic mice that express wild-type human keratin 18 (termed TG2) have been described previously (16, 17). Antibodies used were mouse monoclonal antibody (mAb) L2A1 (18), rabbit anti-human K8/18 antibody 8592 (17), and Troma I rat anti-mouse K8 mAb (Developmental Studies Hybridoma Bank, University of Iowa). The L44 and L45 antibodies are described below. Other reagents and supplies were okadaic acid (LC Services, Woburn, MA), polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), Freund’s adjuvant, griseofulvin, aphidicolin, and protein kinase A (Sigma), immobilized protein A and BCA protein determination kit (Pierce), enhanced chemiluminescence reagent (Amersham Corp.), protein kinase Ca (PanVera Corp., Madison, WI), calcium/calmodulin-dependent kinase II, p34, MAPK (New England Biolabs, Cambridge, MA), anisomycin (Calbiochem), and etoposide (Bristol-Myers, Princeton, NJ).

Cell Culture and Animal Studies—For okadaic acid (OA) treatment, HT29 cells were incubated with culture medium containing 1 µg/ml OA for 2 h at 37 °C. Heat stress was done by culturing cells (~50% confluent and in log phase) at 42 °C for 24 h. To induce apoptosis, cells were treated with 10 µM anisomycin (16 h) or with 200 µM etoposide for 4 h followed by switching to normal culture medium without drug for 48 h. Cell cycle G2/M stage enriched HT29 cells were generated by arresting cells at G2/S using aphidicolin and then washing off the drug and culturing for 10 h (19). This synchronization typically resulted in 70–90% G2/M enriched cells (not shown). Cell cycle analysis was done as described (19). Griseofulvin (GF) feeding (1.25% w/w) or control diet feeding was done for 17 days exactly as described using Balb/c or TG2 transgenic mice (13). Partial hepatectomy was carried out as described (13) followed by harvesting of the livers for immunoprecipitation and immunostaining. Sham hepatectomies were done in a manner identical to the experimental hepatectomy (i.e., anesthesia, abdominal wall and peritoneal incision, exposure of the liver, and closure of the incisions) except that liver tissue was not resected.

Generation of Phosphoepitope-specific Monoclonal Antibodies—Hyperphosphorylated keratins (K8/18/19) were purified by immunoprecipitation, using mAb L2A1-Sepharose-protein A beads, from cultured HT29 cells that were treated with okadaic acid (1 µg/ml, 2 h). The keratins were eluted from the covalently coupled antibody beads using 9 M urea followed by dialysis in PBS. Balb/c mice were immunized intraperitoneally using 20 µg of purified keratins mixed with complete Freund’s adjuvant. The immunizations were repeated four times using incomplete Freund’s adjuvant at 4–6-week intervals. Three days before fusion (20), mice received a booster (20 µg) via tail vein injection. Hybridoma culture supernatants were screened by Western blotting using detergent lysates of HT29 cells that were cultured in the presence or absence of OA. One hybridoma clone (LJ4) reacted preferentially with OA-treated HT29 cell lysates as compared with non-OA-treated HT29 cells. Cells from hybridoma clones of interest were cloned by limiting dilution. mAb LJ4, which reacted equally with phosphorylated and non-phosphorylated K8 and K18) and with mAb LJ4. Similar results to the LJ5 antibody were obtained if L2A1 was used for blotting (not shown).

FIG. 1. Specificity of the LJ4 antibody. A, HT29 cells were cultured at 37, 42 (24 h), or at 37 °C in the presence of okadaic acid (OA) (1 µg/ml, 2 h). An equal number of cells was solubilized with 1% Nonidet P-40 for 30 min followed by immunoprecipitation using anti-K8/18 mAb L2A1 or using mAb LJ4. Immunoprecipitates (i.p.) were analyzed by SDS-PAGE followed by staining with Coomassie Blue. Note co-immunoprecipitation of 14-3-3 proteins after heat stress and OA treatment and of the heat-inducible (hsp70, 72 kDa) and constitutively expressed (hsc70, 73 kDa) heat shock proteins 70 (hsp/c70) as previously reported (24, 26). The LJ4 antibody precipitates more HK8-like species (versus K8) as compared with the L2A1 antibody. B, HT29 cells, treated as in A, were solubilized with 2% SDS-containing sample buffer to obtain a total cell lysate. An equal amount of protein was loaded per lane for immunoblotting with mAb LJ5 (which recognizes total phosphorylated and nonphosphorylated K8 and K18) and with mAb LJ4. Similar results to the LJ5 antibody were obtained if L2A1 was used for blotting (not shown).
phoretically transferred to PVDF membranes. Western blotting was done as described (19). Dot blots were performed using a nitrocellulose membrane. Peptides (0.5 μg in 1 μl) were spotted on the nitrocellulose membrane followed by immunoblotting as done for the Western blotting except that the blocking buffer consisted of 3% bovine serum albumin in PBS (instead of 5% non-fat milk powder in PBS).

Peptide Synthesis and Enzyme-linked Immunosorbent Assay (ELISA)—Peptide synthesis was carried out as described (19) using an automated 9050 Milligen Peptide Synthesizer. The ELISA was done by coating microtiter plates overnight (22 °C) with serial 2-fold dilutions of peptides (0.125 to 4 μg in 100 μl/well) in 100 mM sodium carbonate (pH 9.6). The plates were then rinsed twice with washing buffer (10 mM sodium phosphate (pH 7.2), 0.05% Tween 20). Blocking was performed for 2 h (22 °C) using 200 μl/well of the coating buffer containing 1% bovine serum albumin. After washing, 100 μl/well of mAb (cell culture supernatant diluted 1:5 in washing buffer) were added in the presence or absence of peptides and incubated for 1 h, followed by washing four times. Horseradish peroxidase-conjugated rabbit anti-mouse antibody (100 μl/well, 1:1000 dilution in washing buffer) was added (1 h). Wells were then washed four times followed by the addition of 100 μl/well of the peroxidase substrate (30 min) and measuring the absorbance at 405 nm.

In Vitro Phosphorylation and Immunofluorescence Staining—K8/18 immunoprecipitates, which contain minimally phosphorylated K8/18, were obtained from HT29 cells by isolation (24). This preparation, with 1% Nonidet P-40, spinning, and then solubilization of the pellet with 1% Empigen followed by immunoprecipitation. After heating (95 °C, 1 min) to destroy any associated kinase activity, precipitates were phosphorylated using the following kinases: cAMP-dependent protein kinase, protein kinase Ca, calcium/calmodulin-dependent kinase, p34cdc2, or MAPK (Erk2) (2 h, 30 °C). The buffers used for each kinase reaction were those recommended by the kinase supplier. For phosphorylation of the synthetic peptides, equal amounts of individual peptides (200 μg in 400 μl of 50 mM Tris-HCl (pH 7.4)) were incubated in MAPK buffer (containing 200 μM ATP) with 100 units of MAPK for 2 h at 30 °C. Immunofluorescence staining was carried out exactly as described (19).

Construction of the Ser-73 K8 Mutant and Cell Transfections—A Ser-73 to Ala K8 mutant was generated using a Transformer20 kit (Clontech, Palo Alto, CA). The cDNA for human K8 was used as a template as described (25). Mutation was confirmed by sequencing followed by subcloning into the pMRB101 mammalian expression vector with an HCMV promoter-directed expression. Transfection was done in baby hamster kidney cells using LipofectAMINE as recommended by the manufacturer.

RESULTS

Characterization of mAb LJ4 That Specifically Recognizes a Hyperphosphorylated Form of K8—The initial purpose of our study was (i) to generate mAbs that specifically recognize phosphorylated K8 or K18 using a strategy of immunizing mice with hyperphosphorylated keratins, and (ii) to identify the phosphorylation sites that are recognized by such antibodies and study the biologic context of their phosphorylation. Hyperphosphorylated keratins were purified from human colonic cultured HT29 cells that were pretreated with the phosphatase inhibitor okadaic acid. This antigen source should enrich for physiologically relevant phosphorylation sites given that treatment of HT29 cells with okadaic acid results in massive hyperphosphorylation of K8/18 (19). Using this approach, a mAb termed LJ4 was generated that preferentially immunoblotted a single species upon inducing hyperphosphorylation of keratins in cultured cells using heat stress or okadaic acid treatment (Fig. 1B). The increased reactivity of the antibody was not related to keratin protein levels since an independent antibody (LJ5) that recognizes phosphorylated and nonphosphorylated keratins (not shown) afforded similar levels of reactivity (Fig. 1B).

Using immunoprecipitation, mAb LJ4 recognizes the K8/18/19 complex that is also immunoprecipitated by mAb L2A1 (Fig. 1A), which in turn recognizes the total keratin pool (18). When compared with L2A1 precipitates, LJ4 preferentially precipitates a species that migrates slightly slower than K8 (Fig. 1A), compare lanes a–c with d–e). This slower migrating species is similar to what we previously termed HK8 (i.e. hyperphosphorylated form of K8).

FIG. 2. The LJ4 antibody recognizes HK8, a distinct hyperphosphorylated form of K8. A, individual HK8, K8, K18, and K19 were purified from HT29 cells that were cultured at 37 and 42 °C as described under “Materials and Methods.” 1 μg of each purified keratin was loaded onto an SDS-PAGE gel followed by transfer to a PVDF membrane and then immunoblotted using mAb LJ4. After blotting, the membrane was stained with Coomassie Blue. Note that although equal amounts of keratin were loaded onto the gel (not shown), a relatively small fraction of HK5 and K8 (that were purified from the 42 °C treated cells) transferred to the membrane. B, individually purified K8 and HK8 (isolated from 42 °C treated cells as in A) were mixed with K18 (isolated from cells grown at 37 °C and used as an internal standard). Samples were subjected to isoelectric focusing in the horizontal dimension and SDS-PAGE in the vertical dimension, followed by Coomassie staining. A duplicate sample to that in panel b was transferred to a PVDF membrane followed by immunoblotting using mAb LJ4 (membrane staining after immunoblotting is similar to that in panel b, not shown). In panels a–d, the numbers 1–7 correspond to K8 and HK8 isoforms, and the letters a and b correspond to K18 isoforms. The schematic in panel d shows a representation of the phosphorylated and non-phosphorylated K8 and HK8 isoforms.

10 μM pepstatin, A, 10 μM leupeptin, 25 μg/ml aprotinin, and 0.5 μg/ml OA (45 min, 4 °C). After spinning (16,000 × g, 30 min, 4 °C), the supernatants were used for immunoprecipitation (6 h, 4 °C) with antibodies that are covalently conjugated to protein A-Sepharose. To test the blocking effect of phosphorylated and nonphosphorylated synthetic peptides, antibody beads were first incubated with 100 μg of individual peptides in 200 μl of PBS for 1 h followed by the addition of cell lysates (500 μl) and then immunoprecipitation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22) was performed using 10% polyacrylamide gels under nonreducing conditions. Two-dimensional gel electrophoresis was carried out as described (23). For Western blotting, tissue or cell lysates, immunoprecipitates, or purified keratin proteins were resolved on SDS-polyacrylamide gels and then electrotransferred to PVDF membranes. Western blotting was done as described (19). Dot blots were performed using a nitrocellulose membrane. Peptides (0.5 μg in 1 μl) were spotted on the nitrocellulose membrane followed by immunoblotting as done for the Western blotting except that the blocking buffer consisted of 3% bovine serum albumin in PBS (instead of 5% non-fat milk powder in PBS).
perphosphorylated K8; Ref. 11). In fact, in some experiments only HK8-like and K18 species are noted after precipitation with the LJ4 antibody (e.g., Fig. 3D, lane a), and the preferential binding of LJ4 with the HK8-like species is also prominent in cells grown at 37 °C (Fig. 1A, lane d) in contrast with a barely discernible HK8 using the L2A1 antibody (Fig. 1A, lane a). Selectivity of the LJ4 antibody to the HK8 species was confirmed by immunoblotting individually purified keratins from cells grown at 37 or 42 °C (Fig. 2A, note the intense signal with less efficiently transferred HK8). The slight reactivity of the antibody with individually purified K8, K18, and K19 from heat-stressed cells is likely to be primarily related to slight degradation of the HK8 species during the purification process since immunoblotting of minimally manipulated total cell lysates (e.g., Fig. 1B) shows minimal cross-reactivity. Analysis of the phosphorylated isoforms of K8 and HK8 that are isolated from 42 °C stressed cells showed a shift by one phospho-isoform (i.e., phospho-isoforms of K8 and HK8 begin with isoforms 2 and 3, respectively, Fig. 2B, panels a–d). The assignment of the phospho-isoforms (versus the non-phosphorylated isoform 1) is based on metabolic labeling with 32PO4, as described in several studies (18, 19, 23). Of note, dephosphorylation of HK8 using alkaline phosphatase generates K8 (11, 12), thereby indicating that HK8 formation occurs via a phosphorylation event.

**Identification of Ser-73 as the K8 phosphorylation site recognized by mAb LJ4**

To identify the phosphorylation site recognized by mAb LJ4, we tested several kinases for their ability to phosphorylate K8 with subsequent formation of HK8. Of several kinases tested (Fig. 3A), only MAPK resulted in substoichiometric formation of HK8 (Fig. 3A, lane f, faint band indicated by a small arrow). Since MAPK is a proline-directed kinase with preferential phosphorylation of serines adjacent to prolines (27), inspection of the K8 amino acid sequence (not shown) suggested one likely candidate region with the sequence, 67VN-QSLLSPVL. A second candidate K8 region, 428GLTSPGL, corresponds to Ser-431 which becomes phosphorylated after epidermal growth factor stimulation but does not generate an HK8-like species (not shown).

**Identification of Ser-73 as the K8 phosphorylation site recognized by mAb LJ4.** 

A, K8/18 immunoprecipitates, obtained from 1% Empigen BB-solubilized HT29 cells, were phosphorylated in vitro with the indicated kinases as described under “Materials and Methods.” After washing off the kinase, immunoprecipitates were separated by SDS-PAGE, transferred to a membrane, and then immunoblotted with mAb LJ4. The immunoblotted membrane was then stained with Coomassie Blue. B, the indicated four peptides were phosphorylated in vitro using MAPK. Peptide 1 corresponds to the K8 sequence containing Ser-73 (beginning with Val-67). Dots in peptides 2–4 indicate amino acids that are identical with peptide 1. The phosphorylated and non-phosphorylated peptides (0.5 μg each) were spotted on a membrane followed by incubation with mAb LJ4, washed, and then incubated with peroxidase-conjugated goat anti-mouse IgG. After rewashing, bound antibodies were visualized using enhanced chemiluminescence. C, binding of the LJ4 antibody to the phosphorylated and non-phosphorylated peptides used in B was tested by an ELISA. Quantitation of binding was estimated by measuring the absorbance at 405 nm. All peptides, except for phosphopeptides 1 and 2, showed near-zero background absorbance. D, HT29 cells, incubated at 42 °C for 24 h, were solubilized using 1% Nonidet P-40. Lysates were added to mAb LJ4 that was preincubated with the indicated peptides or phosphopeptides. Immunoprecipitates were then analyzed by SDS-PAGE followed by staining with Coomassie Blue. E, baby hamster kidney cells were co-transfected with wild-type K8 and K18 or with wild-type K18 and a S73A K8 mutant. After 3 days, K8/18 immunoprecipitates were obtained that were then used for immunoblotting using mAb LJ4. Small arrow indicates HK8 that is faintly seen in lane a but not in lane b.
Several peptides were synthesized that corresponded to altering the two potential phosphorylation sites Ser-70 and Ser-73 (Fig. 3B). Dot blot analysis of these peptides, before and after phosphorylation by MAPK, showed that only phosphopeptides 1 and 2 (Fig. 3B) bound with mAb LJ4, and phosphopeptide 2 showed a slightly higher LJ4 binding by ELISA (Fig. 3). Additional experiments with peptides 1 and 2 (Fig. 3) showed that only phosphopeptide 1 binds with subsequent mAb LJ4 reactivity, can be explained by the preferential binding of LJ4 to phosphopeptide 1 that is that phosphorylated at Ser-73 with resultant formation of HK8/K18, whereas phosphopeptide 2 shows partial inhibition (Fig. 3B). One possible explanation for the preferential binding of LJ4 to phosphopeptide 2 versus 1 is that in vitro phosphorylation at Ser-70 either interferes with binding of the LJ4 antibody and/or with phosphorylation at Ser-73.

Further confirmation that Ser(P)-73 is the HK8 phosphorylation site that is recognized by mAb LJ4 was obtained by generating a Ser-73 to Ala (S73A) K8 mutant. As shown in Fig. 3E, LJ4 binds to HK8 after immunoprecipitation of K8/18 precipitates obtained from transfected wild-type K8 and K18 but does not bind to K8/18 precipitates obtained from wild-type K18 and S73A K8 co-transfectants. In addition, mAb LJ4 reactivity is not affected upon immunoblotting of an S431A K8 mutant that is co-transfected with wild-type K18 (not shown). Taken together, the data in Figs. 1–3 indicate that K8 is phosphorylated in vivo on Ser-73 with resultant formation of HK8 and that \(^{15}\)LpSPLVLE of K8 contains the epitope that is recognized by the LJ4 antibody. The possibility of a constitutive K8 Ser-73 phosphorylation that is coupled to Ser-70 dephosphorylation, with subsequent mLJ4 reactivity, can be excluded since HK8 formation depends on a phosphorylation event (of Ser-73) (e.g. Fig. 3A), and because of the two-dimensional results of Fig. 2 and our previous data that demonstrate K8 hyperphosphorylation upon mitotic arrest and cell stress (11, 12).

**Stress and Apoptosis Are Associated with Phosphorylation of K8 on Ser-73** —We used mAb LJ4 to examine K8/18 filament organization in HT29 cells and in intact animals that are subjected to heat- or drug-induced stress, respectively. In the case of HT29 cells that were cultured at 42 °C, reactivity with the LJ4 antibody was patchy in that some cells reacted intensely with the antibody with surrounding cells showing absent staining (Fig. 4, panels a and d). The majority of cells that bound to the antibody manifested a reorganized dot-like staining pattern (Fig. 4, panel a), which overlapped with the staining pattern observed with a rabbit antibody to K8/18 (not shown). In addition, some cells manifested a filamentous staining pattern after heat stress that was not reorganized in any obvious fashion (not shown).

The effect of stress was also tested in mice, in the form of hepatotoxic stress that is induced after feeding mice the hepatotoxic drug griseofulvin (GF) (13). Using that model, we previously showed that GF feeding of normal mice (e.g. Balb/c) or transgenic mice that overexpress wild-type human K18 (termed TG2) resulted in liver toxicity in association with marked hyperphosphorylation of K8/18. We used this animal model to investigate if GF-induced hepatotoxicity is associated with phosphorylation of mouse K8 since the motif surrounding the Ser(P)-73 position of mouse K8 (73VNQSLL) is very similar to Ser-79 (79VQQLL) of human K8 (11, 12).

We also examined generation of the HK8 species in cells that are induced to undergo apoptosis. This was prompted by the observation of very bright LJ4 staining of HT29 cells that was occasionally seen upon staining colcemid-treated mitotically arrested cells in association with nuclear fragmentation (not shown). The induction of apoptosis by anti-microtubule agents such as colcemid has been described previously (29). Hence, we tested K8 Ser-73 phosphorylation in association with apoptosis by treating HT29 cells with anisomycin or etoposide which are known to induce apoptosis in several tested cell lines (30, 31).
As shown in Fig. 4 (panels c and f), etoposide treatment induced apoptosis in some cells, with nuclear fragmentation and a marked increase in mAb LJ4 reactivity. The increase in LJ4 reactivity is not restricted to cells that have undergone nuclear fragmentation, as clearly demonstrated after treatment of cells with anisomycin that results in intense LJ4 staining in most but not all cells (Fig. 4, panels b and e). K8 Ser-73 phosphorylation, as determined by LJ4 immunoreactivity after anisomycin and etoposide treatment, was also associated with formation of HK8 (confirmed by immunoprecipitation, not shown) but a very similar pattern to that in Fig. 1 at levels that are similar to those obtained after heat stress. The filamentous staining pattern in the majority of anisomycin, and in some of etoposide-treated cells, suggests that HK8 formation is not a late event during the course of apoptosis.

**HK8 Is Also Formed during Mitosis in Cultured Cells and Normal Tissues**—Although prominent levels of HK8-like species form after mitotic arrest of HT29 cells using anti-microtubule agents or okadaic acid (12, 18), other means of obtaining mitotic cells form after mitotic arrest of HT29 cells using anti-microtubule agents such as enrichment after using the DNA polymerase agents or okadaic acid (12, 18), other means of obtaining mitotic cells form after mitotic arrest of HT29 cells using anti-microtubule agents such as enrichment after using the DNA polymerase agents or okadaic acid (12, 18), other means of obtaining mitotic cells form. As shown in Fig. 5A (panels a–d), the LJ4 antibody binds selectively to mitotic cells that are obtained after aphidicolin synchronization or that are visualized in mitotic cells that are exponentially growing after being subcultured without the use of aphidicolin (not shown). However, for unclear reasons, this finding was not uniform in that few clearly mitotic cells did not bind to LJ4 (not shown). Immunoblotting of mitotically enriched HT29 cells resulted in a significant increase in LJ4 binding as compared with G0/G1 cells, although the level of formed HK8 in G0/M synchronized cells was barely visible by Coomassie staining (Fig. 6B, right lane) as compared with G2/M-arrested cells (12). To address K8 Ser-73 phosphorylation in mitosis in the context of a physiologic tissue, we asked if the LJ4 antibody can stain intestinal crypt mitotic cells. As shown in Fig. 6C, intensely bright staining of mitotic cells was noted in the basal crypt compartment that contains the mitotic cells (exemplified by the metaphase cell shown in panel h). Similar results were noted after staining mouse small intestine (not shown). The LJ4 positive-staining mouse colonocytes were of simple epithelial enterocytic lineage as determined by double staining using LJ4 and rabbit anti-K8/18 antibodies (not shown but similar to findings in the liver described below). In addition, we examined mouse livers after partial hepatectomy as another physiologic mitosis model. As shown in Fig. 7A, HK8 becomes detectable by immunoblotting beginning 24 h post-hepatectomy but is not detected in pre- or sham-hepatectomized mice. Immunofluorescence staining confirmed the immunoblot results (Fig. 7B, panels a–c), and double staining of the liver sections showed that the LJ4-positive cells are simple epithelial cells (Fig. 7B, panel d). Furthermore, double nuclear/LJ4 staining (Fig. 7C) showed that the LJ4-positive cells are indeed mitotic.

**DISCUSSION**

**Utility of a Reverse Immunological Approach to Characterize K8 Phosphorylation**—Several approaches have been successfully used to identify in vivo phosphorylation sites of IF proteins as well as hundreds of other proteins. This encompasses the use of several biochemical and analytical techniques including mass spectrometry and/or proteolysis coupled with high performance liquid chromatography (or two-dimensional peptide mapping) separation of peptides then microsequencing. Alternatively, in vitro identified phosphorylation sites that are
associated with specific peptides can be matched with in vivo phosphorylated peptides. The approach utilized in this study was to (a) enhance the stoichiometry of several K8/18 phosphorylation sites by culturing cells in the presence of okadaic acid, (b) immunize mice with the purified K8/18 from these cells, and (c) select antibodies that specifically recognize phosphorylated K8 or K18 epitopes. Any generated antibodies would be predicted to recognize in vivo phosphorylation sites and could then be used as probes for the specific site and to aid in the identification of that site. Using this approach, we generated several additional antibodies to K8 and K18, including antibodies that recognize the already characterized Ser(P)-431 of K8 (14). The sensitivity and utility of these antibodies are exemplified by the discussion below on K8 Ser-73 phosphorylation during mitosis. The general utility of antiphospho-IF protein antibodies has been reviewed (23, 33).

The reverse immunological approach utilized in this study has several limitations that warrant mention. For example, the antigenicity of the generated hyperphosphorylation states and their abundance play an important role in the nature of the generated antibodies. In turn, the abundance of a particular phospho-epitope is related to the type of phosphatase inhibition used. One clear advantage of this approach is that it potentially allows for the enrichment of in vivo relevant phospho-epitopes that may otherwise have too low a stoichiometry to be identified using routine biochemical and analytical means. To that end, enrichment of Ser(P), Thr(P), and Tyr(P) epitopes can be done using a variety of phosphatase inhibitors. One plausible variation of the above described approach is to phosphorylate in vitro an IF protein with a particular kinase, which may phosphorylate in a specific and nonspecific manner one or more in vivo relevant sites and then use the generated phosphoprotein as an immunogen to generate and screen for in vivo relevant anti-phospho-epitope antibodies.

Phosphorylation of Ser-73 during Mitosis and Apoptosis—

The first observation of an HK8 species was in mitotically arrested cultured epithelial cell lines (12, 18). For example, significant and easily detectable levels of HK8 were noted upon G2/M arrest using a variety of agents including okadaic acid, nocodazole, and colcemid. However, we were unable to demonstrate a clearly detectable HK8 species in mitotically enriched cells (up to 90% G2/M cells) that were obtained after aphidicolin synchronization or by mechanical shaking (12), although a barely visible Coomassie-stained band that corresponds to HK8 is occasionally seen. Our data here clearly demonstrate that HK8 does form during mitosis, but its steady state levels are low in cultured HT29 cells. Formation of HK8 was noted within the proliferative compartment of mouse colon (Fig. 6) and small intestine (not shown), in dividing hepatocytes after partial hepatectomy (Fig. 7), and in mitotic HT29 cells (Fig. 6). Demonstration of HK8 during physiologic mitosis in tissues was made feasible using the anti-HK8 antibody that would have otherwise been missed due to its relatively low levels (i.e. few dividing cells in self-regenerating tissues).

The function of the HK8 species during mitosis and the reason(s) for its low basal level during "physiologic" mitosis (in contrast with mitotic arrest) in HT29 cells remain to be investigated. However, our results provide several potential explanations for HK8 accumulation upon mitotic arrest. First, one contributor to HK8 accumulation in G2/M-arrested HT29 cells (which include floating and adherent cells) are the generated apoptotic cells. Second, although clearly apoptotic cells (i.e. those exhibiting nuclear fragmentation) represent only a small fraction of the floater G2/M-arrested cells, most of the remaining cells have reached an irreversible state in that they are "locked" at G2/M and/or an apoptotic pathway. For example, treatment of HT29 cells with anisomycin, which induces apoptosis, is associated with a near-uniform formation of HK8 in most treated cells prior to the generation of nuclear fragments (Fig. 4). This indicates that HK8 formation is an early intermediate event along the pathway of apoptosis. Third, G2/M cell arrest using anti-microtubule agents or okadaic acid can be
Modulation of Human Keratin 8 Phosphorylation at Ser-73

Fig. 7. Partial hepatectomy induces HK8 formation in mitotic hepatocytes. TG2 mice were subjected to sham or partial hepatectomy as described in Materials and Methods. A, livers were harvested at the indicated times from sham or hepatectomized mice and then solubilized in Nonidet P-40. After pelleting nonsolubilized material, lysates were used to immunoprecipitate K8/18 with mAb L2A1. Immunoprecipitates were resolved by SDS-PAGE followed by transfer to a PVDF membrane and then immunoblotting with mAb LJ4. The membrane was then stained with Coomassie Blue to visualize K8 and K18. Note that the HK8 species is difficult to detect by Coomassie staining due to its low levels. B, livers from sham-operated or 60-h post-hepatectomized mice were freshly frozen and then sectioned. Staining of the sections was done using the individual antibodies mouse anti-HK8 (LJ4) or rat anti-mouse K8 (8592) antibodies. For single antibody staining shown in panel d, positive cells appear yellow/green, and the remaining K8/18 staining by antibody 8592 is red. C, liver sections from 60-h post-hepatectomized mice were double stained with YO-PRO-1 which stains DNA green and with LJ4 which stains HK8 red upon visualization with Texas red goat anti-mouse IgG. Cells corresponding to the indicated mitosis stage are highlighted by small arrows.

Considered a form of toxic drug-induced stress. Therefore, one or more of these factors could explain the HK8 accumulation. Hence, formation of low levels of HK8 is normal in the life cycle of a dividing cell, but accumulation of HK8 is abnormal and signals cell stress and/or apoptosis (see below).

With regard to the function of Ser-73 phosphorylation during mitosis, several possibilities can be considered. One possibility is a role in filament organization particularly because this phosphorylation site is located within the H1 domain and is 14 amino acids away from domain 1A of the rod. Mutations within the H1 domain, in residues different than the Ser-73 K8 equivalent, have been identified in K1 (34), K5 (35), and in some cases have been attributed to abnormal filament assembly (34, 36). Although K8 Ser-73 is somewhat similar to Ser-22 of lamin C (37) in that both are phosphorylated during mitosis by a proline-directed kinase, several differences can be noted. For example, Ser-22 of lamin C is only 5 amino acids from the rod (versus 16 amino acids for K8), does not have a similar sequence to that surrounding K8 Ser-73, and does play some role in nuclear lamina reorganization (37). In contrast, phosphorylation of K8 at Ser-73 can maintain a filamentous pattern (e.g., Fig. 4b), and the complete disorganization of the keratin filament network in hepatocytes of GF-fed mice was associated with HK8 formation in only a small subset of the cells (Fig. 5c). Also, although keratins purified from heat-stressed cells (which include K18, K8, and HK8) have a higher soluble component, as compared with K8/18 isolated from non-heat-stressed cells, they do make bona fide filaments after in vitro filament assembly (11). We cannot, however, exclude a more subtle effect by K8 Ser-73 phosphorylation on filament organization. A second possibility is that K8 Ser-73 phosphorylation may positively, or negatively, regulate an interaction with another cellular element.

Phosphorylation of K8 at Ser-73 Is a Marker of Cell Stress in Simple Epithelia—Our results showed that two modalities of stress, heat in cultured HT29 cells (Figs. 1 and 4) and GF-induced hepatotoxicity in mice (Fig. 5), were associated with generation of HK8 (i.e. Ser-73 K8 phosphorylation). We define hepatotoxicity here as a form of stress since we previously showed that GF-associated hyperphosphorylation is not related to mitosis based on the lack of hyperphosphorylation of K18 Ser-52 that occurs in mice after partial hepatectomy (13). However, we cannot exclude the possibility that apoptosis may play some role in the observed generation of HK8 after GF feeding of mice. Although heat stress induces apoptosis in some HT29 cells (not shown) and in the intestine of intact animals (38), formation of HK8 occurs in the majority of cells that exhibit a condensed nuclear staining pattern (e.g., Fig. 4) that differs from that seen after anisomycin or etoposide treatment. Furthermore, heat stress is associated with induction of hsp70 in HT29 cells, which is not the case after anisomycin or etoposide treatment (not shown). In the case of heat-stressed HT29 cells, induction of HK8 occurs at a stage prior to irreversible heat-induced damage and accumulates to a high stoichiometry with stress prolongation (11). This raises the possibility of an adaptive survival-type function for this phosphorylation and indicates that it can serve as a unique marker for cell stress in simple epithelia.

Examination of the K8 sequence that surrounds Ser-73 as compared with other type II keratin sequences shows a high degree of homology (Table I). Our results indicate that Ser-73 is phosphorylated in vivo, as determined by the anti-HK8 antibody reactivity with the phosphorylated and nonphosphorylated peptides and the mutational analysis shown in Fig. 3. The LJ4 reactivity to mouse K8 (which has a Lys in the sequence LSPLK instead of Val-76 in the human K8 sequence 74LLSPLV) (Figs. 5–7), coupled with the lack of LJ4 reactivity with the phosphopeptide . . . . SLLAPLV . . . . but the strong reactivity with the phosphopeptide . . . . ALLSPLV . . . . (Fig. 3),
indicate that the epitope that is recognized by the anti-HK8 LJ4 antibody encompasses the sequence phospho-LLSPL. In
examination of type II keratin residues that correspond to K8 131, 1303–1314
Ser-73 of K8 may also be phosphorylated in settings 18. Chou, C.-F., and Omary, M. B. (1993) J. Biol. Chem. 268, 4465–4472
that have a threonine instead of a serine (Table I). Examination of mouse skin and esophagus that ex-
press K5 and K4, respectively, did not show cross-reactivity, 19. Liao, J., Lowthert, L. A., Ku, N.-O., Fernandez, R., and Omary, M. B. (1995) J. Cell Biol. 131, 1291–1301
possibly due to the Ser → Thr substitution of the epitope. Interestingly, K4/5 and K6 are expressed within tissue compart-
ments that have a regenerative capacity (K4 and K5 in the esophagus and skin, respectively) or in the “stress” context of
intestinal and liver. Therefore, one possible model that 20. Kohler, G., and Milstein, C. (1975) Nature 237, 495–497
will require testing is that the threonines in K4/5/6 that corre-
spond to Ser-73 of K8 may also be phosphorylated in settings 21. Lowthert, L. A., Ku, N.-O., Liao, J., Coullombe, P. A., and Omary, M. B. (1995) Biochem. Biophys. Res. Commun. 206, 370–379
that are similar to those observed for K5.

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